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# Handbook of Neurochemistry and Molecular Neurobiology

*3rd Edition*

## Neuroactive Proteins and Peptides

Springer

# Handbook of Neurochemistry and Molecular Neurobiology

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Abel Lajtha (Ed.)

# Handbook of Neurochemistry and Molecular Neurobiology

## Neuroactive Proteins and Peptides

Volume Editor: Ramon Lim

With 112 Figures and 28 Tables

Springer

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# Preface

## Exploring brain proteins and peptides—past, present, and future*

Proteins carry out a multitude of functions in the biological system. Commensurate with their structural and functional complexities, the exploration of proteins in any organ has never been a simple task, let alone those in the nervous system, where cytoarchitectonic and hierarchical organizations reign supreme. For those who have witnessed the growth in this area over the past few decades, one cannot help but be amazed by the progress made, bringing the field from stone-age chemistry to present-day sophistication of gene technology. Looking back, the study of brain proteins can be roughly divided into three eras.

**I. The pre-biotech era or era of brute-force fractionation.** This period covers the time from the early 20th century to the 1970s. The earliest methods for protein isolation were crude, consisting mainly of extraction and precipitation, yielding information on groups of proteins rather than individual ones. Unfortunately, no meaningful information could be gathered until pure proteins could be isolated and individually studied. But since biologically active proteins are often present in miniscule amounts, purifying individual proteins was analogous to looking for a needle in a haystack. One way to mitigate the difficulty was to start with a source material that could be procured in large quantities, such as pig or bovine brains from a slaughter house, although there was no guarantee that the protein in question would remain intact by the time the brains arrived in the laboratory. It was also advantageous to do a preliminary separation (neurons from glia) or dissection (white from gray matter) or a subcellular fractionation (e.g., obtaining the synaptosomes) before protein purification, although such procedures inevitably reduced the amount of tissue available. Purification of enzymes could be monitored by test-tube assay; other biologically active proteins must be followed by tedious bioassay procedures, severely hindering the progress of the work. Some investigators aimed at obtaining proteins found only in the nervous system, with the assumption that brain-specific proteins must have brain-specific functions. This assumption was only partially true as proteins in common with other organs may have unique roles in the brain, while proteins unique to the brain often turn out to be also present in other organs in smaller amounts. The use of monoclonal antibodies, obtained from a protein mixture, to monitor the final steps of purification, provided yet another strategy. The advent of high-performance liquid chromatography (HPLC) in the 1980s provided a powerful addition to the existing methods of ion-exchange and size-exclusion fractionation techniques. Despite these advances, obtaining a pure protein was laborious and quite often a matter of serendipity and luck. Even so, the purity and identity of the isolated proteins were difficult to ascertain because of the minute quantity of the final product.

**II. Era of biotechnology.** Progress in molecular biology revolutionized the approach to the study of brain proteins. Since DNA is much easier to analyze and manipulate than proteins, the major obstacles in protein chemistry were circumvented by dealing with DNA. Once a protein was partially sequenced, the complementary DNA (cDNA) could be cloned from a cDNA library and the pure, recombinant protein

*Dedicated to Blake W. Moore, who dared to tread in murky waters in the early days of brain proteins.

subsequently expressed in vitro in quantities sufficient for chemical and biological analyses. Even without a partial sequence, cloning could be achieved by monitoring the gene product with antibody or function analysis, such as receptor binding. Sequencing the entire protein became unnecessary as the information could be deduced from the cDNA. Once a protein is identified, the entire family of related members could be found by homology search in the cDNA library. Site-directed mutagenesis and deletions/additions could be made on the DNA and conveniently expressed as modified proteins. Through DNA technology, proteins could be dissected at will to reveal the contribution of each constituting domain. The intracellular function of a protein could be determined by overexpression or underexpression, the former through transgenes (using plasmid or virus carriers) while the latter through gene knockout or RNA interference. Thanks to molecular biology, an entirely new era of protein chemistry has dawned. Brain proteins can now be explored through myriads of approaches never dreamed of before.

**III. The postgenomic era or era of neuroproteomics.** The complete sequencing of the human genome in 2001 propelled the study of brain proteins to a new height. Proteins can now be discovered, complete with amino acid sequences, by scanning the genomic map alone, even before knowing their functions, which can frequently be deduced by homology comparisons from the database. Through microarray technology, high throughput studies are feasible. DNA microarrays are available to determine which genes are activated by a given protein. Protein and antibody microarrays can be purchased to study protein–protein and protein–DNA interactions. With bioinformatics, one can hope to make sense of the intricate relationships of proteins in the nervous system. Today, the classical approach to explore brain proteins has been reversed and turned upside down. What used to be the mainstay of protein chemistry—fractionation, amino acid sequencing, and cumbersome bioassays—are now relegated to a secondary, confirmatory role. For those of us whose research careers span the length of the three eras, one cannot look back without being overwhelmed by a sense of awe and humiliation.

The current volume is a collection of a variety of brain proteins and peptides whose structure and functions are relatively well known. It is meant to provide a glimpse of the field rather than an exhaustive treatise. In a rapidly expanding area such as this, not only that new members are constantly being identified, but also that new functions are quickly being added to the known proteins and peptides. It can be said that bringing a new protein into light is analogous to giving birth to a child, each having a life of its own, growing in its own way frequently with outcomes unexpected to the parents.

Every author and coauthor in this volume is an established expert in the field. Each provides a succinct and up-to-date summary of a protein or peptide as well as their own contributions to the field. In putting this volume together, they have sacrificed their precious time that could have been used for other important activities such as research, teaching, publishing original papers, and grant applications. The editor is deeply appreciative of their dedication. It is a pleasure to see their labor coming to fruition, even though one is cognizant of the fact that a large portion of the content will be outdated within a few years. Hopefully, like the regenerating nerve, this volume will renew itself as time goes by.

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# 1 Nerve Growth Factor and Related Proteins

*J. Regino Perez-Polo*

**Abstract:** The purification of the nerve growth factor (NGF) protein to homogeneity allowed for the subsequent characterization of the remainder of the neurotrophin (NT) family of growth factors and their cognate receptors. The juxtaposition of elegant protein biochemistry and insightful tissue culture approaches to the elucidation of the developmental sequelae responsible for selective innervation and neuronal number regulation led to an understanding of cell death processes and neuronal plasticity in response to growth factor cues and later to trauma to the peripheral nervous system (PNS) and the central nervous system (CNS). An understanding of the key evolution of these concepts is rooted in large measure in the character of the early pioneering investigators in the neurotrophin (NT) field.

**List of Abbreviations:** BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglia; NGF, nerve growth factor; NT, neurotrophin

## 1 Introduction: Discovery of NGF

While the concept of growth factors as proteins responsible for the survival and proliferation of cells is common to the cancer and immunology fields, its analog in the neurosciences can easily be traced to the discovery and early characterization of the nerve growth factor (NGF) protein, the founding member of the neurotrophin (NT) family of proteins. The NGF and related NTs not only play roles in the cell survival of neurons but also in their migration, neurite elongation, neurotransmitter expression, and synaptic maturation both in the developmental process and in the maintenance of phenotypes specific to well-defined areas of the peripheral nervous system (PNS) and the central nervous system (CNS). Also, while NGF activity was originally described as neuronal-specific, the presence of NGF receptors on nonneuronal glial and immune-like cells and the subsequent demonstration of effects on these nonneuronal cells is consistent with similarities among the NT and cytokine gene families most likely reflecting evolutionary events (Perez-Polo, 1990; Aloe, 2004). It would be hard to exaggerate the importance of NGF to our understanding of those processes that balance the need for homeostasis with the growth requirements of development. In addition, the particular cast of scientists involved in the early years of the discovery, purification, and characterization of the NGF protein sometimes reads like an Indiana Jones script, inclusive of experiments in cellars away from the persecution of Gestapo agents in Turin, Italy, during World War II followed by trips to South America smuggling tumor-bearing mice (Cowan, 2001; Aloe, 2004).

At the heart of the NGF story there are three individuals: Rita Levi-Montalcini and Viktor Hamburger on the biology side and Stanley Cohen on the biochemistry side. They brought together the skills and insights of developmental science tradition, explant culture techniques, and a then newly ascendant protein biochemistry armoire in a way that only serendipity and historical accidents could have accomplished. Deprived of a position at the University of Torino during the early years of the World War II, Rita Levi-Montalcini was forced to rely on the chicken embryo as a tool to determine the forces that made innervation of tissues a well-choreographed event. After the war, this student of Professor Giuseppe Levi, and Viktor Hamburger, the student of Professor Hans Spemann, were united when as a result of the publication of the work carried out by Rita Levi-Montalcini in a cellar, where eggs not used in research provided more direct sustenance, he invited her in 1947 to St. Louis to work in his department. Over the next decade the careful analyses of experiments with chicken embryos and later explanted dorsal root ganglia (DRG) exposed to tumor explant extracts led to the discovery of a protein that could elicit neuronal outgrowth. At the time the absence of pure reagents led to the use of snake venom to abolish phosphodiestarase activities as a way of eliminating nucleic acids as contributors to the neurite-extending activity and establishing proteins as likely agents of neurite-promoting activity. The salivary origin of the mouse sarcoma tumors 37 and 180, rich in this biological activity, led to the choice by Stanley Cohen of the mouse submaxillary gland as a potential source of the factor. Retired male breeders were used as a measure of economy, given their low cost. These sexually mature male random-bred mice would yield as much as 1% of their protein content as NGF protein. Had females, containing less than 0.01% of the protein when compared with sexually mature male mice, been used, the history of the Nobel Prize might be different. Nevertheless, the key event was the establishment of the DRG in vitro assay, which provided a semiquantitative assay useful in the screening of tissues and fractionation that made purification of isolated NT proteins possible.

## 2 The NGF Bioassay

The ability to isolate and culture chick DRG for days at a time led to the development of a semiquantitative assay based on the extension of neurites from cultured explants of DRG from 8-day-old chicken embryos in response to the application of extracts containing "nerve growth promoting activity." This fairly easy assay allowed for the purification of the NGF protein from mouse sarcoma (Levi-Montalcini and Hamburger, 1953), as well as from submaxillary gland of adult male mouse (Varon et al., 1967; Bochini and Angeletti, 1969), snake venom (Angeletti, 1970; Perez-Polo et al., 1978), guinea pig prostrate (Harper et al., 1979), and human placenta (Goldstein et al., 1978; Walker et al., 1980). In time, two different forms of NGF were isolated: a highly basic protein of 26,500 Da and a subunit-containing complex made up of three different protein dimers in a hexamer configuration stabilized by zinc, reminiscent of other zinc-containing hexamers with important signaling properties such as insulin and β-amyloid protein.

## 3 The Neurotrophic Hypothesis

Interestingly, early studies suggested that part of the growth promoting activity present in NGF, in addition to its neurotropic activity, was a stimulation of neuronal proliferation, now confirmed for CNS (Barnabe-Heider and Miller, 2003). In time, elegant experiments showed that the differences in the sizes of the well-contained explants and the numbers of cells present in the presence of NGF or in rats or mice injected with NGF was due to effects of NGF on developmental cell death. The ability to control neuronal cell survival in developing PNS by the application of NGF or antibodies to NGF led to the elucidation and direct testing of the neurotrophic hypothesis (Levi-Montalcini and Angeletti, 1966; Hamburger and Oppenheim, 1982). That is, careful counting of neurons present in well-defined sensory, sympathetic, and enteric ganglia as first NGF and then other members of the NGF family of factors were either added or withdrawn via treatment with antibodies showed that in all cases there was a considerable decrease in the number of neurons associated with innervation of target organs that could be circumvented via the application of appropriate cognate growth factors or exaggerated by treatment with antibodies to these same growth factors (Hamburger et al., 1981; Oppenheim, 1981).

The neurotrophic hypothesis states that competition for target-derived trophic factors by neurons during well-defined windows of development characterized by the rapid growth provides a mechanism for the accurate innervation of target tissues during the development of the nervous system and the elimination of those neurons that are not necessary. This developmental plasticity is a response to variations in phenotype and the vagaries of events during development that allow for a very high level of circuit pattern formation without the necessity to "hard wire" nervous system via the transfer, storage, and expression of precise genetic information. The properties of neurons that perish apoptotically during development, the transient or permanent nature of their dependence on those trophic growth factors at the termination of the developmental sequelae, or the role of these growth factors in adult neuronal function or responses to injury can vary greatly. However, in spite of these variations, there are certain common features that are now accepted.

The strategy for establishing the final neural net that allows a nervous system to process sensory signals, establish memory engrams, and generate responses ranging from the endocrine and motor to the sensation of self-awareness depends in some fashion on the principle of the neurotrophic hypothesis, perhaps best illustrated during the early neuronal development of the sympathetic nervous system. Early in development there is an overproduction of precursor sympathetic neurons, sympathicoblasts, that when exposed to the NGF, in the absence of glucocorticoids, extend neurites oriented to the NGF source. Over time there is a decrease in the ambient levels of NGF even as the extended growth cones contact target tissues that synthesize and release the reduced amounts of NGF. Synaptic contact between the neuronal growth cones that display NGF receptors takes place, and there is binding of nearby NGF molecules to these receptors followed by internalization and the eventual retrograde transport to the neuronal soma of both encapsulated NGF–receptor complexes and activated NGF receptors (Miller and Kaplan, 2002). Those sympathicoblasts whose axons fail to reach a target become growth factor deprived, experience neurite

pruning, and perish via apoptosis, the fate of half of all sympathetic neurons during development. This is not a unique event as the fate of neuronal apoptosis during development is common to almost all neuronal populations (Oppenheim, 1991).

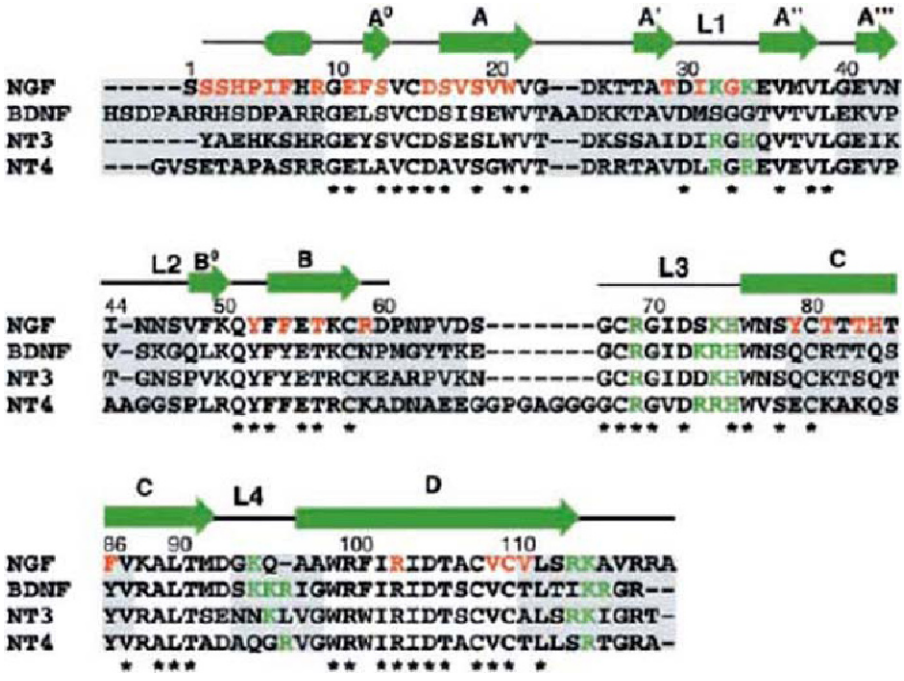
Experiments that support the neurotrophic hypothesis have shown that an overproduction of neurons early in development is followed by a significant decrease in their number as maturity is reached, that the synthesis of neurotrophic substances takes place at tissues that are targets of innervation, but not at the neurons that innervate them, and that cognate high-affinity neurotrophic receptors are present in the innervating neurons but not in their target tissues. Furthermore, reducing the levels of the neurotrophic factors and their cognate receptors, or interrupting the retrograde transport of NT results in the death of the innervating neurons, an event that can be abrogated by the external application of the appropriate growth factors to the innervating neurons (Johnson, 1978; Johnson et al., 1980; Oppenheim et al., 1982; Levi-Montalcini, 1987; Smeyne et al., 1994).

## 4 NGF Protein Structure

The biological activity of NGF is associated exclusively with the highly basic protein subunit, 2.5S or β-NGF, here called NGF, which stimulates the differentiation and survival of both sensory and sympathetic neurons (Levi-Montalcini and Hamburger, 1951; Greene and Shooter, 1980; Thoenen and Barde, 1980) and affects certain populations of cholinergic neurons in the CNS. In several animal models, intraventricular infusion of NGF has been shown to prevent the loss of cholinergic neurons in the septohippocampal lesions (Hefti, 1986; Williams et al., 1986; Kromer, 1987) and to restore cognitive function in aged rats when brain levels of NGF were reduced (Fischer et al., 1987). NGF is part of a family of related growth factors known as the NTs (◉ *Figure 1-1*). There are four members of the NT family: NGF, brain-derived neurotrophic factor (BDNF),

**Figure 1-1**

**Sequence alignment of the neurotrophins. Numbering refers to NGF. Conserved residues are marked with an asterisk. Regions of low sequence homology are shaded. Adapted from Wiesmann and Vos (2001)**



NT-3, and NT-4/5 (Levi-Montalcini and Hamburger, 1953; Cohen, 1959; Bochini and Angeletti, 1969; Angeletti and Bradshaw, 1971; Perez-Polo et al., 1972; Barde et al., 1982; Ernsfors et al., 1990; Hon et al., 1990; Kaisho et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Berkemeier et al., 1991; Hallbook et al., 1991; Ip et al., 1992; Ibanez, 1995; Shooter, 2001) that share a 50–60% sequence homology (Shooter, 2001) and 25% sequence homology to proinsulin (Barde and Thoenen, 1982). The NTs in turn belong to the cysteine knot superfamily (Chao and Bothwell, 2002), so called because of ring structures made up of intracellular disulfide bridges that form a tightly packed "cysteine knot" that allows for homodimers with extensive surfaces in contact between antiparallel disposed monomers and a strong noncovalent bond between dimer members ($K_d \sim 10^{-13}$ M for NGF). Neurotrophins are typically very basic small proteins (pI $\sim$ 9–10; mol. wt. $\sim$ 12–14). For one such NT, NGF, the biologically active dimer (β-NGF) can be isolated as part of a heterohexamer made up of the β-NGF dimer and two other protein dimer kallikreins, one active, γ-NGF, and one inactive, α-NGF, which together with two zinc atoms form a stable equilibrium complex ($\alpha_2\beta_2\gamma_2Zn_2$) called 7S NGF based on its equilibrium sedimentation constant (Shooter, 2001).

Murine 7S NGF is a stable multimer within a pH range of 5–9 and at concentrations consistent with its dissociation equilibrium constant ($K_d = 10^{-9}$ M) (Shooter, 2001). Dissociation and association of the 7S complex serves regulatory functions given that (whereas cross-linked β-NGF is biologically active) the cross-linked 7S multimer is not active and the equilibrium dissociation constant for 7S NGF is in the same range as the binding equilibrium constant of NTs for the $p75^{NTR}$ receptor (Chao and Bothwell, 2002). The α-NGF subunit stabilizes the 7S NGF complex but has no other known biological function (Thomas et al., 1981a), and the γ-NGF subunits are arginine-specific esteropeptidases of the serine family that process the pro-β-NGF precursor yielding the mature NGF form (Thomas et al., 1981b). The significant levels of NT precursor forms present throughout adulthood may serve a complex regulatory function given the reported widespread presence of pro-NTs and the selective cleavage of pro-NGF by γ-NGF and of pro-BDNF by the matrix metalloproteinase MMP-7 but not by MMP-2 or MMP-3 (Lee et al., 2001). The nature and processing of NT-3 and NT-4/5 are not known.

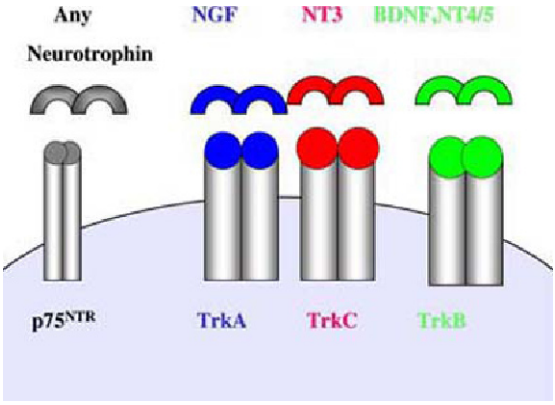
There appear to be elevated ambient levels of pro-NTs, as compared with the mature forms, consistent with there being a regulatory role for pro-NT species (Lee et al., 2001). This is based in part on evidence that pro-NGF has a higher affinity for NGF $p75^{NTR}$ receptor when compared with NGF (Lee et al., 2001). This could have proapoptotic consequences. Given that $p75^{NTR}$ enhances the affinity of the other NGF receptor, tropomyosin-related tyrosine kinase (TrkA), for NGF while decreasing the affinity of TrkA for NT-3, it may be that a role of pro-NTs is to selectively affect developmental sequelae in individuals in response to environmental cues, providing a more flexible developmental program based on the binding of pro-NTs to p75–sortilin complexes (Kalb, 2005). A precise elaboration of the role of the different states of processing of the NTs or of the high-molecular-weight forms of NTs—characterized for NGF in mouse submaxillary gland and snake venom—is lacking.

## 5 The NT Receptors

The first obligatory action of a neurotrophin (NGF, BDNF, NT-3, NT4/5) in the regulation of neuronal cell death is binding to its cognate receptors (Bibel et al., 1999). There are two kinds of NT receptors: $p75^{NTR}$ and Trk (Meakin and Shooter, 1992; Bibel et al., 1999). The $p75^{NTR}$ receptor belongs to the tumor necrosis factor (TNF), fas antigen receptor (Fas) family of receptors, all of which contain an intracellular death domain and are able to trigger ceramide signaling and NF-κB activation (Dobrowsky et al., 1994; Carter et al., 1996; Taglialatela et al., 1997, 1998). The ability of $p75^{NTR}$ to mediate both cell survival and cell death of neurons and glia depends on its interaction with Trk receptors ($p140^{TrkA}$, $p145^{TrkB}$, and $p145^{TrkC}$) (Radeke et al., 1987; Rodriguez-Tebar, 1990; Middlemas et al., 1991; McDonald et al., 1995; Taglialatela et al., 1996; Dechant, 2001; Hirata et al., 2001; Miller and Kaplan, 2001; Chao and Bothwell, 2002). The $p75^{NTR}$ receptor has an equilibrium dissociation constant of $\sim 10^{-9}$ M and when both $p75^{NTR}$ and Trk are present on cells, the $p75^{NTR}$ receptors are present in much larger numbers compared with Trk. Also, when both are present the combined equilibrium dissociation constant typically measured is in the $\sim 10^{-11}$ M range. The Trk receptors belong to the tyrosine kinase family of receptors that share amino acid sequence

homology with the tropomyosin-related tyrosine kinase. They are commonly called TrkA, TrkB, and TrkC (*Figure 1-2*). The Trk receptors display differential binding to the different NTs (McDonald et al., 1995; Chao and Bothwell, 2002). While the $p75^{NTR}$ receptors appear to bind equally to any of the NTs, the Trk receptors do show some specificity with TrkA preferentially binding to NGF, TrkB preferentially binding to BDNF and NT-4/5, and TrkC preferentially binding to NT-3. There are also nonfunctional truncated TrkB and TrkC receptors, likely to quench NT action (Middlemas et al., 1991) and mediate separate intracellular signaling cascades (Kalb, 2005). Although $p75^{NTR}$ binds all of the NTs, there may also be selective activation of ceramide signaling by NGF but not the other NTs. BDNF and NT-3 Trk receptors are widely distributed in the nervous system as compared with the more restricted distribution of TrkA receptors. In addition, the intracellular fates of internalized NT receptors, which may be controlled via regulated receptor proteolysis, could have regulatory importance (Kalb, 2005). It is clear that both $p75^{NTR}$ and the different Trks activate intracellular cascades that share elements and thus engage in crosstalk. While a detailed analysis of these is beyond the scope of this chapter, it is likely that some of the apparent difficulty in reconciling observations as to the role of these elements in cell death and phenotypic expression may be due to the lack of acuity of the studies to date. For example, while both $p75^{NTR}$ and TrkA activate the transcription factor NF-κB, which NF-κB dimers are involved in the activities stimulated by the two receptors has not been established. In addition to the cleavage of receptors into inactive truncated forms, which may have different or unknown biological activities, there is also evidence for secretase cleavage of $p75^{NTR}$, which may also play a role in development (Zampieri et al., 2005).

**Figure 1-2**
**The neurotrophin receptors**




## 6 Biological Activity of NTs: Specificity and Plasticity

In the CNS, NT action is very specific. NGF is synthesized by glutaminergic hippocampal neurons in the proximity of nerve terminals belonging to basal forebrain cholinergic neurons expressing $p75^{NTR}$ receptors (Radeke et al., 1987; Gu et al., 1988; Rodriguez-Tebar et al., 1990; Middlemas et al., 1991; McDonald et al., 1995; Rossner et al., 1995, 1997a, b; Yu et al., 1995, 1996; Taglialatela et al., 1996; Dechant, 2001; Hirata et al., 2001; Miller and Kaplan, 2001; Chao and Bothwell, 2002; Naumann et al., 2002). The $p75^{NTR}$ receptors present on the synaptic terminals of projecting basal forebrain cholinergic neurons in turn bind and internalize NGF, providing trophic support to basal forebrain cholinergic neurons. Interruption of this retrograde transport selectively kills the cholinergic neurons and impairs cognitive function. Exogenous NGF can overcome the effects of hippocampus-derived NGF.

Studies using NT and NT-receptor-null mice would suggest that abolition of the TrkA gene disturbs sensory neurons in the periphery to a greater extent than the TrkA-bearing neurons in the CNS (basal

forebrain cholinergic neurons and striatal cholinergic interneurons) that appear to express both TrkB receptors (Crowley et al., 1994; Koliatsos et al., 1994; Smeyne et al., 1994). Given that both TrkB and TrkC are widely distributed in both the CNS and the PNS, it is not surprising that in null mice lacking individual NT or NT receptor genes the absence of a growth factor, or its receptor, is compensated by the expression of others (Ernfors et al., 1994a, b; Jones et al., 1994; Conover et al., 1995). Thus, although there are specific developmental stages for the maximal expression of the different NTs, null mice studies would suggest that there is also a great deal of plasticity present during development.

## References


Aloe L. 2004. Rita Levi-Montalcini: the discovery of nerve growth factor and modern neurobiology. Trends Cell Biol 14: 395-399.

Angeletti RH. 1970. Nerve growth factor from cobra venom. Proc Natl Acad Sci USA 65: 668-674.

Angeletti RH, Bradshaw RA. 1971. Nerve growth factor from mouse submaxillary gland: amino acid sequence. Proc Natl Acad Sci USA 68: 2417-2420.

Barde YA, Edgard D, Thoenen H. 1982. Purification of a new neurotrophic factor from mammalian brain. EMBO J 1: 549-553.

Barnabe-Heider F, Miller FD. 2003. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci 23: 5149-5160.

Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, et al. 1991. Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron 7: 857-866.

Bibel M, Hoppe E, Barde YA. 1999. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18: 616-622.

Bocchini V, Angeletti PU. 1969. The nerve growth factor: purification as a 30,000-molecular-weight protein. Proc Natl Acad Sci USA 64: 787-794.

Carter BD, Kaltschmidt C, Kaltschmide B, Offenhäuser N, Böhm-Matthaei R, et al. 1996. Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75. Science 272: 542-545.

Chao MV, Bothwell M. 2002. Neurotrophins: to cleave or not to cleave. Neuron 33: 9-12.

Cohen S. 1959. Purification and metabolic effects of a nerve growth-promoting protein from snake venom. J Biol Chem 234: 1129-1137.

Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, et al. 1995. Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature 375: 235-238.

Cowan WM. 2001. Viktor Hamburger and Rita Levi-Montalcini: the path to the discovery of nerve growth factor. Ann Rev Neurosci 24: 551-600.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, et al. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76: 1001-1011.

Dechant G. 2001. Molecular interactions between neurotrophin receptors. Cell Tissue Res 305: 229-238.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannum YA. 1994. Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 65: 1596-1599.

Ernfors P, Ibez CF, Ebendal T, Olson L, Persson H. 1990. Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. Proc Natl Acad Sci USA 87: 5454-5458.

Ernfors P, Lee KF, Jaenisch R. 1994a. Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature 368: 147-150.

Ernfors P, Lee KF, Kucera J, Jaenisch R. 1994b. Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell 77: 503-512.

Fischer W, Wictorin K, Bjorklund A, Williams LR, Varon S, et al. 1987. Intracerebral infusion of nerve growth factor ameliorates cholinergic neuron atrophy and spatial memory impairments in aged rats. Nature 339: 65-68.

Goldstein LD, Reynolds CP, Perez-Polo JR. 1978. Isolation of human nerve growth factor from placental tissue. Neurochem Res 3: 175-183.

Greene LA, Shooter EM. 1980. Nerve growth factor biochemistry, synthesis and mechanism of action. Annu Rev Neurosci 3: 353-402.

Gu Z, Yu J, Perez-Polo JR. 1988. Long term changes in brain cholinergic markers and NGF levels after partial immunolesion. Brain Res 801: 190-197.

Hallböök F, Ibáñez CF, Persson H. 1991. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron 6: 845-858.

Hamburger V, Oppenheim RW. 1982. Cell death. Neurosci Commun 39-55.

Hamburger V, Brunso-Bechtold JK, Yip JW. 1981. Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J Neurosci 1: 60-71.

Harper GP, Barde YA, Burnstock G, Carstairs J, Dennison ME, et al. 1979. Guinea pig prostate is a rich source of nerve growth factor. Nature 279: 160-162.

Hon A, Liebrock J, Bailey K, Barde Y-A. 1990. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature 344: 339-341.

Hefti F. 1986. Nerve growth factor promotes survival of septal cholinergic neurons after fimbrial transection. J Neurosci 6: 2155-2162.

Hirata H, Hibasami H, Yoshida T, Ogawa M, Matsumoto M, et al. 2001. Nerve growth factor signaling of p75 induces differentiation and ceramide-mediated apoptosis in Schwann cells cultured from degenerating nerves. Glia 36: 245-258.

Ibáñez CF. 1995. Neurotrophic factors: from structure–function studies to designing effective therapeutics. Trends Biotechnol 13: 217-227.

Ip NY, Ibáñez CF, Nye SH, McClain J, Jones PF, et al. 1992. Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA 889: 3060-3064.

Johnson EM. 1978. Destruction of the sympathetic nervous system in neonatal rats and hamsters by vinblastine: prevention by concomitant administration of nerve growth factor. Brain Res 141: 105-118.

Johnson EM, Gorin PM, Brandeis LD, Pearson J. 1980. Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. Science 210: 916-918.

Jones KR, Fariñas I, Backus C, Reichardt LF. 1994. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76: 989-999.

Kaisho Y, Yoshimura K, Nakaham K. 1990. Cloning and expression of a cDNA encoding a novel human neurotrophic factor. FEBS Lett 266: 187-191.

Kalb R. 2005. The protean actions of neurotrophins and their receptors on the life and death of neurons. Trends Neurosci 28: 5-11.

Koliatsos VE, Price DL, Gouras GK, Cayouette MH, Burton LE, et al. 1994. Highly selective effects of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 on intact and injured basal forebrain magnocellular neurons. J Comp Neurol 343: 247-262.

Kromer LF. 1987. Nerve growth factor treatment after brain injury prevents neuronal death. Science 235: 214-216.

Lee R, Kermani P, Teng KK, Hempstead BL. 2001. Regulation of cell survival by secreted proneurotrophins. Science 294: 1945-1948.

Levi-Montalcini L. 1987. The nerve growth factor 35 years later. Science 237: 1154.

Levi-Montalcini R, Angeletti PU. 1966. Second symposium on catecholamines. Modification of sympathetic function. Immunosympathectomy. Pharmacological Reviews 18(1): 619-28.

Levi-Montalcini R, Hamburger V. 1951. Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J Exp Zool 116: 321-361.

Levi-Montalcini R, Hamburger V. 1953. A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. J Exp Zool 123: 233-287.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, et al. 1990. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247: 1446-1451.

McDonald NQ, Chao MV. 1995. Structural determinants of neurotrophin action. J Biol Chem 270: 19669-19672.

Meakin SO, Shooter EM. 1992. The nerve growth factor family of receptors. Trends Neurosci 15: 323-331.

Middlemas DS, Lindberg RA, Hunter T. 1991. trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol 11: 143-153.

Miller FD, Kaplan DR. 2001. On Trk for retrograde signaling. Neuron 32: 767-770.

Miller FD, Kaplan DR. 2002. TRK makes the retrograde. Science 295: 1471-1473.

Naumann T, Casademunt E, Hollerbach E, Hofmann J, Dechant G, et al. 2002. Complete deletion of the neurotrophin receptor $p75^{NTR}$ leads to long-lasting increases in the number of basal forebrain cholinergic neurons. J Neurosci 22: 2409-2418.

Oppenheim RW. 1981. Cell death of motoneurons in the chick embryo spinal cord. V. Evidence on the role of cell death and neuromuscular function in the formation of specific peripheral connections. J Neurosci 1(2): 141-151.

Oppenheim RW. 1991. Cell death during development of the nervous system. Ann Rev Neurosci 14: 453-501.

Oppenheim RW, Maderdrut JL, Wells DJ. 1982. Cell death of motoneurons in the chick embryo spinal cord. VI. Reduction of naturally occurring cell death in the thoracolumbar column of terni by nerve growth factor. J Comp Neurol 210: 174-189.

Perez-Polo JR. 1990. The nerve growth factor receptor. Trophic factors and the Nervous System. L.A. Horrocks, editor. New York: Raven Press; pp. 107-118.

Perez-Polo JR, Bomar H, Beck C, Hall K. 1978. Nerve growth factor from *Crotalus adamenteus* snake venom. J Biol Chem 253: 6140-6148.

Perez-Polo JR, de Jong WW, Strauss D, Shooter EM. 1972. The physical and biological properties of 7S and beta-NGF from the mouse submaxillary gland in functional and structural proteins of the nervous system. Advances in Experiment. Medicine and Biology. Davidson A, editor. New York: Plenum Press; 32:91

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325: 593-597.

Rodriguez-Tebar A, Dechant G, Barde Y-A. 1990. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. Neuron 4: 487-492.

Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, et al. 1990. Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4: 767-773.

Rossner S, Schliebs R, Härtig W, Perez-Polo JR, Bigl V. 1997a. Selective induction of c-Jun and NGF in reactive astrocytes after cholinergic degenerations in rat basal forebrain. Neuroreport 8: 2199-2202.

Rossner S, Schliebs R, Perez-Polo JR, Wiley RG, Bigl V. 1995. Differential changes in cholinergic markers from selected brain regions after specific immunolesion of rat cholinergic basal forebrain system. J Neurosci Res 40: 31-43.

Rossner S, Woertwein G, Gu Z, Yu J, Schliebs R, et al. 1997b. Cholinergic control of nerve growth factor in adult rats: evidence from cortical cholinergic deafferentation and chronic drug treatment. J Neurochem 69: 947-953.

Shooter EM. 2001. Early days of the nerve growth factor proteins. Annu Rev Neurosci 24: 601-629.

Smeyne RJ, Klein R, Schnapp A, Long LK, Brynat S, et al. 1994. Severe sensory and sympathetic neuropathy in mice carrying a disrupted trk/NGF receptor gene. Nature 365: 246-249.

Taglialatela G, Hibbert CJ, Werrbach-Perez K, Perez-Polo JR. 1996. Suppression of $p140^{trkA}$ does not abolish nerve growth factor rescue of apoptotic PC12 cells and enables brain-derived neurotrophic factor to promote cell survival. J Neurochem 66: 1826-1835.

Taglialatela G, Kauffman JA, Trevino A, Perez-Polo JR. 1998. Central nervous system DNA fragmentation induced by the inhibition of nuclear factor kappa B. Neuroreport 9: 489-493.

Taglialatela G, Robinson R, Perez-Polo JR. 1997. Inhibition of nuclear factor kappa B (NF-κB) activity induces NGF-resistant apoptosis in PC12 cells. J Neurosci Res 47: 155-162.

Thoenen H, Barde YA. 1980. Physiology of nerve growth factor. Physiol Rev 60: 1284-1335.

Thomas KA, Silverman RE, Jeng I, Baglan NC, Bradshaw RA. 1981a. Electrophoretic heterogeneity and polypeptide chain structure of the γ-subunit of mouse submaxillary 7S nerve growth factor. J Biol Chem 256c: 9147-9153.

Thomas KA, Baglan NC, Bradshaw RA. 1981b. The amino acid sequence of the γ-subunit of mouse submaxillary gland 7S nerve growth factor. J Biol Chem 256: 9156-9166.

Varon S, Nomura J, Shooter EM. 1967. The isolation of the mouse growth factor protein in a high molecular weight form. Biochemistry 6: 2202-2209.

Walker P, Weichsel ME Jr, Fische DA. 1980. Human nerve growth factor: lack of immunoreactivity with mouse nerve growth factor. Life Sci 26: 195-200.

Wiesmann C, Vos AM. 2001. Nerve growth factor: structure and function. Cell Mol Life Sci 58: 748-759.

Williams LR, Varon S, Peterson GM, Wictorin K, Fischer W, et al. 1986. Continuous infusion of nerve growth factor prevents forebrain neuronal death after fimbrial–fornix transection. Proc Natl Acad Sci USA 83: 9231-9235.

Yu J, Pizzo DP, Hutton LA, Perez-Polo JR. 1995. Role of the cholinergic system in the regulation of neurotrophin synthesis. Brain Res 705: 247-254.

Yu J, Wiley RG, Perez-Polo JR. 1996. Altered NGF protein levels in different brain areas after immunolesion. J Neurosci Res 43: 213-223.

Zampieri N, Xu C-F, Neubert TA, Chao MV. 2005. Cleavage of p75 neurotrophin receptor by α-secretase and γ-secretase requires specific receptor domains. J Biol Chem 280: 14563-14571.

# 2 Signaling Through the Neurotrophin Receptors

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**Abstract:** Neurotrophins are a family of growth factors critical for the development and functioning of the nervous system. Members of the neurotrophin family initiate downstream signaling via two types of neurotrophin receptors: Trks (TrkA, TrkB, and TrkC) and p75. This chapter reviews what is known about the structures and functions of Trk and p75: from ligand specificity and binding, initiation and regulation of intracellular signaling cascades, and crosstalks with other signaling pathways to the biological responses in the nervous system.

**List of Abbreviations:** A2A, adenosine A2A receptor; AMPA, alpha-amino-5-methyl isoxazole-4-propionate; ARMS, ankyrin repeat-rich membrane spanning protein; Bad, Bcl2 antagonist of cell death; BDNF, brain-derived neurotrophic factor; CaM kinase, calcium/calmodulin-dependent protein kinase; Cdc42, cell division cycle 42; CHK, Csk homologous kinase; CIPA, congenital insensitivity to pain and anhidrosis; CNS, central nervous system; CNTF, ciliary neurotrophic factor; CREB, cAMP response element binding protein; DAG, diacylglycerol; DRG, dorsal root ganglion; Egr-1, early growth response-1; ERK, extracellular signal-regulated kinases; FADD, Fas-associated death domain; Frs2, fibroblast growth factor receptor substrate 2; Gab, Grb2-associated binder-1; GPCR, G-protein-coupled receptor; Grb2, growth factor receptor-binding protein-2; GRK2, G-protein-coupled receptor kinase 2; GSK3β, glycogen synthase kinase 3-β; Ig domain, immunoglobulin-like domain; IκB, inhibitor of kappa-B; IL-6, interleukin-6; IP3, inositol 1,4,5-trisphosphate; JNK, Jun N-terminal kinase; LIF, leukemia inhibitory factor; LINGO-1, LRR- and Ig-domain-containing Nogo receptor interacting protein; LTP, long-term potentiation; MAG, myelin-associated glycoprotein; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor-2; MEK, MAPK/ERK kinase; MKP-1, mitogen-activated protein kinase phosphatase 1; NADE, $p75^{NTR}$-associated cell death executor; $Na_v1.9$, voltage-gated sodium channel 1.9; NF-κB, nuclear factor kappa-B; NGF, nerve growth factor; Ngr, Nogo-66 receptor; NMDA, *N*-methyl-D-aspartate; NRAGE, neurotrophin receptor interacting MAGE homolog; NRIF, neurotrophin receptor interacting factor; NT, neurotrophin; OMgp, oligodendrocyte–myelin glycoprotein; PAC1, PACAP-preferring; PACAP, pituitary adenylate cyclase-activating polypeptide; PDK-1, phosphoinositide-dependent protein kinase; PI3K, phosphatidylinositol-3 kinase; PKC, protein kinase C; PLC-γ1, phospholipase C-gamma 1; PNS, peripheral nervous system; PP1, Src kinase inhibitor; PTB, phosphotyrosine binding; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RhoGDI, RhoGDP dissociation inhibitor; RIP-2, receptor interacting protein-2; RT-PCR, reverse transcriptase-polymerase chain reaction ; S1P, sphingosine-1-phosphate; SCG, superior cervical ganglion; SH2, Src-homology 2; Shc, SH2-containing collagen-related proteins; SHP-1, SH2-containing phosphatase 1; SOS, son of sevenless; SphK1, sphingosine kinase type 1; STAT3, signal transducer and activator of transcription 3; TNF, tumor necrosis receptor; TRADD, tumor necrosis factor receptor 1-associated death domain protein; TRAF6, TNF-receptor-associated factor 6; Trk, tropomyosin-related kinase; TRPC3, transient receptor potential cation channel 3; VR1, vanilloid receptor 1

## 1 Introduction

Discovery of the family of neurotrophic factors known as the neurotrophins represents one of the major steps in understanding how neurons develop and differentiate to acquire their unique function and morphology. Since the identification of the first neurotrophin nerve growth factor (NGF) in 1953, much of the research efforts have focused on understanding the downstream signaling events and the biological effects of these proteins. Neurotrophins are a family of neurotrophic factors implicated as key players in regulating neuronal survival and development. To date, several neurotrophins have been identified including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT4/5) (Ip et al., 1992; Ip and Yancopoulos, 1994). Neurotrophin-6/7 (NT-6/7) is a recent addition to the neurotrophin family isolated in lower vertebrates (Götz et al., 1994; Lai et al., 1998).

## 2 Discovery of the Neurotrophin Receptors

For a long period, characterization of the neurotrophin receptors presented a formidable, albeit urgent, task. Given that neurotrophins are factors that may utilize cell surface receptors for signal transduction, initial studies aimed to characterize the binding characteristics of NGF receptors using $^{125}$I-NGF in kinetics studies (Sutter et al., 1979; Landreth and Shooter, 1980). Back in the early 1980s, it had already been demonstrated that neurons exhibit two saturable binding sites for NGF, thereby prompting the idea that there exist two populations of NGF receptors, namely the high-affinity and the low-affinity receptors (Sutter et al., 1979; Landreth and Shooter, 1980).

The first NGF receptor, known as the p75 low-affinity NGF receptor, was identified and sequenced in 1986 (Chao et al., 1986; Johnson et al., 1986; Radeke et al., 1987). Nonetheless, p75 was later found to display no intrinsic kinase activity, which is in contrast to a previous observation showing that NGF treatment induces rapid tyrosine kinase activation in PC12 cells (Maher, 1988; Kaplan et al., 1991b). In addition, NGF-mediated induction of c-fos transcription was shown to depend upon binding to the high-affinity site ($K_d \approx 10^{-11}$ M). p75, on the other hand, only associates with NGFs with low affinity ($K_d \approx 10^{-9}$ M) (Hempstead et al., 1989). These observations collectively suggest that the biological responsiveness to NGF was mediated by other NGF receptors.

The continued quest was rewarded by the isolation of Trk in 1989, also known as gp140$^{\text{proto-trk}}$ or gp140$^{\text{TrkA}}$. Trk, the founding member of the Trk family of receptor tyrosine kinase, is now referred to as TrkA to distinguish it from the other members of the family, namely TrkB and TrkC. Trk was serendipitously identified during a search for transforming genes in human colon cancer in 1986 (Martin-Zanca et al., 1986). The isolated oncogene *trk* was named tropomyosin-related kinase for bearing a stretch of a nonmuscle tropomyosin sequence after somatic rearrangement. The presence of a tyrosine kinase domain in the *trk* oncogene indicates that the *trk* protooncogene may code for a novel member of tyrosine kinase. Determination of the sequence for the trk protooncogence in 1989 revealed that it encodes a receptor tyrosine kinase (Martin-Zanca et al., 1989), therefore suggesting that it may serve as a trophic factor receptor.

Soon after the characterization of the *trk* protooncogene, TrkA was found to serve as a transducing receptor for NGF in the total absence of p75 (Kaplan et al., 1991a; Klein et al., 1991a; Squinto et al., 1991). TrkA-mediated induction of downstream signaling such as PLC$\gamma$ phosphorylation strongly suggests that TrkA is the cognate receptor for NGF. This observation was corroborated by the findings that TrkB and TrkC, members of the same family, were identified as cognate receptors for other neurotrophins including BDNF and NT-3, respectively (Glass et al., 1991; Klein et al., 1991b).

In this chapter we describe in detail the subtypes of neurotrophin receptors, the cascades of signaling events initiated following neurotrophin receptor activation, and the functional significance of neurotrophin receptor signaling.

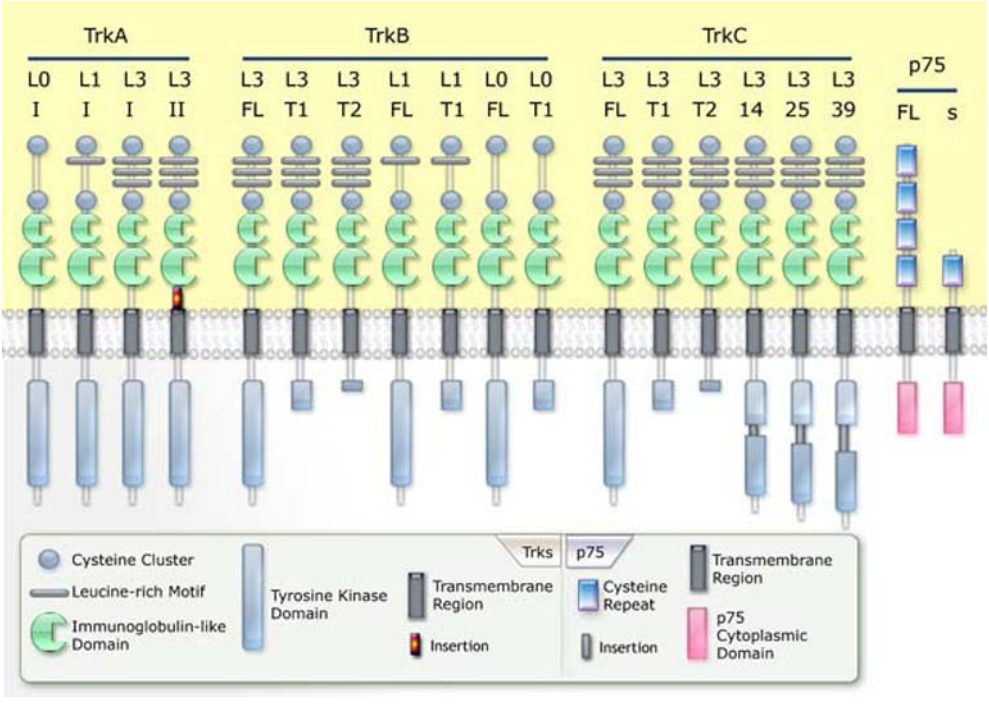
## 3 The Neurotrophin Receptors

### 3.1 Structure of Trks

Although TrkA, TrkB, and TrkC were transcribed from separate loci, they share high degree of sequence homology and are similar structurally. Congruent with other receptor tyrosine kinases, Trks comprise an extracellular ligand-binding region, a single transmembrane region, and a cytosolic region containing a tyrosine kinase domain. The extracellular domain is crucial for ligand recognition, ligand binding, and receptor dimerization. It is characterized by the presence of two cysteine-rich domains (domains 1 and 3), which sandwich three tandem leucine-rich motifs (domain 2), followed by two C2-type immunoglobulin-like (Ig) domains (domains 4 and 5) (Schneider and Schweiger, 1991). Among these domains, the C2-type immunoglobulin-like domain proximal to the membrane (domain 5) has been shown to mediate ligand binding (❯ *Figure 2-1*; Wiesmann et al., 1999; Naylor et al., 2002).

**Figure 2-1**

**Isoforms of Trk family receptors. TrkA isoforms have identical intracellular region, but different extracellular region. Three of them contain either three (L3I), one (L1I), or no (L0I) leucine-rich motif. Another TrkA isoform with three leucine-rich motifs is characterized by an insertion between the second immunoglobulin-like domain (Ig2) and the transmembrane region (L3II). TrkB isoforms with three leucine-rich motifs include TrkB-FL (L3FL), T1 (L3T1), and T2 (L3T2). TrkB-FL contains an intracellular tyrosine kinase domain, but Trk-T1 and T2 only have a short protein sequence of 11 and 9 amino acids, respectively. Isoforms with one (L1FL and L1T1) or no (L0FL and L0T1) leucine rich motifs are also found for TrkB-FL and T1. All TrkC isoforms identified contain three leucine rich motifs. TrkC-FL (L3FL) contains a cytosolic kinase domain, but T1 (L3T1) and T2 (L3T2) do not contain a kinase domain, which is replaced by a short amino acid sequence. Also, isoforms of TrkC with various amino acids insert (14, 25, and 39; denoted as L314, L325, and L339) in the kinase domain are also identified. For p75, one isoform (s-p75$^{NTR}$) characterized by the deletion of three of the four cysteine repeats has been identified**




In addition to the five domains described above, the extracellular region of Trk also contains consensus sites for N-glycosylation. TrkA, for example, contains 13 of these sites (Martin-Zanca et al., 1989). Inhibition of glycosylation was shown to prevent the localization of Trks to the membrane surface and induce constitutive activation of the Trk tyrosine kinase in the absence of ligand binding (Watson et al., 1999; Mutoh, 2000). These studies suggest that in addition to engaging in ligand binding, modulation in the extracellular domain may also play an important regulatory role in receptor function.

Adjacent to the extracellular domain is the hydrophobic transmembrane domain, which serves as an anchorage for the receptor to the plasma membrane. The intracellular region, on the other hand, comprises a juxtamembrane region, a tyrosine kinase domain followed by a short carboxy-terminal tail. The tyrosine kinase domain is indispensable for signal initiation and propagation. Tyrosine residues located in the intracellular region are autophosphorylated upon ligand binding, which serves as crucial docking sites for downstream signaling components (reviewed in Ip and Yancopoulos, 1994; Segal, 2003). The signaling cascades will be discussed in more detail later in the chapter.

## 3.2 Expression Profiles and Isoforms of Trks

### 3.2.1 TrkA

*trkA* encodes a 140-kDa glycoprotein ($gp140^{trkA}$), which can be found in the central nervous system (CNS), the peripheral nervous system (PNS) and the immune system. TrkA receptors are expressed in the cholinergic neurons of the basal forebrain and several subtypes of ganglia including the sensory cranial and spinal ganglia, the sympathetic ganglia, the dorsal root ganglia (DRGs), and the retinal ganglion cells (Martin-Zanca et al., 1990; Holtzman et al., 1992; Carroll et al., 1992; Schecterson and Bothwell ,1992; Mu et al., 1993; Zanellato et al., 1993).

Four splicing variants of TrkA exhibiting distinctive extracellular and intracellular regions were previously found in rat pheochromocytoma PC12 cells and thymus. Two TrkA isoforms were found in PC12 cells, the first one being the original TrkA found by Martin-Zanca's group in 1989 and a second isoform containing a six-amino-acid insertion between the Ig2 domain and the transmembrane region. To classify these receptors, the original TrkA (without the insertion) is designated as TrkAI and the isoform (with the insertion) as TrkAII (Barker et al., 1993). Functional studies showed that the two isoforms display no significant differences in ligand binding or NGF-induced signaling transduction in fibroblast cells. However, NT-3 elicits a higher activation response compared with NGF in TrkAII-overexpressing PC12 cells, in contrast to the usual preference for NGF observed in TrkAI isoform (Clary and Reichardt, 1994). This suggests that the insertion in the extracellular domain may modulate ligand selectivity. Expression profiles of these two isoforms are also nonoverlapping in vivo, with TrkAI expressed mainly in nonneuronal tissues and TrkAII expressed predominantly in neuronal tissue.

The other two TrkA isoforms were identified in the thymus. They are distinguished from the original TrkAI by the presence of various leucine-rich region truncations. The original TrkAI contains three leucine-rich regions and is therefore also referred to as TrkAL3. The other two isoforms are referred to as TrkAL0 and TrkAL1 for containing either none or only one of the leucine-rich motifs. These isoforms are expressed almost exclusively in the thymus, with their functional significance not fully understood at this stage. Nonetheless, the lack of a leucine-rich region may participate in regulate the ligand specificity of these two isoforms (Dubus et al., 2000). Please refer to ❯ *Figure 2-1* for a complete list of the TrkA isoforms.

### 3.2.2 TrkB

In 1989, Klein et al. identified *trkB* complementary DNA (cDNA) by screening a mouse brain cDNA library using a probe that encodes human *trk* tyrosine kinase. The function of TrkB, nonetheless, remained unclear until the identification of TrkB as a receptor for the neurotrophins in 1991 (Klein et al., 1991b; Squinto et al., 1991). *trkB* encodes a 145-kDa glycoprotein ($gp145^{trkB}$), which exhibits differential spatial and temporal expression profiles during development (Klein et al., 1989). *trkB* transcripts are detected most abundantly in the nervous system, and to a lesser extent, in the lung, muscle, and ovaries. In situ hybridization analysis and immunostaining showed that TrkB is widely expressed in the brain including the cerebral cortex, hippocampus, dentate gyrus, striatum, brainstem, spinal cord, as well as the spinal and cranial ganglia, paravertebral trunk of the sympathetic nervous system, and various innervation pathways. During development, both the transcripts and proteins of TrkB are detected from embryonic to adult stages (Fryer et al., 1996; Yan et al., 1997). In early embryogenesis, *trkB* is also expressed in neuroepithelium and neural crest cells (Klein et al., 1990).

To date, seven truncated isoforms of *trkB* have been identified. Two receptors without cytoplasmic kinase domain were screened from adult rat cerebellar cDNA library and were named TrkB-T1 and TrkB-T2. Compared with full-length TrkB (TrkB-FL), they have the same extracellular domain and transmembrane region, but the intracellular domain is replaced with an unidentical short C-terminal sequence (Middlemas et al., 1991). Various isoforms of TrkB receptors are widely and abundantly expressed in the nervous system, but the localization varies for each isoform. Within the nervous system, TrkB-FL is expressed ubiquitously

in neuronal cells, but is absent in nonneuronal cells. TrkB-T1 can be found in both neuronal and nonneuronal tissues such as astrocytes, oligodendrocytes, and Schwann cells, whereas TrkB-T2 is mainly expressed in the neuronal region, overlapping with that of TrkB-FL (Armanini et al., 1995). Expression of various TrkB isoforms also changes during development, with TrkB-FL proteins initially detected in the embryonic stages and truncated TrkB predominates in late postnatal and adult stages (Fryer et al., 1996).

Like TrkA isoforms, TrkB isoforms may also differ by having only one (TrkBL1) or complete absence (TrkBL0) of leucine-rich regions in the extracellular domain. Reverse transcriptase-polymerase chain reaction (RT-PCR) study demonstrated that both TrkB-FL and TrkB-T1, but not TrkB-T2, can encode isoforms bearing three (L3), one (L1), or no leucine-rich motif (L0) in extracellular domains (❯ *Figure 2-1*). The L1 and L0 variants do not bind to TrkB ligands including BDNF, NT-3, and NT-4, and cannot maintain survival and morphological transformation in fibroblasts (Ninkina et al., 1997).

### 3.2.3 TrkC

Another member was added to the Trk family when Lamballe et al. (1991) isolated *trkC* by screening an adult porcine brain cDNA library with a catalytic domain of the human *trk* DNA sequence in 1991. Like *trkA* and *trkB*, *trkC* encodes a glycoprotein of about 145 kDa ($gp145^{trkC}$), which is preferentially expressed in the brain, particularly in the hippocampus, cerebral cortex, the granular cell layer of the cerebellum, spinal cord motorneurons (Lamballe et al., 1991; Merlio et al., 1992), and various neural crest and ganglia (Tessarollo et al., 1993).

A total of six isoforms with truncation or insertion in the intracellular domain have been identified for TrkC (❯ *Figure 2-1*). Like TrkB, two TrkC isoforms exhibit truncated kinase domain (TrkC-T1 and TrkC-T2), which is replaced by a short C-terminal sequence. Three other isoforms are characterized by different lengths of insertions (TrkC-14, TrkC-25, and TrkC-39) in the intracellular domain. These insertions interfere with the major autophosphorylation site of the kinase domain of these receptors (Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993). This interruption may underlie the observed failure of these isoforms to mediate proliferation in fibroblasts or neuronal differentiation in PC12 cells, despite induction of rapid phosphorylation of the tyrosine residues upon ligand binding. Expression of TrkC isoforms remained concentrated in the nervous system. Interestingly, only the truncation isoforms are expressed in astrocytes, peripheral nerve, and nonneural tissues (Lamballe et al., 1993; Valenzuela et al., 1993; Guiton et al., 1995).

## 3.3 Structure and Expression Profile of p75

p75, although not a member of the Trk family receptors, also binds to neurotrophins and functions as an indispensable component of neurotrophin signaling. The structure and biological functions of p75 are quite dissimilar compared with Trks. p75 belongs to the tumor necrosis factor (TNF) family receptors and was first identified as an NGF receptor in 1986 (Chao et al., 1986; Johnson et al., 1986). It encodes a 75-kDa glycoprotein, which can form homodimers or heterodimers with Trk receptor in vivo. The nomenclature of p75 has been revised to low-affinity NGF receptor, $p75^{NTR}$, because it was found to associate with NGF via low-affinity binding (Sutter et al., 1979; Johnson et al., 1986; Radeke et al., 1987). Being a member of the TNF family, it is structurally similar to other TNF members, consisting of four cysteine-rich domains in the extracellular region, a transmembrane domain, and an internal death domain lacking any kinase domain (Johnson et al., 1986). Within the internal death domain, the intracellular juxtamembrane domain is also called the Chopper domain for its ability to initiate cell death in DRG cells (Barrett and Barlett, 1994). The cysteine-rich domains in the extracellular region are essential for binding to the neurotrophins, and the cytoplasmic region is implicated in the activation of nuclear factor kappa-B (NF-κB) and triggering of apoptosis (Liepinsh et al., 1997).

The expression of p75 is developmentally regulated. During development, it is highly expressed during embryonic and postnatal stages, with reduced expression in the adult stage. p75 is expressed abundantly in various neuronal populations, including the striatal neurons and spinal cord motor neuron, as well as in the sympathetic and sensory ganglia. Interestingly, expression of p75 was found to partially overlap with that of Trk, correlating with its ability to form heterodimers with Trks. For example, abundant levels of p75 were found in basal forebrain where TrkA is also highly expressed (Buck et al., 1987; Ernfors et al., 1988, 1989; Cohen-Cory et al., 1989; Gibbs et al., 1989; Lu et al., 1989; Mobley et al., 1989; Friedman et al., 1991; Liepinsh et al., 1997).

For a long time it was believed that the *p75* locus expresses only a single, full-length receptor. Recently, it was demonstrated that there exists a truncated isoform of p75, named s-p75$^{NTR}$, characterized by the absence of three of the four cysteine-rich repeats in the extracellular domain (*Figure 2-1*; von Schack et al., 2001). Like p75, s-p75$^{NTR}$ is expressed in the brain and spinal cord, although the expression levels are much reduced compared with that of full-length p75. Deletion of the cysteine-rich repeats renders s-p75$^{NTR}$ unable to bind NGF. Nonetheless, both full-length p75 and s-p75$^{NTR}$-null mice display neuronal loss in sensory neurons and Schwann cells, therefore suggesting that both isoforms are associated with maintaining neuronal survival (von Schack et al., 2001).

# 4 Neurotrophin Receptor Signaling

## 4.1 Neurotrophins and Their Receptors

Despite the structural similarity of TrkA, TrkB, and TrkC, they display rather remarkable selectivity for their ligands. TrkA serves as the cognate receptor for NGF, although it also associates with NT-3 with low affinity (Kaplan et al., 1991a; Klein et al., 1991a; Clary and Reichardt, 1994). In addition, NT-6/7 has also been shown to utilize TrkA for signal transduction (Lai et al., 1998; Nilsson et al., 1998). TrkB, on the other hand, serves as a cognate receptor for BDNF and NT4/5. NT-3 again interacts weakly with TrkB (Berkemeier et al., 1991; Klein et al., 1991b, 1992; Soppet et al., 1991; Squinto et al., 1991; Lai et al., 1996). TrkC interacts selectively with NT-3 (Cordon-Cardo et al., 1991; Lamballe et al., 1991). Finally, all neurotrophins interact with p75, a low-affinity NGF receptor (Barbacid, 1995; Bothwell, 1995; Lewin and Barde, 1996; Huang and Reichardt, 2003). The ligand selectivity for the Trks and p75 is summarized in *Figure 2-2*.

## 4.2 Ligand Binding

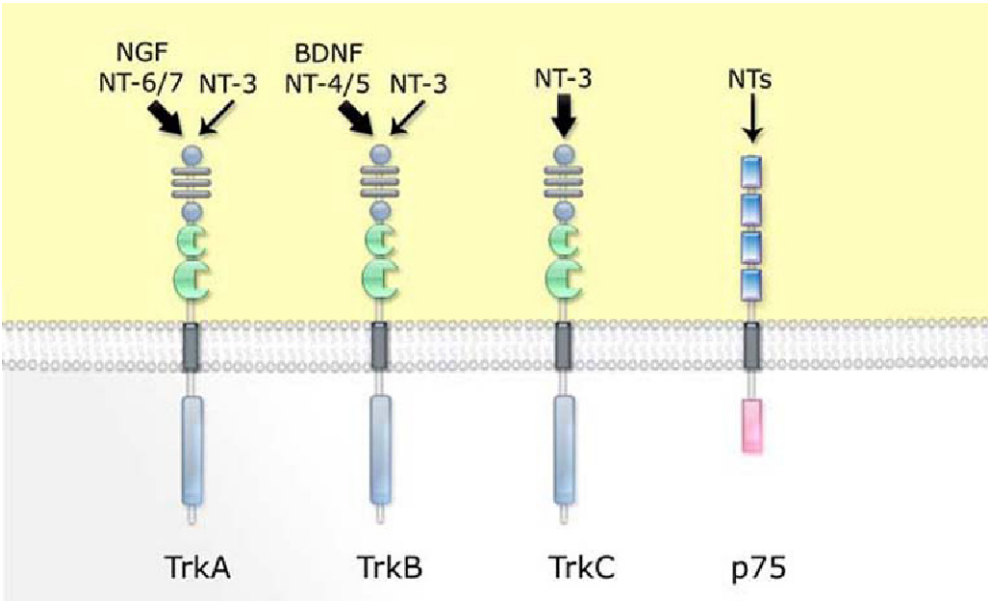
### 4.2.1 Ligand Selectivity and Ligand Binding for Trks

To understand how Trk receptors interact selectively with their cognate ligands, the extracellular region of Trk has been extensively examined. In a series of domain swapping and chimeric receptor studies, the two C2-type Immunoglobuline-like domains of TrkA were shown to be essential for the binding of NGF (Perez et al., 1995). The Ig2 (domain 5) of TrkB and TrkC can also regulate the specificity and binding of the ligands (Urfer et al., 1995). Furthermore, determination of the 3D structure of the TrkA–NGF complex consolidated the role of TrkA domain 5 in neurotrophin binding (Ultsch et al., 1999; Wiesmann et al., 1999). Binding of NGF to TrkA induces TrkA dimerization, with NGF–TrkA complex existing as a symmetric structure in a 2:2 stoichiometric ratio (Wiesmann et al., 1999).

In addition to domain 5, other modification in the extracellular domain has also been shown to affect receptor activation. Mutation of proline residue 203 of TrkA, situated between the leucine-rich regions and the Ig1 domain of Trk, to alanine enhances receptor dimerization, and triggers the autophosphorylation of the tyrosine residues on the receptors in the absence of ligand binding (Arevalo et al., 2001). This observation suggests that this proline residue may take part in modulating receptor activation subsequent to ligand binding.

**Figure 2-2**

**Ligand specificities of Trk family receptors. Neurotrophins bind to specific Trk receptors. NGF and NT-6/7 interact with TrkA whereas BDNF and NT415 interact with TrkB with high affinity (thick arrow). NT-3 binds strongly to TrkC, but also interacts with TrkA and B with lower affinity (*thin arrow*). Most neurotrophins (NTs) bind to p75 with low affinity**




### 4.2.2 Ligand Binding for p75

p75 receptor, in contrast to Trks, interacts with all neurotrophins. Recent evidence suggests that whereas all neurotrophins bind to p75, they associate with p75 with different affinities. BDNF was found to associate and dissociate from p75 at a much slower rate compared with NGF and NT-3 (Rodriguez-Tebar et al., 1992). In addition, although all neurotrophins bind to p75 via a cluster of positively charged residues on the neurotrophins, the residues involved in the association are different for the different neurotrophins (Ryden et al., 1995; Ryden and Ibanez, 1996). A recent study characterizing the crystal structure of NGF complexed with p75 revealed that the cysteine-rich domain of p75 forms an asymmetric complex with NGF in a 1:2 stoichiometric ratio (He and Garcia, 2004). This is in contrast to the dimerization of TrkA observed following association with NGF. Trk dimerization has been shown to play a crucial role in initiating the downstream signaling cascade. It is therefore rather surprising to observe that the binding of NGF to p75 allosterically distorts the conformation of the NGF homodimers, thereby preventing association with a second p75. Nonetheless, this conformational change opens up a potential interaction site for other receptor components. Thus making the physical interaction between Trk and p75 possible (He and Garcia, 2004; Zampieri and Chao, 2004).

Recently, Lee et al. (2001) have proposed that the pro-forms of neurotrophins show a higher affinity to p75 than the mature neurotrophins, suggesting that proneurotrophin may be the primary ligand for p75. However, instead of promoting trophic support, these pro-neurotrophins induce p75-dependent apoptosis in sympathetic neurons (Lee et al., 2001) and oligodendrocytes (Beattie et al., 2002; Lu, 2003). The relative importance of pro-neurotrophins and neurotrophins in mediating p75 signaling will remain an interesting area for further investigation.

## 4.3 Signaling Cascades Downstream of Trk Activation

What happen to the Trk receptors after their cognate ligands come into contact with the extracellular domains of the receptors? Like other receptor tyrosine kinases, Trk receptors dimerize in response to ligand binding (Jing et al., 1992). This dimerization triggers the transactivation of the tyrosine kinase domain, and initiates a massive cascade of signaling events. Some of these signaling pathways have been shown to mediate crucial functions of Trk signaling, while for some the mechanism of activation and functional importance are just beginning to be understood. In the following section, we briefly summarize the myriad of molecules recruited to propagate and diversify Trk signaling following ligand binding and receptor activation.

Immediately following transactivation of the tyrosine kinase domain of the Trk receptor, several tyrosine residues present in the cytoplasmic domains of the receptors become phosphorylated. In the case of TrkA, NGF binding induces phosphorylation of Y490, Y670, Y674, Y675, and Y785 (Ip and Yancopoulos, 1994; Segal, 2003). Tyrosine residues present in TrkB and TrkC at equivalent positions are also phosphorylated upon ligand binding. For simplicity, we will adopt the nomenclature for the TrkA tyrosines in the following description.

Among the five tyrosines, Y670, Y674, and Y675 are in the activation loop of the kinase domain. Y490 and Y785, on the other hand, are located outside the kinase domain (Ip and Yancopoulos, 1994; Segal, 2003). The phosphorylation of these residues is required not only for the full transactivation of the Trk receptors, but also for serving as crucial docking sites for adaptor proteins indispensable for the initiation of Trk downstream signaling. Association between the activated Trk receptor and the adaptor proteins occur mostly via the phosphotyrosine-binding (PTB) or src-homology 2 (SH2) domain of the adaptor proteins. Upon association, these adaptor proteins are usually phosphorylated by Trk itself, which then triggers several intracellular signaling pathways including the Ras-MAPK, phosphatidylinositol-3 kinase (PI3-kinase), and PLC-$\gamma$ pathways (Stephens et al., 1994).

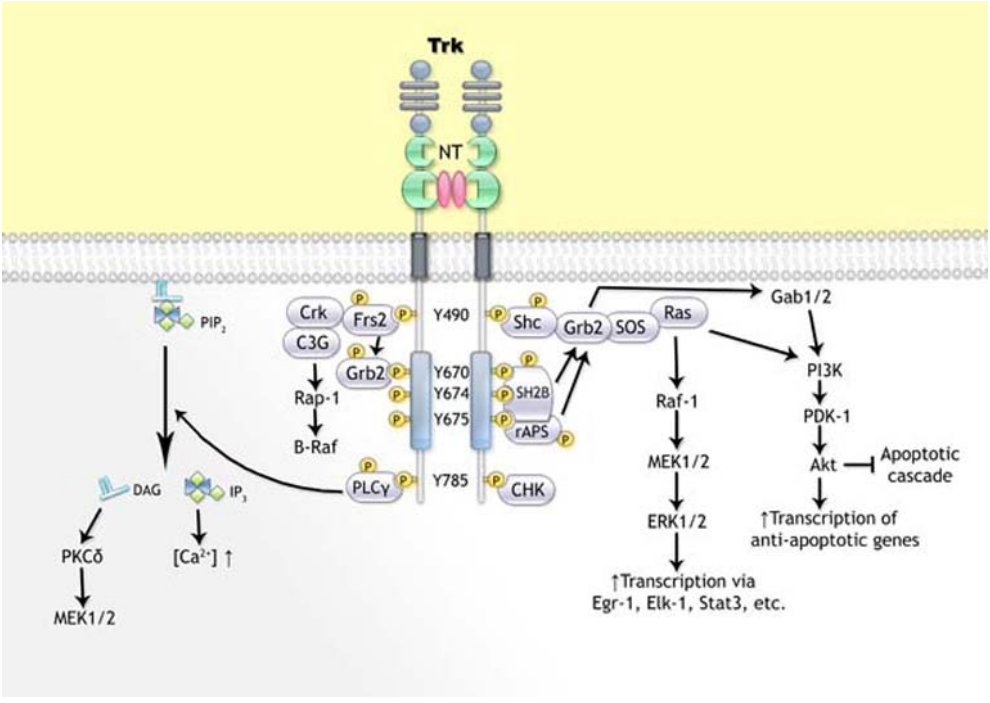
### 4.3.1 Ras-MAPK Signaling

Among these signaling pathways, activation of the Ras-MAPK pathway involves the most complex network of adaptor proteins and signaling molecules, with multiple converging and diverging points within the signaling cascade. All pathways eventually converge on the activation of small GTPase Ras, which then stimulates the three-tiered activation of the MAPK pathways. Upon Trk activation, Ras activation is accomplished by the recruitment of SH2-containing collagen-related proteins (Shc) to phospho-Y490 via the PTB domain of Shc. The bound Shc is then phosphorylated by Trk, which prompts its association with growth factor receptor-binding protein-2 (Grb2) and (son of sevenless (SOS); see ❯ *Figure 2-3*). Recruitment of SOS, an exchange factor for Ras, results in Ras activation (Atwal et al., 2000). It should be noted, however, that in addition to associating with the activated Trk receptor by binding to phosphorylated Shc, Grb2 can also bind directly to activated Trk (❯ *Figure 2-3*).

Alternatively, Ras activation is achieved by interaction with the SH2 domain of SH2-B or rAPS, or via the PTB domain of fibroblast growth factor receptor substrate 2 (Frs2; also known as SNT); (also known as SNT). Both SH2B and rAPS interact with the phosphotyrosines in the activation loop of the activated Trks (Qian et al., 1998; Qian and Ginty, 2001). Frs2, on the other hand, associates with Y490 of activated Trk. All three adaptor proteins are then phosphorylated upon their association with activated Trk, and each can recruit Grb2 to initiate Ras activation via the Grb2–SOS complex (Qian et al., 1998; Meakin et al., 1999). Activation of Ras results in the sequential activation of the MAPK superfamily, the MAPK kinase kinase Raf, MAPK/ERK kinase (MEK), and MAPK extracellular signal-regulated kinase (ERK). Indeed, following Trk activation, Ras induces the phosphorylation and activation of MAPKKK Raf-1 and B-Raf (Lange-Carter et al., 1993; Vaillancourt et al., 1994). Both Raf-1 and B-Raf then trigger the activation of the MAPK kinase MEK1/2, followed by activation of the MAP kinase ERK1/2. The activated ERK1/2 then translocates to the nucleus to activate several transcription factors including early growth response-1

**Figure 2-3**

**Schematic diagram of neurotrophin-induced Trk signaling pathways. After binding of the neurotrophin, dimerization of two Trk receptors triggers the activation of the kinase domain, thereby resulting in *trans*-phosphorylation of tyrosine residues on the neighboring receptor. Positional information for the tyrosine residues listed in the figure corresponds to the tyrosines phosphorylated in activated TrkA. The phosphotyrosines then provide docking sites for various adaptor proteins or signaling molecules including Shc, PLCγ-1, Frs2, SH2B, rAPS, and CHK to activate MAPK, PI3K, and PLCγ signaling pathways. Please refer to Sections 4.4 for detail description of the pathways**

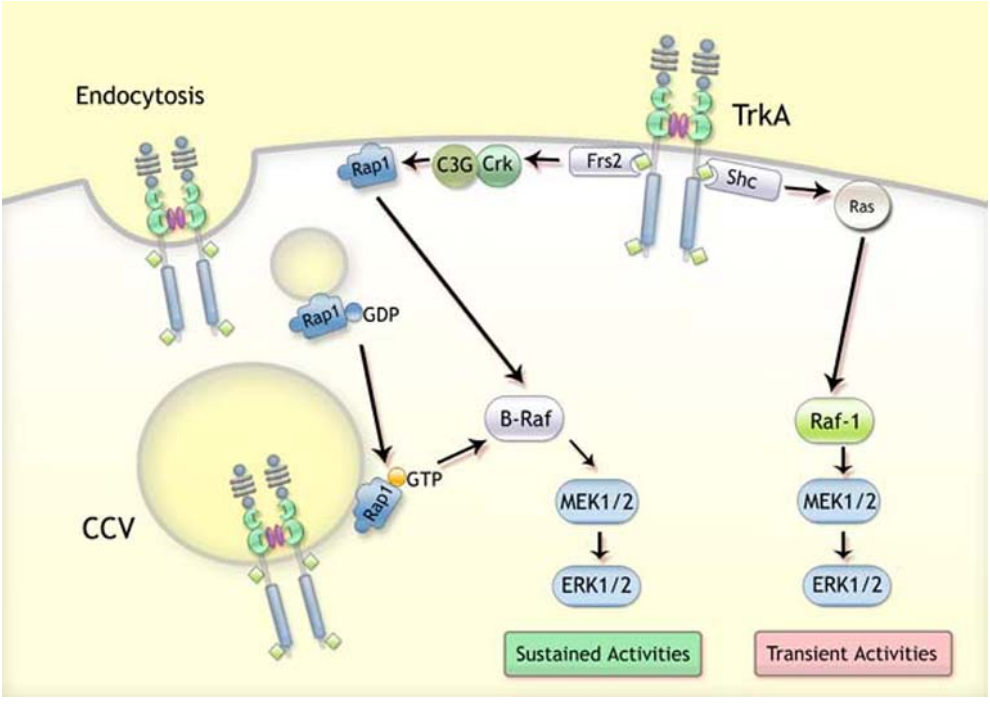



(Egr-1) and Elk-1 (Marais and Marshall, 1996; Harada et al., 2001; Levkovitz and Baraban, 2002). Activation of Egr-1, for example, induces the transcription of cyclin-dependent kinase 5 (Cdk5) activator p35 to facilitate neurite outgrowth in PC12 cells (Harada et al., 2001). In addition, ERK activation was also shown to be required for NGF-induced STAT3 phosphorylation at serine727 in PC12 cells (Ng and Ip, manuscript submitted). NGF-induced activation of ERK hence takes part in regulating a myriad of transcription factors crucial for mediating NGF-stimulated differentiation and neurite outgrowth.

It should be noted that NGF can also induce ERK activation independently of Ras activation. Phosphorylation of Frs2 following association with Y490 of activated TrkA activates the formation of the Crk–C3G complex. It then induces the activation of ERK via another small GTPase Rap1 (◉ *Figure 2-4*; York et al., 1998; Kao et al., 2001). Remarkably, activation of ERK via Ras and Rap1 represents distinctive phases of ERK activation. A recent paper showed that the sustained activation of ERK induced by NGF can actually be deciphered as two phases: the initial rapid or transient phase, followed by a sustained phase of ERK activation (York et al., 1998). The use of dominant-negative Ras demonstrated that the two phases are mediated by distinct signaling pathways. The initial transient phase is mediated by Ras signaling, while the sustained activation of ERK1/2 occurs through Rap1/B-Raf/MEK (Kao et al., 2001). Furthermore, it was recently shown that Rap1 activation following NGF treatment is localized to endosomes and requires TrkA internalization (◉ *Figure 2-4*; York et al., 2000; Wu et al., 2001). These observations suggest that endocytosis may also play a key role in the activation of Trk signaling.

**Figure 2-4**

**Rapid and sustained activation of ERK by Trk. NGF-induced sustained and rapid activation of ERK occur by distinct signaling cascade downstream of TrkA activation. NGF-induced TrkA phosphorylation at the plasma membrane activates rapid phosphorylation of ERK1/2 through Ras and B-Raf/MEK pathway. Sustained activation of ERK1/2, on the other hand, is activated when TrkA associates with Frs2, resulting in activation of Rap-1 via the C3C/Crk complex. Alternatively, NGF induces the formation of clathrin-coated vesicles (CCV) and induces activation of Rap1 at the endosomal vesicles during endocytosis. The activated Rap1 then triggers the sustained activation of ERK1/2**




Finally, NGF treatment also activates other MAPK signaling such as p38 MAPK and Jun N-terminal kinase (JNK), although the mechanisms of activation remain unclear. NGF-induced activation of p38 MAPK is implicated in the regulation of neurite outgrowth (Morooka and Nishida, 1998; Xing et al., 1998). Inhibition of p38 MAPK by a specific inhibitor SB203580 or by expression of dominant-negative constructs of the p38 MAPK pathway blocks neurite outgrowth in PC12 cells (Morooka and Nishida, 1998). In addition, p38 MAPK-mediated phosphorylation of paxillin, a focal adhesion adaptor protein, is important in NGF-induced neurite outgrowth in PC12 cells. Inhibition of this phosphorylation attenuates neurite outgrowth in NGF-stimulated PC12 cells (Huang et al., 2004). These observations collectively suggest that p38 may also play a crucial role in neurite outgrowth. On the other hand, JNK activation downstream of Trk activation in PC12 cells has also been implicated in stress-induced apoptosis as well as in NGF-induced neurite formation (Maroney et al., 1999; Takeda et al., 2000).

### 4.3.2 PI3-Kinase Signaling

Another pathway activated following Trk activation is the PI3-kinase pathway. Activation of the PI3-kinase signaling functions as a crucial pathway in relaying pro-survival signaling from Trk activation (Rodriguez-Viciana et al., 1994; Vaillant et al., 1999; Vanhaesebroeck and Waterfield, 1999). There are at least two pathways by which Trks initiate PI3-kinase activation. The first pathway involves the recruitment

of Grb2-associated binder-1 (Gab1) and Grb2-associated binder-2 (Gab2) by phosphorylated Grb2 (Holgado-Madruga et al., 1997; Vaillant et al., 1999; Liu and Rohrschneider, 2002). Gab1 and Gab2 are then phosphorylated, which triggers their association with the regulatory subunit of PI3-kinase, p85, and activation of the kinase. Alternatively, the catalytic subunit of PI3-kinase can interact directly with Ras to initiate PI3-kinase activation. Phosphatidylinositides generated by PI3-kinase then activate phosphoinositide-dependent protein kinase (PDK-1), which acts together with the phosphatidylinositides to stimulate the downstream protein kinase Akt (❯ *Figure 2-3*).

Akt activation downstream of PI3-kinase stimulation represents a key mechanism through which cell survival is promoted by the PI3-kinase pathway. Upon activation, Akt phosphorylates and modulates the activity of several proteins to promote survival (Datta et al., 1999). For example, Akt-mediated phosphorylation of Bcl2 antagonist of cell death (Bad) inhibits its ability to promote cytochrome *c* release and subsequent caspase activation (Datta et al., 1997). In addition, Akt was shown to phosphorylate procaspase-9 to inhibit its activation (Cardone et al., 1998; Datta et al., 1999). Akt also regulates the expression of several antiapoptotic genes through phosphorylating transcriptional factors of the Forkhead family (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999). Furthermore, Akt increases NF-κB-promoted gene transcription by phosphorylating the NF-κB inhibitory binding partner, IκB. Finally, Akt-induced phosphorylation of glycogen synthase kinase 3-β (GSK3β) inactivates it, thereby preventing GSK3β-promoted apoptosis (Pap and Cooper, 1998; van Weeren et al., 1998).

Accumulating evidence indicates that PI3-kinase may also take part in modulating neuronal architecture. PI3-kinase was shown to mediate the activation of small GTPases Cdc42/Rac/Rho family, which regulates actin polymerization (Kjoller and Hall, 1999; Bishop and Hall, 2000). In addition, activation of Akt appears to affect axon diameter and branching (Markus et al., 2002). Localized Trk-promoted activation of Ras and PI-3 kinase also promotes cell motility (Sachdev et al., 2002; Weiner et al., 2002) and growth cone steering (Song and Poo, 1999; Ming et al., 2002). These observations suggest that PI3-kinase signaling may mediate more diverse functions of Trks other than promoting neuronal survival.

### 4.3.3 PLC-γ1 Signaling

Activation of phospholipase C-gamma 1 (PLC-γ1) signaling occurs after direct association of PLC-γ1 with the phosphorylated Y785 of activated TrkA (❯ *Figure 2-3*; Kaplan and Miller, 2000). When activated, PLC-γ1 provokes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce two secondary messenger molecules: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers $Ca^{2+}$ release from intracellular stores, resulting in the activation of $Ca^{2+}$-regulated enzymes such as calcium/calmodulin-dependent protein kinases (CaM kinases) and $Ca^{2+}$-regulated isoform of protein kinase C (PKC). DAG, on the other hand, stimulates DAG-regulated PKC isoforms. For example, PKCδ is activated in PC12 cells after NGF treatment (Corbit et al., 1999), which was shown to be required for NGF-induced neurite outgrowth and ERK1/2 activation. In addition, PLC-γ1 activity is required for NGF-regulated MAPK signaling pathway (Rong et al., 2003).

Recent studies suggest that activation of PLC-γ downstream of Trk activation may also take part in modulating electrical activity in neuronal cells. Stimulation of TrkB subsequent to a pulse of BDNF treatment triggers PLC-γ activation, which in turn increases intracellular $Ca^{2+}$ level, followed by activation of CaM kinases and stimulated glutamate release (Lessmann, 1998). Moreover, mice homozygous for the mutation at the PLC-γ docking site display significant deficiencies in the induction of both the early and late phases of hippocampal CA1 long-term potentiation (LTP) (Minichiello et al., 2002). It therefore appears that Trk-mediated PLC-γ signaling pathway may serve a critical role in neurotrophin-evoked electrical activity and neural plasticity.

### 4.3.4 Other Adaptor Molecules

In addition to the relatively well-characterized pathways discussed in previous sections, several adaptor molecules have also been shown to associate with Trk receptor but the significance of downstream signaling

remains obscure. Csk homologous kinase (CHK) associates via its SH2 domain with Y785 of TrkA upon NGF stimulation. While the downstream signaling initiated has not been fully characterized, overexpression of CHK in PC12 cells was shown to enhance the ERK1/2 activity. Furthermore, blocking the action of CHK by CHK-specific antibodies suppresses NGF-induced differentiation, suggesting that CHK may also take part in mediating NGF-induced neurite outgrowth in PC12 cells (Yamashita et al., 1999). SHP-2, on the other hand, associates with TrkA, although the binding site for SHP-2 on Trks has not been determined (Goldsmith and Koizumi, 1997). Overexpression of SHP-2 enhances survival of dopaminergic neurons in the presence of BDNF (Takai et al., 2002), implicating a role of SHP-2 in regulating Trk-induced survival signaling. Finally, c-Abl was recently found to interact with the juxtamembrane region of TrkA, although the binding does not require TrkA phosphorylation (Yano et al., 2000). The functional significance of this interaction remains unknown.

## 4.4 Transport and Signaling via Internalized Neurotrophin–Trk Complex

Subsequent to ligand binding and activation of downstream signaling, neurotrophin–receptor complex is internalized via the formation of clathrin-coated pits, followed by dynamin-mediated endosytosis. In PC12 cells, TrkA activation by NGF causes rapidly internalization of the receptor (Beattie et al., 2000). In addition to the requirement of clathrin and dynamin, a novel protein named Pincher (pinocytic chaperone) may also be involved in mediating NGF and TrkA endocytosis in vitro (Shao et al., 2002). Internalized Trk receptors can then either be transported, recycled to the membrane surface for a second round of activation (Grimes et al., 1996; Zapf-Colby and Olefsky, 1998), or degraded in lysosomal and/or proteosomal degradation system (Sorkin and Waters, 1993; Sommerfeld et al., 2000).

The importance of internalized neurotrophin–receptor complex signaling in mediating Trk function is barely beginning to be unraveled. Certain aspects of Trk signaling require internalization of Trk. As mentioned previously, sustained activation of ERK1/2 subsequent to Trk activation, for example, may require endocytosis of the neurotrophin–Trk complex (York et al., 2000). On the other hand, transport of endocytosed neurotrophin–receptor complex is required for the initiation of responses of the cell body to target-derived neurotrophins. All of the endocytosed neurotrophin receptors, TrkA (Ehlers et al., 1995), TrkB (von Bartheld et al., 1996; Pease et al., 2000), TrkC (Helke et al., 1998), and the p75 receptor (Curtis et al., 1995), are retrogradely transported in vivo. Activation of Trks at the axon terminals initiates neurotrophin–Trk complex endocytosis and formation of clathrin-coated vesicles (CCV). Internalized vesicles are then transported to the cell body based on a dynein-dependent mechanism (Bhattacharyya et al., 2002). This transport process has been demonstrated to be important for mediating the retrograde survival signal from the axon terminals in the PNS. When distal axons of sensory or sympathetic neurons are stimulated by neurotrophins, transported neurotrophin–receptor complex was shown to induce ERK5 and PI3-kinase signaling in the cell body (Kuruvilla et al., 2000; Watson et al., 2001). Activated ERK5 is then translocated to the nucleus, followed by phosphorylation of cAMP response element binding protein (CREB) and myocyte enhancer factor-2 (MEF2), thereby enhancing neuronal survival (Watson et al., 2001). Finally, PI-3 kinase appears to also play an important role in retrograde transport because inhibition of PI3-kinase at the nerve terminal inhibits neurotrophin retrograde transport (Bartlett et al., 1997, 2002).

## 4.5 Negative Regulators of Trk Signaling

In addition to the stimulation of signaling cascade for signal propagation, Trk activation was also found to initiate several feedback mechanisms to limit Trk activation. This is important for the maintenance of responsiveness to the next wave of stimulation, and to prevent overamplification of downstream signals. Of the several feedback machineries initiated, Trk-activation-induced internalization of Trk serves as one of the mechanisms to temporally shut down response to excess ligands. In addition, tyrosine dephosphorylation of the receptors may also contribute to preventing further Trk activation. Transient association of

SH2-containing phosphatase 1 (SHP-1) with the phospho-Y490 of TrkA is reported after NGF treatment in PC12 cells and sympathetic neurons (Goldsmith and Koizumi, 1997; Marsh et al., 2003). SHP-1 was observed to directly dephosphorylate TrkA to regulate its activity (Marsh et al., 2003). Furthermore, overexpression of another phosphatase, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), in PC12 cells attenuates TrkA activation upon NGF treatment, resulting in a marked inhibition of neurite outgrowth (Musatov et al., 2004). This finding suggests that PTEN has a negative regulatory effect on TrkA signaling.

The third mechanism for negative regulation of Trk signaling is mediated by deactivation of Trk-induced downstream signaling molecules through dephosphorylation. For example, upregulation of mitogen-activated protein kinase phosphatase 1 (MKP-1) by NGF has been reported in dissociated embryonic sympathetic neurons or fibroblasts transfected with TrkA (Peinado-Ramon et al., 1998; Rosini et al., 2004). MKP-1 then inactivates ERK1/2 by dephosphorylation, thereby attenuating Trk-induced MAPK signaling.

Finally, ligand-induced downregulation of Trk expression serves as another mechanism for limiting Trk signaling. Downregulation of TrkB protein and messenger RNA (mRNA) was observed after a long period (ranging from 30 min to 24 h) of BDNF treatment in primary neuronal cultures, within the midbrain and retina in vivo (Frank et al., 1996, 1997; Chen and Weber, 2004). The cytoplasmic domain and a short sequence in the intracellular juxtamembrane domain of TrkB are essential for down-regulation of TrkB (Sommerfeld et al., 2000). However, prolonged exposure of NGF does not lead to downregulation of TrkA. On the contrary, treatment of NGF-responsive basal forebrain cultures with NGF was found to increase TrkA mRNA levels (Kojima et al., 1994). This observation suggests that even though TrkA and B are structurally similar, their expression and downregulation are regulated by distinctive mechanisms.

## 4.6 Signaling Cascade Downstream of p75

Unlike Trk receptors, p75 does not contain a kinase domain. As such, transmission of extracellular signal by p75 upon ligand binding depends on its association with other cell surface receptors (e.g., Trk receptors and Nogo receptor) or adaptor proteins in the cytoplasm (e.g., TRAF, RhoA, ankyrin repeat-rich membrane spanning protein (ARMS)). To date, a wide range of molecules of dissimilar structure and properties have been identified to interact with p75, but the signaling cascade initiated is far from clear. Identification of the downstream signaling and functions of p75 therefore remains a continued challenge in the field of neurotrophin receptor research.

Most p75-interacting proteins were identified only within the past 5 years. Current knowledge is focused mostly on the structural aspect of the interacting proteins, with the functional significance and signaling components not fully characterized. Neurotrophin receptor interacting factor (NRIF) was among the first to be identified. It is characterized by the presence of five zinc finger domains, two Kruppel boxes, and a nuclear localization signal. NRIF was shown to associate with p75 via the juxtamembrane and death domain of p75 (Casademunt et al., 1999). Another p75-interacting protein, $p75^{NTR}$-associated cell death executor (NADE), was first identified as a p75-interacting protein using yeast two-hybrid screening. It exhibits a nuclear export signal and two ubiquitination sites and was observed to interact with p75 via the death domain of p75 (Mukai et al., 2000). Neurotrophin receptor interacting MAGE homolog (NRAGE) is yet another protein observed to interact with p75 via its juxtamembrane domain (Salehi et al., 2000). Furthermore, p75 was shown to interact with small GTPase RhoA, which binds to the intracellular domain of p75. It is suggested that the association between RhoA and p75 plays an important role in p75-mediated neurite outgrowth (Yamashita et al., 1999).

A member of the TNF family, p75 was observed to associate with TNF-receptor-associated factor (TRAF6), which apparently has important functions in a number of TNF-receptor-mediated signalings (Bradley and Pober, 2001). NGF induces the association of p75 with TRAF6, which in turns regulates NF-κB activation (Khursigara et al., 1999). In addition, it has recently been shown that p75 associates with a number of kinases. Receptor interacting protein-2 (RIP-2) is a serine/threonine kinase that binds to p75

upon NGF treatment, leading to NF-κB activation (Khursigara et al., 2001). On the other hand, p75 associates with the β catalytic subunit of cAMP-dependent protein kinase (PKACβ) (Higuchi et al., 2003). NGF activation of p75 results in a transient increase in intracellular cAMP levels (Knipper et al., 1993; Higuchi et al., 2003), which is associated with the activation of this PKA variant, resulting in phosphorylation of p75 itself at Ser 304.

It should be noted that in addition to the full-length form, p75 can also exist as truncated forms by proteolytic processing. On the surface of Schwann cells, full-length p75 can be cleaved by unknown membrane metalloproteinase(s) that release a free-floating extracellular domain, and an intracellular domain linked to the membrane via the transmembrane region (Zupan et al., 1989; DiStefano et al., 1993). The function of this soluble extracellular domain remains unclear, but it has been suggested that the production of this cleavage form of p75 increases after peripheral nerve injury and is developmentally regulated (DiStefano et al., 1991). Moreover, intracellular proteolysis of p75 can also occur within or near the transmembrane region, resulting in the generation of a soluble cytoplasmic domain of p75. The proteolytic cytoplasmic release of intracellular domain of p75 is mediated by α- and γ-secretases, and is modulated by TrkA and TrkB signaling (Kanning et al., 2003). The cleaved C-terminal fragment of p75, although lacking its extracellular domain for ligand binding, can still interact with TrkA and TrkB, suggesting an important role of this truncated p75 in Trk signaling. The truncated and intracellular forms of p75 alone may also mediate neuronal cell death in the absence of Trk (Coulson et al., 2004).

Finally, p75 activation was observed to induce the hydrolysis of sphingomyelin to ceramide (Dobrowsky et al., 1994). The biological effect of p75-induced ceramide production, nonetheless, remains controversial. While some studies suggest that ceramide promotes differentiation and survival, others report that it is correlated with JNK activation and apoptosis. The precise function of p75-mediated ceramide production therefore remained to be elucidated (Roux and Barker, 2002).

## 4.7 Crosstalks Between Trk and p75 Signaling

Although Trks and p75 are structurally unrelated and appear to initiate different subsets of downstream signaling, increasing evidence suggests that crosstalks between Trk and p75 signaling are common, and may serve important functions. Their reciprocal modulations occur either via direct association with each other, or through other adaptor proteins that interact with both Trks and p75. In this section, we briefly review the mechanisms of crosstalks between Trk and p75 signaling.

### 4.7.1 Trk/p75 Dimerization

Since the identification of the high-affinity binding site for NGF, it was observed that association of NGF with neither TrkA nor p75 pertains to the affinity described for the high-affinity site (Kaplan et al., 1991a). Rather, expression of both TrkA and p75 is required for the formation of the high-affinity NGF binding site (Hempstead et al., 1989, 1991). A recent study showed that the number of high-affinity sites formed is regulated by the relative ratio of TrkA and p75 receptors. When TrkA and p75 are expressed at an almost equimolar ratio, the highest number of high-affinity sites is formed (Hempstead et al., 1992; Esposito et al., 2001). The affinity of TrkA for NGF is therefore significantly enhanced in the presence of p75. Interestingly, the association of p75 with Trks is not limited to TrkA. In fact, p75 was found to associate with all members of Trks (Bibel et al., 1999). Interaction of Trks and p75 was found to occur via the transmembrane and cytoplasmic domain of both receptors, although the detailed mechanisms remain enigmatic (Esposito et al., 2001). In addition to enhancing the affinity of Trks for their cognate ligands, the association of p75 with Trks also regulates their ligand specificity. For example, TrkB exhibits a much stronger selectivity for BDNF over NT-3 in the presence of p75 (Bibel et al., 1999). On the other hand, association of TrkA with p75 was shown to lower the affinity p75 exhibits for NGF (Ross et al., 1998). These observations collectively suggest that in addition to mediating separate downstream signaling, Trks and p75 may reciprocally regulate the function and signaling of each other through direct interaction.

### 4.7.2 Interaction Through Other Adaptor Proteins

Besides directly associating with each other, crosstalks of p75 and Trk can occur via other proteins. ARMS and p62 are two recently identified proteins that may serve as the link between the two pathways.

ARMS was first identified as a p75-interacting protein using yeast two-hybrid screening (Kong et al., 2001). Interestingly, ARMS was later found to interact also with Trks (Arevalo et al., 2004). ARMS is coexpressed frequently with Trk and p75 and it is suggested that it interacts with TrkA, TrkB, and TrkC directly through their transmembrane domains (Arevalo et al., 2004), while ARMS interacts with the Chopper domain of p75 (Kong et al., 2001). A ternary complex can be formed between Trk, p75, and ARMS (Chang et al., 2004). Interestingly, increasing ARMS expression results in decreased association of TrkA with p75, suggesting that ARMS might play an important role in regulating interactions between p75 and Trk receptors.

In PC12 cells, treatment with NGF induces tyrosine phosphorylation of ARMS, whereas in primary cultures of cortical neurons, treatment with BDNF also causes ARMS phosphorylation (Kong et al., 2001; Arevalo et al., 2004). Phosphorylated ARMS then recruits the Crk–C3G complex, which stimulates Rap-1-dependent sustained ERK activation. In addition to functioning as a neurotrophin receptor interacting protein, ARMS is also a substrate of protein kinase D (Iglesias et al., 2000). Moreover, ARMS is also identified as a downstream target of ephrin receptors (Kong et al., 2001), which implies that ARMS may also facilitate ephrin receptor signaling and neurotrophin receptor signaling crosstalks in neurons.

A recent report showed that atypical PKC-interacting protein, p62, also interacts with TrkA. p62 typically interacts with atyptical PKC to phosphorylate IκB kinase and leads to the activation of transcription factor NF-κB. Geetha and Wooten (2003) found that p62 interacts with TrkA at the juxtamembrane region. NGF-dependent localization of p62 is observed in the endosomal compartment, suggesting that p62 may act as a shuttling protein for routing activated atypical PKC to endosomes (Samuels et al., 2001). p62 also binds TRAF6 to interact with p75. TRAF6–p62 complex may therefore serve as a link to facilitate p75 and TrkA interaction (Wooten et al., 2001).

## 5 Crosstalks with Other Signaling Pathways

### 5.1 G Protein Signaling

In addition to the crosstalks observed between Trk and p75 signaling, neurotrophin signaling was also shown to cooperate with other signaling pathways. The G-protein-coupled receptor (GPCR) pathway, for instance, was found to initiate Trk activation in the absence of neurotrophin binding (Lee et al., 2002a). Activation of Trk via the GPCR pathway involves adenosine and the neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP), which leads to Trk activation via $A_{2A}$ receptor and PACAP-preferring (PAC1) receptor, respectively (Lee and Chao, 2001; Lee et al., 2002b). In contrast to neurotrophin-mediated activation, transactivation of Trk by GPCR signaling is slower. For example, at least 1 h of PACAP treatment is required to activate TrkA in PC12 cells (Lee et al., 2002b). In addition, similar to Trk activation downstream of neurotrophin binding, adenosine treatment of PC12 cells promotes the phosphorylation of Shc and PLC-γ1. Activation of PI3-kinase also occurs via transactivation by adenosine and PACAP. Trk transactivation via this pathway results in a long-lasting Akt activation and thereby promotes neuronal cell survival after trophic factor withdrawal (Lee et al., 2002b). These observations demonstrate that activation of Trk via neurotrophins and GPCR pathways initiates similar downstream signaling, albeit with different kinetics. Neurotrophins and GPCR pathways may hence serve as parallel pathways for activation of Trk signaling.

The mechanism by which GPCR activates Trk in the absence of neurotrophin remains unclear. For Trk transactivation by adenosine, Src kinase is implicated because Src kinase inhibitor, PP1, markedly attenuates adenosine-elicited tyrosine phosphorylation of TrkA (Lee et al., 2002b). In addition, Trk transactivation may also involve new protein synthesis or gene activation (Lee et al., 2002a). Transactivation of TrkA receptors by PACAP, on the other hand, can be inhibited with an intracellular calcium chelator,

BAPTA/AM, implicating a calcium-dependent pathway in its activation mechanism (Lee et al., 2002b). Rajagopal et al. (2004) demonstrated that transactivation of Trk receptor by adenosine, adenosine agonists, and PACAP occurs exclusively in an intracellular location and partly involves the Golgi apparatus.

It should be noted that crosstalks between Trk and GPCRs can also occur in the presence of NGF. Activation of TrkA by NGF stimulates sphingosine kinase type 1 (SphK1, Edsall et al., 1977), which then phosphorylates sphingosine to form sphingosine-1-phosphate (S1P). S1P, an important lipid mediator (Spiegel and Milstien, 2002), is the ligand for five GPCRs, designated $S1P_1$–$S1P_5$. Transactivation of $S1P_1$ by NGF in $S1P_1$-overexpressing PC12 cells markedly enhances neurite extension, suggesting that S1P receptors may function downstream of NGF/Trk receptor signaling to modulate neuronal differentiation (Toman et al., 2004).

Finally, NGF-induced activation of ERK1/2 in PC12 cells may also involve a classical G protein signaling pathway as ERK1/2 activation by NGF can be partially blocked by pertussis toxin (which inactivates the G proteins G(i/o); Rakhit et al., 2001). This is further supported by the finding that overexpression of G-protein-coupled receptor kinase 2 (GRK2) potentiates NGF-induced ERK1/2 activation in PC12 cells. Moreover, GRK2 is constitutively associated with the TrkA receptor. These observations suggest that G protein may also take part in regulating NGF-induced activation of ERK1/2.

### 5.2 Neuropoietic Cytokine Signaling

Another pathway that was demonstrated to exhibit a certain extent of crosstalk with neurotrophin receptor signaling is the neuropoietic cytokine-initiated signaling. Differentiation of neuronal progenitors into postmitotic neurons or regulation of developmental apoptosis often requires collaboration between different classes of neurotrophic factors (Ip and Yancopoulos, 1996). Induction of apoptosis in cultured sympathetic neurons by leukemia inhibitory factor (LIF), for example, is dependent on concurrent p75 signaling (Savitz and Kessler, 2000). For the precursor cells of sensory neurons a combination of LIF and NGF is needed for the differentiation (Murphy et al., 1993). Furthermore, ciliary neurotrophic factor (CNTF) can cooperate with NGF to enhance production of postmitotic NGF-dependent neurons in *trk*-transfected MAH cells (Ip et al., 1994). Aside from promoting neuronal differentiation, neurotrophins and neuropoietic cytokines have also been shown to regulate neuronal phenotypes. For example, NGF, LIF, and CNTF were demonstrated to regulate the cholinergic phenotype such as ChAT and VAChT expression in a coordinated fashion under a variety of physiological and pathological conditions (Berse et al., 1999). Taken together, these findings suggest that neurotrophins and interleukin-6 (IL-6)-family cytokines synergistically affect the differentiation program of the neuronal progenitors.

In addition to their synergistic property in modulating neuronal differentiation, the two families of neurotrophic factors may also regulate their own expression pattern and downstream signaling in a reciprocal manner. Expression of LIFR, one of the receptor component shared by IL-6-family cytokines, is specifically induced by NGF in PC12 cells, which was shown to exert a negative regulatory effect on neurite extension and branching (Ng et al., 2003). On the other hand, IL-6-family cytokines treatment can also regulate the expression of neurotrophins. For example, exposure of rat cortical astrocytes to human recombinant CNTF was found to increase the level of mRNA for NGF (Semkova and Krieglstein, 1999).

## 6 Functions of Neurotrophin Receptors

### 6.1 Functions of Trk Receptors

#### 6.1.1 Maintenance of Neuronal Survival

It has long been established that the neurotrophins are key survival factors for neurons during development. In addition, neurotrophins influence the differentiation and proliferation of neural crest-derived neuronal precursors. Nonetheless, different subpopulations of neurons respond to different members of the

neurotrophin family, possibly due to the expression of different neurotrophin receptors at the time of development. In particular, both sympathetic and sensory neurons undergo developmental changes in response to NGF. Subpopulations of sensory neurons, on the other hand, respond instead to BDNF or NT3 for maintenance of neuronal survival. The dependence of different neuronal subpopulations on different neurotrophins and the corresponding Trk signaling is revealed by the strikingly similar phenotypes exhibited by neurotrophin and Trk knockout mice. Both *trkA* and *NGF* mutant mice exhibit complete loss of sympathetic and sensory neurons (Crowley et al., 1994; Smeyne et al., 1994). Mice lacking NT4/5, another cognate ligand for TrkA, also exhibit a loss of sensory neurons in the nodose–petrosal and geniculate ganglia (Conover et al., 1995; Liu et al., 1995). *BDNF*- and *trkB*-deficient mice, on the other hand, show malfunctions in the vestibular system and neuron loss in trigeminal, nodose ganglia and DRGs. Motor neuron loss is also observed in *trkB* knockout mice (Klein et al., 1993; Jones et al., 1994). Finally, *NT-3*- and *trkC*-deficient mice have an extraordinary phenotype of abnormal movements and postures with a deficiency in proprioceptive neurons (Ernfors et al., 1994; Klein et al., 1994).

In addition to findings from biochemical studies or targeted gene disruption analysis, naturally occurring mutation in TrkA also indicates a crucial requirement of TrkA in the maintenance of neuronal survival. TrkA mutations have been identified as the cause of a human syndrome, congenital insensitivity to pain and anhidrosis (CIPA), or hereditary sensory and autonomic neuropathy type IV (Indo et al., 1996; Mardy et al., 1999; Indo, 2002). Because of the lack of sympathetic neurons and small unmyelinated nociceptive sensory neurons, CIPA patients exhibit defects in thermoregulation and insensitivity to pain, which often result in injuries, self-mutilation, and death-causing episodes of hyperpyrexia (Indo et al., 1996; Shatzky et al., 2000).

### 6.1.2 Synaptic Transmission and Neural Plasticity

In addition to their classical effects on neuronal survival, neurotrophins have also been shown recently to take part in regulating neuronal morphology and synaptic plasticity. Trk signaling is implicated in inducing morphological changes through the regulation of both dendritic and axonal arborizations in vivo to regulate precise network formation (Gallo and Letourneau, 1998; Schinder and Poo, 2000; Yacoubian and Lo, 2000). Neurotrophins have chemotrophic effects on growth cones and can also function to protect them from inhibitory guidance cues (Gundersen and Barrett, 1979, 1980; Cai and Reed, 1999). Local activation of Trk signaling maintains the advance of sympathetic neuron growth cones (Campenot, 1977). In addition, Song and Poo (1999) demonstrated that gradient of NGF is necessary for growth cone turning. These observations collectively suggest that Trk signaling can have regulatory roles on the formation of neuronal network.

Neurotrophins also exert effects on the modulation of neuronal excitability and synaptic transmission (Rudy et al., 1987; Lohof et al., 1993; Kang and Schuman, 1995; Lesser et al., 1997; Lai and Ip, 2003). TrkB activation potentiates *N*-methyl-D-aspartate (NMDA) responsiveness by increasing the channel open probability (Levine and Kolb, 2000). Conversely, electrical activity has also been shown to modestly increase the expression of TrkB (Castren et al., 1992). Furthermore, the surface expression of TrkB is elevated by depolarization in superior cervical ganglion (SCG) and nodose ganglion neurons (Meyer-Franke et al., 1998). Trk receptors may also modulate neuronal excitability and signal transduction through interaction with other ion channels. For example, capsaicin (vanilloid receptor 1, VR1) receptor, a heat-activated ion channel, is activated through NGF binding to TrkA (Chuang et al., 2001). Coimmunoprecipitation studies revealed that VR1 associates with TrkA and PLC$\gamma$ to form a complex. In addition, BDNF binding to TrkB was shown to produce a rapid influx of cations through transient receptor potential cation channel 3 (TRPC3) that is dependent on activation of PLC$\gamma$. An interaction between TrkB and TRPC3 ion channels was also observed (Li et al., 1999). Furthermore, both voltage-gated sodium channel 1.9 ($Na_v1.9$) and alpha-amino-5-methyl isoxazole-4-propionate (AMPA) receptor activities can also be modified by activated TrkB (Blum et al., 2002; Itami et al., 2003; Du and Poo, 2004). While the significance for the interaction between Trks and these ion channels may not have been completely characterized, these observations suggest that Trk signaling may also regulate signal transduction across the synapse.

Finally, emerging evidence indicates that neurotrophins are also involved in the induction of hippocampal LTP, which is important for learning and memory (Figurov et al., 1996; Korte et al., 1996; Chen et al., 1999). Conditional knockout of TrkB in postnatal forebrain results in mice exhibiting a severe impairment in stressful spatial learning (Minichiello et al., 1999). Hippocampal LTP is also inhibited by target disruption of the PLCγ docking site on TrkB (Minichiello et al., 2002).

### 6.1.3 Neural and Glial Migration During Development

Increasing evidence indicates that Trk signaling also mediates the ability of neurotrophins to regulate neural and glial migration during development. Medina et al. (2004) recently reported that removal of TrkB in developing cortex delays neuronal migration, in addition to reducing the number of myelinated axons in the corpus callosum. Furthermore, BDNF and NT-3 were shown to function coherently to facilitate Schwann cell migration and myelination. Activation of TrkC by NT-3 results in activation of the JNK pathway via the Rho GTPases Rac1 and Cdc42 to enhance Schwann cell migration. BDNF-mediated activation of p75, on the other hand, results in activation of RhoA via Src kinase to limit Schwann cell migration. Interestingly, upon the completion of Schwann cell migration, NT-3-mediated activation of TrkC inhibits myelination, while activation of p75 by BDNF enhances myelination (Cosgaya et al., 2002; Yamauchi et al., 2003, 2004). A second report also demonstrated that BDNF overexpression enhances myelin sheath thickness, further confirming an important role of BDNF signaling in myelin formation (Tolwani et al., 2004). Schwann cell migration and myelination is therefore tightly regulated by the relative abundance of neurotrophins and neurotrophin receptor subtypes. In fact, it was observed that when myelination is initiated, the inhibitory action of NT-3 is removed by a reduction in its expression. Once active myelination is initiated, the action of BDNF is removed by increased levels of TrkB-T1 (Cosgaya et al., 2002). In addition to regulating Schwann cell migration and myelination, NT-3-induced activation of TrkC was also shown to be required for oligodendrocyte differentiation (Hu et al., 2004). Taken together, these observations suggest a major role of neurotrophin signaling in regulating migration and differentiation of both neuron and glia during development.

### 6.1.4 Higher Cognitive Function

Recent evidence reveals that Trk signaling may also be implicated in higher cognitive function. CIPA patients with TrkA mutations exhibit mental retardation in addition to other symptoms, reflecting a higher cognitive function of TrkA signaling (Indo et al., 1996; Shatzky et al., 2000). In addition, psychosocial stress has been shown to markedly alter NGF and BDNF levels, both in plasma and in selected brain areas, including the hypothalamus and hippocampus. NGF levels are also enhanced by emotional stress induced by parachute jumping in human volunteers (Alleva and Santucci, 2001). A recent study suggests that BDNF may also be involved in psychiatric disorders such as the pathophysiology of depression (Neves-Pereira et al., 2002; Sklar et al., 2002), implying a functional role of Trk signaling in neuropsychiatric function. Furthermore, BDNF-mediated activation of TrkB in the amygdale was found to be required for fear conditioning (Rattiner et al., 2004). Together, these findings suggest a role of neurotrophins in mediating both short- and long-term effects of experience on brain structure and function.

### 6.1.5 Other Functions

Trk signaling may also take part in regulating functions as diverse as energy balance regulation and tumor malignancy. Xu et al. (2003) found that BDNF–TrkB serves as downstream components in the melanocortin-4 receptor-mediated control of energy balance. Hence, mouse mutants expressing decreased amounts of TrkB showed hyperphagia and maturity-onset obesity (Xu et al., 2003). TrkC expression, on the other hand, was found to correlate with overall survival among patients suffering from

medulloblastomas. Patients with tumors expressing high levels of TrkC mRNA had significantly longer intervals without disease progression and a more favorable overall survival (Segal et al., 1994). The biological action of TrkC activation was interestingly found to induce apoptosis in medulloblastoma, thereby accounting for the observed clinical outcome (Kim et al., 1999).

## 6.2 Functions of p75

Contrary to the function of Trk receptors, the function of p75 has remained a little obscure. The lack of an intracellular kinase domain makes the identification of signaling downstream of p75 more difficult, thereby limiting data on its potential function. Nonetheless, generation of knockout mice lacking p75 provided some important clues on the potential function of p75, especially during development. The first *p75* knockout mice generated was constructed by targeted disruption of exon 3 of *p75* (Lee et al., 1992). Mutant mice are viable and fertile but developed skin defects and ulcers. Immunohistochemistry revealed a lack of calcitonin gene-related peptide and substance-P-expressing peripheral sensory nerve fibers. p75 expression is upregulated in different parts of Müller glial cells but not in photoreceptors after retinal degeneration (Harada et al., 2000). In p75-deficient mice, phototoxicity-induced photoreceptor apoptosis is reduced compared with that in wild-type mice, suggesting that p75 may take part in initiating photoreceptor apoptosis in multiple forms of retinitis pigmentosa.

Soon after the generation of the first *p75* knockout mice, another isoform of *p75* lacking exons 1–3 was identified. To fully understand the significance of p75 signaling, von Schack et al. (2001) constructed another *p75* mutant mice by completely deleting exon 4 of *p75*, thereby preventing expression of both isoforms. There are significant phenotypic differences between the exon 3 and the exon 4 *p75* knockout mice. Mice with deletion of *p75* exon 4 are smaller in size compared with the exon 3-deleted counterpart. They also show serious loss of peripheral sensory neurons and peripheral innervation. Besides, unlike *p75* exon 3 knockout mice that are viable, mice with *p75* exon 4 deletion show partial perinatal death probably due to abnormal blood vessel formation (von Schack et al., 2001). It therefore appears that similar to Trk, p75 may also take part in regulating neuronal death, in addition to modulating other biological functions such as vessel formation and target innervation.

### 6.2.1 Neuronal Death/Survival

With p75 being a member of the TNF family and bearing a death domain, it was predicted that p75 may function as a death receptor. Indeed, *p75* exon 3 knockout mice exhibit reduced apoptosis in the retina (Lee et al., 1992). In addition, expression of p75 was elevated in multiple cell types following injury (reviewed in Roux and Barker, 2002). Consistent with this observation, injury-induced neuronal death is alleviated in *p75* exon 3 knockout mice or when p75 expression is reduced by antisense oligonucleotides (Cheema et al., 1996; Ferri and Bisby, 1999). Furthermore, overexpression of p75 in primary cortical neurons, PC12 cells, or glioma cells leads to activation of JNK and caspase activation (Gu et al., 1999; Wang et al., 2001; Harrington et al., 2002; Bhakar et al., 2003). While increasing evidence linked p75 with the initiation of apoptosis, the mechanisms implicated remained obscure. Another member of the TNF family, Fas, activates the apoptotic machinery in response to external apoptotic stimuli by recruitment of Fas-associated death domain (FADD) and tumor necrosis factor receptor 1-associated death domain protein (TRADD) via its death domain, thereby activating caspase-8. p75-induced apoptosis, nonetheless, was found to involve no caspase-8, nor does it require association with FADD or TRADD. Instead, activation of caspase-9 was observed (Wang et al., 2001). This observation argues against the importance of the death domain in p75-mediated cell death. In corroboration with this hypothesis, a 29-amino-acid sequence in the intracellular juxtamembrane domain of p75 named Chopper was found to be required and sufficient for p75-initiated cell death (Coulson et al., 2000).

The death domain of p75 is crucial for the association of p75 with some of its interacting proteins including NRIF and NADE, which has been suggested to take part in mediating the pro-apoptotic property

of p75. NRIF, for example, has been suggested to function in p75-mediated apoptosis (Casademunt et al., 1999) and serves as a transcription factor in p75 signaling (Gentry et al., 2004). In NRIF knockout mice, p75-dependent cell death of retinal cells is reduced during early development. Furthermore, activation of p75 by neurotrophins fails to induce apoptosis in sympathetic neuron cultures taken from NRIF knockout mice (Casademunt et al., 1999; Gentry et al., 2004). These observations suggest that NRIF is required for p75-induced apoptosis. The relative importance of the death domain and the Chopper domain in the pro-apoptotic property of p75 therefore requires further clarification.

NADE, on the other hand, was found to initiate apoptosis and caspase activation when overexpressed together with p75 in 293T cells (Mukai et al., 2000). Another p75-interacting protein NRAGE induces robust JNK activation and caspase activation in PC12 cells (Salehi et al., 2002). These observations collectively indicate that one of the mechanisms through which p75 initiates apoptosis is by recruitment of proapoptotic interacting partners. The circumstances under which p75 prefers one interacting protein over the other will provide essential information on how the function of p75 is regulated.

It should be noted that in addition to promoting apoptosis, p75 has also been suggested to favor neuronal survival. While this may seem rather surprising, this hypothesis is consistent with the absence of peripheral sensory neurons in the *p75* exon 4 knockout mice, suggesting that p75 is crucial for maintaining neuronal survival for this subpopulation of neurons. The ability of p75 to enhance neuronal survival is associated with the increase in NF-κB activation observed downstream of p75. In the absence of TrkA, NGF binds to p75 and activates NF-κB in rat Schwann cells (Dobrowsky and Carter, 1998). In both embryonic neurons and sympathetic neurons, neurotrophins have been shown to trigger p75-dependent NF-κB activation. How p75 activation results in NF-κB induction has not been completely elucidated, but recent evidence indicates that it may involve association with some of its interacting proteins. Association of p75 with TRAF6, for example, was shown to mediate the downstream NF-κB activation (Khursigara et al., 1999). On the other hand, binding of RIP-2 similarly leads to NF-κB activation (Khursigara et al., 2001). It therefore appears that p75 may promote both apoptosis and survival, depending on the cellular context.

### 6.2.2 Nerve Regeneration and Neurite Outgrowth

In addition to modulating neuronal survival and death, p75 has also been implicated in modulating neurite elongation via its interaction with the Nogo receptor. The proteins Nogo-A, oligodendrocyte–myelin glycoprotein (OMgp), and myelin-associated glycoprotein (MAG) have all been identified as inhibitory components present in CNS myelin, acting through the same Nogo receptor (Ngr). The glycosyl phosphatidylinositol (GPI) linkage of Ngr suggests the requirement for additional transmembrance proteins as transducer for the inhibitory signal inside the cells. Recently, p75 was identified as a potential coreceptor for Ngr (Wang et al., 2002; Wong et al., 2002). Both Ngr and p75 are coexpressed extensively in the developing rat nervous system. Ngr was also found to physically interact with p75 via their extracellular domain upon ligand binding. Activation of the p75–Ngr complex increases RhoA activity, which is crucial for the ability of the p75–Ngr complex to inhibit axonal elongation (Yamashita and Tohyama, 2003). p75 interacts with the RhoGDP dissociation inhibitor (RhoGDI) to enable RhoA activation (Yamashita and Tohyama, 2003). This interaction is further enhanced by MAG and Nogo. Using a blocking peptide to inhibit the interaction between RhoGDI and p75, the inhibitory cues of CNS regeneration can be blocked, suggesting that RhoA activity is indispensable for the inhibitory cues elicited by p75/Ngr signaling. Recently, a novel protein LINGO-1, was also shown to be required for p75/Ngr-mediated activation of RhoA (Mi et al., 2004).

Since activated p75 can modulate RhoA activity, it is highly possible that functional p75 may also regulate the dynamic actin polymerization at the growth cones, thereby controlling growth cone advance. Binding of neurotrophins to Trk and p75 receptors on growth cones triggers changes in actin filament dynamics and growth cone behaviors. Activation of Trks mediates local accumulation of actin filaments via increase in Rac1 activity, whereas p75 activation causes local reduction of RhoA signaling that promotes lengthening of filopodia (Gallo and Letourneau, 2004).

## 7 Future Perspectives

It has been almost 20 years since the identification of the first neurotrophin receptor. With the advances in our research techniques, some of our previous understanding on neurotrophin receptor signaling has been consolidated, while at the same time novel aspects of the receptor functions have been unraveled. The three-dimensional structures of TrkA and p75 in association with NGF, for example, have revealed the structural bases underlying the specificity in neurotrophin recognition and receptor binding (He and Garcia, 2004). However, new insights from recent studies also point to a new direction in neurotrophin research. Whereas most of our present knowledge on neurotrophin receptor signaling is based on studies using bath-applied neurotrophin, recent studies indicate that localized administration of neurotrophin to the axonal terminals and cell bodies leads to distinct signaling mechanisms (Watson et al., 2001). Thus, it will be interesting to apply the present findings from bath cultures, such as downstream signaling mechanisms, Trk- and p75-binding partners, to delineate the neurotrophin receptor functions in different subcellular microenvironments, such as pre- or postsynaptic regions. In addition, considerable evidence has drawn our attention to the functional roles of Trk receptors in synaptic formation, transmission, and plasticity. Future investigations will provide a better understanding on the involvement of neurotrophin receptor signaling in higher cognitive functions.

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## References

Alleva E, Santucci D. 2001. Psychosocial vs. "physical" stress situation in rodents and humans. Role of neurotrophins. Physiol Behav 73: 313-320.

Arevalo JC, Conde B, Hempstead BI, Chao MV, Martin-Zanca D. et al. 2001. A novel mutation within the extracellular domain of TrkA causes constitutive receptor activation. Oncogene 20: 1229-1234.

Arevalo JC, Yano H, Teng KK, Chao MV. 2004. A unique pathway for sustained neurotrophin signaling through an ankyrin-rich membrane-spanning protein. EMBO J 23: 2358-2368.

Armanini MP, McMahon SB, Sutherland J, Shelton DL, Phillips HS. 1995. Truncated and catalytic isoforms of trkB are co-expressed in neurons of rat and mouse CNS. Eur J Neurosci 7: 1403-1409.

Atwal JK, Massie B, Miller FD, Kaplan DR. 2000. The TrkB–Shc site signals neuronal survival and local axon growth via MEK and P13-kinase. Neuron 27: 265-277.

Barbacid M. 1995. Neurotrophic factors and their receptors. Curr Opin Cell Biol 7: 148-155.

Barker PA, Lomen-Hoerth C, Gensch EM, Meakin SO, Glass DJ, et al. 1993. Tissue-specific alternative splicing generates two isoforms of the trkA receptor. J Biol Chem 268: 15150-15157.

Barrett GL, Bartlett PF. 1994. The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. Proc Natl Acad Sci USA 91: 6501-6505.

Bartlett SE, Reynolds AJ, Weible M II, Hendry IA. 2002. Phosphatidylinositol kinase enzymes regulate the retrograde axonal transport of NT-3 and NT-4 in sympathetic and sensory neurons. J Neurosci Res 68: 169-175.

Bartlett SE, Reynolds AJ, Weible M II, Heydon K, Hendry IA. 1997. In sympathetic but not sensory neurones, phosphoinositide-3 kinase is important for NGF-dependent survival and the retrograde transport of 125I-betaNGF. Brain Res 761: 257-262.

Beattie MS, Harrington AW, Lee R, Kim JY, Boyce SL, et al. 2002. ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neuron 36: 375-386.

Beattie EC, Howe CL, Wilde A, Brodsky FM, Mobley WC. 2000. NGF signals through TrkA to increase clathrin at the plasma membrane and enhance clathrin-mediated membrane trafficking. J Neurosci 20: 7325-7333.

Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, et al. 1991. Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron 7: 857-866.

Berse B, Lopez-Coviella I, Blusztajn JK. 1999. Activation of TrkA by nerve growth factor upregulates expression of the cholinergic gene locus but attenuates the response to ciliary neurotrophic growth factor. Biochem J 342: 301-308.

Bhakar AL, Howell JL, Paul CE, Salehi AH, Becker EB, et al. 2003. Apoptosis induced by p75NTR overexpression requires Jun kinase-dependent phosphorylation of Bad. J Neurosci 23: 11373-11381.

Bhattacharyya A, Watson FL, Pomeroy SL, Zhang YZ, Stiles CD, et al. 2002. High-resolution imaging demonstrates dynein-based vesicular transport of activated Trk receptors. J Neurobiol 51: 302-312.

Bibel M, Hoppe E, Barde YA. 1999. Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18: 616-622.

Biggs W. III, Meisenhelder J, Hunter T, Cavenee WK, Arden KC. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc Natl Acad Sci USA 96: 7421-7426.

Bishop AL, Hall A. 2000. Rho GTPases and their effector proteins. Biochem J 348: 241-255.

Blum R, Kafitz KW, Konnerth A. 2002. Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9. Nature 419: 687-693.

Bothwell M. 1995. Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 18: 223-253.

Bradley JR, Pober JS. 2001. Tumor necrosis factor receptor-associated factors (TRAFs). Oncogene 20: 6482-6491.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, et al. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96: 857-868.

Buck CR, Martinez HJ, Black IB, Chao MV. 1987. Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. Proc Natl Acad Sci USA 84: 3060-3063.

Cai H, Reed RR. 1999. Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. J Neurosci 19: 6519-6527.

Campenot RB. 1977. Local control of neurite development by nerve growth factor. Proc Natl Acad Sci USA 74: 4516-4519.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, et al. 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science 282: 1318-1321.

Carroll SL, Silos-Santiago I, Frese SE, Ruit KG, Milbrandt J, et al. 1992. Dorsal root ganglion neurons expressing trk are selectively sensitive to NGF deprivation in utero. Neuron 9: 779-788.

Casademunt E, Carter BD, Benzel I, Frade JM, Dechant G, et al. 1999. The zinc finger protein NRIF interacts with the neurotrophin receptor p75(NTR) and participates in programmed cell death. EMBO J 18: 6050-6061.

Castren E, Zafra F, Thoenen H, Lindholm D. 1992. Light regulates expression of brain-derived neurotrophic factor mRNA in rat visual cortex. Proc Natl Acad Sci USA 89: 9444-9448.

Chang MS, Arevalo JC, Chao MV. 2004. Ternary complex with Trk, p75, and an ankyrin-rich membrane spanning protein. J Neurosci Res 78: 186-192.

Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, et al. 1986. Gene transfer and molecular cloning of the human NGF receptor. Science 232: 518-521.

Cheema SS, Barrett GL, Bartlett PF. 1996. Reducing p75 nerve growth factor receptor levels using antisense oligonucleotides prevents the loss of axotomized sensory neurons in the dorsal root ganglia of newborn rats. J Neurosci Res 46: 239-245.

Chen G, Kolbeck R, Barde YA, Bonhoeffer T, Kossel A. 1999. Relative contribution of endogenous neurotrophins in hippocampal long-term potentiation. J Neurosci 19: 7983-7990.

Chen H, Weber AJ. 2004. Brain-derived neurotrophic factor reduces TrkB protein and mRNA in the normal retina and following optic nerve crush in adult rats. Brain Res 1011: 99-106.

Chuang HH, Prescott ED, Kong H, Shields S, Jordt SE, et al. 2001. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. Nature 411: 957-962.

Clary DO, Reichardt LF. 1994. An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. Proc Natl Acad Sci USA 91: 11133-11137.

Cohen-Cory S, Dreyfus CF, Black IB. 1989. Expression of high- and low-affinity nerve growth factor receptors by Purkinje cells in the developing rat cerebellum. Exp Neurol 105: 104-109.

Conover JC, Erickson JT, Katz DM, Bianchi LM, Poueymirou WT, et al. 1995. Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. Nature 375: 235-238.

Corbit KC, Foster DA, Rosner MR. 1999. Protein kinase Cdelta mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells. Mol Cell Biol 19: 4209-4218.

Cordon-Cardo C, Tapley P, Jing SQ, Nanduri V, O'Rourke E, et al. 1991. The trk tyrosine protein kinase mediates the

mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66: 173-183.

Cosgaya JM, Chan JR, Shooter EM. 2002. The neurotrophin receptor p75NTR as a positive modulator of myelination. Science 298: 1245-1248.

Coulson EJ, Reid K, Baca M, Shipham KA, Hulett SM, et al. 2000. Chopper, a new death domain of the p75 neurotrophin receptor that mediates rapid neuronal cell death. J Biol Chem 275: 30537-30545.

Coulson EJ, Reid K, Shipham KM, Morley S, Kilpatrick TJ, et al. 2004. The role of neurotransmission and the Chopper domain in p75 neurotrophin receptor death signaling. Prog Brain Res 146: 41-62.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts-Meek S, et al. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76: 1001-1011.

Curtis R, Adryan KM, Stark JL, Park JS, Compton DL, et al. 1995. Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron 14: 1201-1211.

Datta SR, Brunet A, Greenberg ME. 1999. Cellular survival: a play in three Akts. Genes Dev 13: 2905-2927.

Datta SR, Dudek H, Tao X, Masters S, Fu H, et al. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231-241.

Di Stefano PS, Chelsea DM, Schick CM, McKelvy JF. 1993. Involvement of a metalloprotease in low-affinity nerve growth factor receptor truncation: inhibition of truncation in vitro and in vivo. J Neurosci 13: 2405-2414.

Di Stefano PS, Clagett-Dame M, Chelsea DM, Loy R. 1991. Developmental regulation of human truncated nerve growth factor receptor. Ann Neurol 29: 13-20.

Dobrowsky RT, Carter BD. 1998. Coupling of the p75 neurotrophin receptor to sphingolipid signaling. Ann N Y Acad Sci 845: 32-45.

Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA. 1994. Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265: 1596-1599.

Du J, Poo M. 2004. Rapid BDNF-induced retrograde synaptic modification in a developing retinotectal system. Nature 429: 878-883.

Dubus P, Parrens M, El-Mokhtari Y, Ferrer J, Groppi A, et al. 2000. Identification of novel trkA variants with deletions in leucine-rich motifs of the extracellular domain. J Neuroimmunol 107: 42-49.

Edsall LC, Pirianov GG, Spiegel S. 1977. Involvement of sphingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation. J Neurosci 17: 6952-6960.

Ehlers MD, Kaplan DR, Price DL, Koliatsos VE. 1995. NGF-stimulated retrograde transport of trkA in the mammalian nervous system. J Cell Biol 130: 149-156.

Ernfors P, Hallbook F, Ebendal T, Shooter EM, Radeke MJ, et al. 1988. Developmental and regional expression of beta-nerve growth factor receptor mRNA in the chick and rat. Neuron 1: 983-996.

Ernfors P, Henschen A, Olson L, Persson H. 1989. Expression of nerve growth factor receptor mRNA is developmentally regulated and increased after axotomy in rat spinal cord motoneurons. Neuron 2: 1605-1613.

Ernfors P, Lee KF, Kucera J, Jaenisch R. 1994. Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell 77: 503-512.

Esposito D, Patel P, Stephens RM, Perez P, Chao MV, et al. 2001. The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. J Biol Chem 276: 32687-32695.

Ferri CC, Bisby MA. 1999. Improved survival of injured sciatic nerve Schwann cells in mice lacking the p75 receptor. Neurosci Lett 272: 191-194.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B. 1996. Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381: 706-709.

Frank L, Ventimiglia R, Anderson K, Lindsay RM, Rudge JS. 1996. BDNF down-regulates neurotrophin responsiveness, TrkB protein and TrkB mRNA levels in cultured rat hippocampal neurons. Eur J Neurosci 8: 1220-1230.

Frank L, Wiegand SJ, Siuciak JA, Lindsay RM, Rudge JS. 1997. Effects of BDNF infusion on the regulation of TrkB protein and message in adult rat brain. Exp Neurol 145: 62-70.

Friedman WJ, Olson L, Persson H. 1991. Temporal and spatial expression of NGF receptor mRNA during postnatal rat brain development analyzed by in situ hybridization. Brain Res Dev Brain Res 63: 43-51.

Fryer RH, Kaplan DR, Feinstein SC, Radeke MJ, Grayson DR, et al. 1996. Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. J Comp Neurol 374: 21-40.

Gallo G, Letourneau PC. 1998. Localized sources of neurotrophins initiate axon collateral sprouting. J Neurosci 18: 5403-5414.

Gallo G, Letourneau PC. 2004. Regulation of growth cone actin filaments by guidance cues. J Neurobiol 58: 92-102.

Geetha T, Wooten MW. 2003. Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. J Biol Chem 278: 4730-4739.

Gentry JJ, Barker PA, Carter BD. 2004. The p75 neurotrophin receptor: multiple interactors and numerous functions. Prog Brain Res 146: 25-39.

Gibbs RB, McCabe JT, Buck CR, Chao MV, Pfaff DW. 1989. Expression of NGF receptor in the rat forebrain detected with in situ hybridization and immunohistochemistry. Brain Res Mol Brain Res 6: 275-287.

Glass DJ, Nye SH, Hantzopoulos P, Macchi MJ, Squinto SP, et al. 1991. TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell 66: 405-413.

Goldsmith BA, Koizumi S. 1997. Transient association of the phosphotyrosine phosphatase SHP-2 with TrkA is induced by nerve growth factor. J Neurochem 69: 1014-1019.

Götz R, Koster R, Winkler C, Raulf F, Lottspeich F, et al. 1994. Neurotrophin-6 is a new member of the nerve growth factor family. Nature 372: 266-269.

Grimes ML, Zhou J, Beattie EC, Yuen EC, Hall DE, et al. 1996. Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. J Neurosci 16: 7950-7964.

Gu C, Casaccia-Bonnefil P, Srinivasan A, Chao MV. 1999. Oligodendrocyte apoptosis mediated by caspase activation. J Neurosci 19: 3043-3049.

Guiton M, Gunn-Moore FJ, Glass DJ, Geis DR, Yancopoulos GD, et al. 1995. Naturally occurring tyrosine kinase inserts block high affinity binding of phospholipase C gamma and Shc to TrkC and neurotrophin-3 signaling. J Biol Chem 270: 20384-20390.

Gundersen RW, Barrett JN. 1979. Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science 206: 1079-1080.

Gundersen RW, Barrett JN. 1980. Characterization of the turning response of dorsal root neurites toward nerve growth factor. J Cell Biol 87: 546-554.

Harada T, Harada C, Nakayama N, Okuyama S, Yoshida K, et al. 2000. Modification of glial-neuronal cell interactions prevents photoreceptor apoptosis during light-induced retinal degeneration. Neuron 26: 533-541.

Harada T, Morooka T, Ogawa S, Nishida E. 2001. ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. Nat Cell Biol 3: 453-459.

Harrington AW, Kim JY, Yoon SO. 2002. Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis. J Neurosci 22: 156-166.

He XL, Garcia KC. 2004. Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. Science 304: 870-875.

Helke CJ, Adryan KM, Fedorowicz J, Zhuo H, Park JS, et al. 1998. Axonal transport of neurotrophins by visceral afferent and efferent neurons of the vagus nerve of the rat. J Comp Neurol 393: 102-117.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV. 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. Nature 350: 678-683.

Hempstead BL, Rabin SJ, Kaplan L, Reid S, Parada LF, et al. 1992. Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. Neuron 9: 883-896.

Hempstead BL, Schleifer LS, Chao MV. 1989. Expression of functional nerve growth factor receptors after gene transfer. Science 243: 373-375.

Higuchi H, Yamashita T, Yoshikawa H, Tohyama M. 2003. PKA phosphorylates the p75 receptor and regulates its localization to lipid rafts. EMBO J 22: 1790-1800.

Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ. 1997. Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci USA 94: 12419-12424.

Holtzman DM, Li Y, Parada LF, Kinsman S, Chen CK, et al. 1992. p140trk mRNA marks NGF-responsive forebrain neurons: evidence that trk gene expression is induced by NGF. Neuron 9: 465-478.

Hu X, Jin L, Feng L. 2004. Erk1/2 but not PI3K pathway is required for neurotrophin 3-induecde oligodendrocyte differentiation of post-natal neural stem cells. J Neurochem 90: 1339-1347.

Huang C, Borchers CH, Schaller MD, Jacobson K. 2004. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. J Cell Biol 164: 593-602.

Huang EJ, Reichardt LF. 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72: 609-642.

Iglesias T, Cabrera-Poch N, Mitchell MP, Naven TJ, Rozengurt E, et al. 2000. Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. J Biol Chem 275: 40048-40056.

Indo Y. 2002. Genetics of congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV. Clinical, biological and molecular aspects of mutations in TrkA(NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. Clin Auton Res 12 (Suppl. 1): 120-132.

Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, et al. 1996. Mutations in the TrkA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. Nat Genet 13: 485-488.

Ip NY, Boulton TG, Li Y, Verdi JM, Birren SJ, et al. 1994. CNTF, FGF, NGF collaborate to drive the terminal differentiation of MAH cells into postmitotic neurons. Neuron 13: 443-455.

Ip NY, Ibanez CF, Nye SH, McClain J, Jones PF, et al. 1992. Mammalian neurotrophin-4: structure, chromosomal

localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA 89: 3060-3064.

Ip NY, Stitt TN, Tapley P, Klein R, Glass DJ, et al. 1993. Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells. Neuron 10: 137-149.

Ip NY, Yancopoulos GD. 1994. Neurotrophic factor receptors: just like other growth factor and cytokine receptors? Curr Opin Neurobiol 4: 400-405.

Ip NY, Yancopoulos GD. 1996. The neurotrophins and CNTF: two families of collaborative neurotrophic factors. Annu Rev Neurosci 19: 491-515.

Itami C, Kimura F, Kohno T, Matsuoka M, Ichikawa M, et al. 2003. Brain-derived neurotrophic factor-dependent unmasking of "silent" synapses in the developing mouse barrel cortex. Proc Natl Acad Sci USA 100: 13069-13074.

Jing S, Tapley P, Barbacid M. 1992. Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron 9: 1067-1079.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, et al. 1986. Expression and structure of the human NGF receptor. Cell 47: 545-554.

Jones KR, Farinas I, Backus C, Reichardt LF. 1994. Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell 76: 989-999.

Kang HJ, Schuman EM. 1995. Neurotrophin-induced modulation of synaptic transmission in the adult hippocampus. J Physiol Paris 89: 11-22.

Kanning KC, Hudson M, Amieux PS, Wiley JC, Bothwell M, et al. 2003. Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability. J Neurosci 23: 5425-5436.

Kao S, Jaiswal RK, Kolch W, Landreth GE. 2001. Identification of the mechanisms regulating the differential activation of the MAPK cascade by epidermal growth factor and nerve growth factor in PC12 cells. J Biol Chem 276: 18169-18177.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF. 1991a. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252: 554-558.

Kaplan DR, Martin-ZancaD, Parada LF. 1991b. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature 350: 158-160.

Kaplan DR, Miller FD. 2000. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10: 381-391.

Khursigara G, Bertin J, Yano H, Moffett H, Di Stefano PS, et al. 2001. A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2. J Neurosci 21: 5854-5863.

Khursigara G, Orlinick JR, Chao MV. 1999. Association of the p75 neurotrophin receptor with TRAF6. J Biol Chem 274: 2597-2600.

Kim JY, Sutton ME, Lu DJ, Cho TA, Goumnerova LC, et al. 1999. Activation of neurotrophin-3 receptor TrkC induces apoptosis in medulloblastomas. Cancer Res 59: 711-719.

Kjoller L, Hall A. 1999. Signaling to Rho GTPases. Exp Cell Res 253: 166-179.

Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M. 1991a. The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65: 189-197.

Klein R, Lamballe F, Bryant S, Barbacid M. 1992. The trkB tyrosine protein kinase is a receptor for neurotrophin-4. Neuron 8: 947-956.

Klein R, Martin-Zanca D, Barbacid M, Parada LF. 1990. Expression of the tyrosine kinase receptor gene trkB is confined to the murine embryonic and adult nervous system. Development 109: 845-850.

Klein R, Nanduri V, Jing SA, Lamballe F, Tapley P, et al. 1991b. The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66: 395-403.

Klein R, Parada LF, Coulier F, Barbacid M. 1989. trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO J 8: 3701-3709.

Klein R, Silos-Santiago I, Smeyne RJ, Lira SA, Brambilla R, et al. 1994. Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature 368: 249-251.

Klein R, Smeyne RJ, Wurst W, Long LK, Auerbach BA, et al. 1993. Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell 75: 113-122.

Knipper M, Beck A, Rylett J, Breer H. 1993. Neurotrophin induced cAMP and IP3 responses in PC12 cells. Different pathways. FEBS Lett 324: 147-152.

Kojima M, Ikeuchi T, Hatanaka H. 1994. Nerve growth factor induces trkA mRNA expression in cultured basal forebrain cholinergic neurons from 17-day fetal rats. Neurosci Lett 169: 47-50.

Kong H, Boulter J, Weber JL, Lai C, Chao MV. 2001. An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. J Neurosci 21: 176-185.

Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, et al. 1999. Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398: 630-634.

Korte M, Staiger V, Griesbeck O, Thoenen H, Bonhoeffer T. 1996. The involvement of brain-derived neurotrophic factor in hippocampal long-term potentiation revealed by gene targeting experiments. J Physiol Paris 90: 157-164.

Kuruvilla R, Ye H, Ginty DD. 2000. Spatially and functionally distinct roles of the PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron 27: 499-512.

Lai KO, Fu WY, Ip FC, Ip NY. 1998. Cloning and expression of a novel neurotrophin, NT-7, from carp. Mol Cell Neurosci 11: 64-76.

Lai KO, Glass DJ, Geis D, Yancopoulos GD, Ip NY. 1996. Structural determinants of Trk receptor specificities using BDNF-based neurotrophin chimeras. J Neurosci Res 46: 618-629.

Lai KO, Ip NY. 2003. Central synapse and neuromuscular junction: same players, different roles. Trends Genet 19: 395-402.

Lamballe F, Klein R, Barbacid M. 1991. trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66: 967-979.

Lamballe F, Tapley P, Barbacid M. 1993. trkC encodes multiple neurotrophin-3 receptors with distinct biological properties and substrate specificities. EMBO J 12: 3083-3094.

Landreth GE, Shooter EM. 1980. Nerve growth factor receptors on PC12 cells: ligand-induced conversion from low- to high-affinity states. Proc Natl Acad Sci USA 77: 4751-4755.

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL. 1993. A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260: 315-319.

Lee FS, Chao MV. 2001. Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc Natl Acad Sci USA 98: 3555-3560.

Lee R, Kermani P, Teng KK, Hempstead BL. 2001. Regulation of cell survival by secreted proneurotrophins. Science 294: 1945-1948.

Lee KF, Li E, Huber LJ, Landis SC, Sharpe AH, et al. 1992. Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell 69: 737-749.

Lee FS, Rajagopal R, Chao MV. 2002a. Distinctive features of Trk neurotrophin receptor transactivation by G protein-coupled receptors. Cytokine Growth Factor Rev 13: 11-17.

Lee FS, Rajagopal R, Kim AH, ChangPC, Chao MV. 2002b. Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. J Biol Chem 277: 9096-9102.

Lesser SS, Sherwood NT, Lo DC. 1997. Neurotrophins differentially regulate voltage-gated ion channels. Mol Cell Neurosci 10: 173-183.

Lessmann V. 1998. Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. Gen Pharmacol 31: 667-674.

Levine ES, Kolb JE. 2000.Brain-derived neurotrophic factor increases activity of NR2B-containing *N*-methyl-D-aspartate receptors in excised patches from hippocampal neurons. J Neurosci Res 62: 357-362.

Levkovitz Y, Baraban JM. 2002. A dominant negative Egr inhibitor blocks nerve growth factor-induced neurite outgrowth by suppressing c-Jun activation: role of an Egr/c-Jun complex. J Neurosci 22: 3845-3854.

Lewin GR, Barde YA. 1996. Physiology of the neurotrophins. Annu Rev Neurosci 19: 289-317.

Li HS, Xu XZ, Montell C. 1999. Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. Neuron 24: 261-273.

Liepinsh E, Ilag LL, Otting G, Ibanez CF. 1997. NMR structure of the death domain of the p75 neurotrophin receptor. EMBO J 16: 4999-5005.

Liu HM, Lei HY, Kao KP. 1995. Correlation between NGF levels in wound chamber fluid and cytological localization of NGF and NGF receptor in axotomized rat sciatic nerve. Exp Neurol 132: 24-32.

Liu Y, Rohrschneider LR. 2002. The gift of Gab. FEBS Lett 515: 1-7.

Lohof AM, Ip NY, Poo MM. 1993. Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. Nature 363: 350-353.

Lu B. 2003. Pro-region of neurotrophins: role in synaptic modulation. Neuron 39: 735-738.

Lu B, Buck CR, Dreyfus CF, Black IB. 1989. Expression of NGF and NGF receptor mRNAs in the developing brain: evidence for local delivery and action of NGF. Exp Neurol 104: 191-199.

Maher PA. 1988. Nerve growth factor induces protein-tyrosine phosphorylation. Proc Natl Acad Sci USA 85: 6788-6791.

Marais R, Marshall CJ. 1996. Control of the ERK MAP kinase cascade by Ras and Raf. Cancer Surv 27: 101-125.

Mardy S, Miura Y, Endo F, Matsuda I, Sztriha L, et al. 1999. Congenital insensitivity to pain with anhidrosis: novel mutations in the TrkA (NTRK1) gene encoding a high-affinity receptor for nerve growth factor. Am J Hum Genet 64: 1570-1579.

Markus A, Zhong J, Snider WD. 2002. Raf and akt mediate distinct aspects of sensory axon growth. Neuron 35: 65-76.

Maroney AC, Finn JP, Bozyczko-Coyne D, O'Kane TM, Neff NT, et al. 1999. CEP-1347 (KT7515), an inhibitor of JNK activation, rescues sympathetic neurons and neuronally differentiated PC12 cells from death evoked by three distinct insults. J Neurochem 73: 1901-1912.

Marsh HN, Dubreuil CI, Quevedo C, Lee A, Majdan M, et al. 2003. SHP-1 negatively regulates neuronal survival by functioning as a TrkA phosphatase. J Cell Biol 163: 999-1010.

Martin-Zanca D, Barbacid M, Parada LF. 1990. Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev 4: 683-694.

Martin-Zanca D, Hughes SH, Barbacid M. 1986. A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature 319: 743-748.

Martin-Zanca D, Oskam R, Mitra G, Copeland T, Barbacid M. 1989. Molecular and biochemical characterization of the human trk proto-oncogene. Mol Cell Biol 9: 24-33.

Meakin SO, Mac Donald JI, Gryz EA, Kubu CJ, Verdi JM. 1999. The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. J Biol Chem 274: 9861-9870.

Medina DL, Sciarretta C, Calella AM, Bohlen und Halback von O, Unsicker K, et al. 2004. TrkB regulates neocortex formation through the Shc/PLCγ-mediated control of neuronal migration. EMBO J 23: 3803-3814.

Merlio JP, Ernfors P, Jaber M, Persson H. 1992. Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system. Neuroscience 51: 513-532.

Meyer-Franke A, Wilkinson GA, Kruttgen A, Hu M, Munro E, et al. 1998. Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. Neuron 21: 681-693.

Mi S, Lee X, Shao Z, Thill G, Ji B, et al. 2004. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. Nat Neurosci 7: 221-228.

Middlemas DS, Lindberg RA, Hunter T. 1991. trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol 11: 143-153.

Ming GL, Wong ST, Henley J, Yuan XB, Song HJ, et al. 2002. Adaptation in the chemotactic guidance of nerve growth cones. Nature 417: 411-418.

Minichiello L, Calella AM, Medina DL, Bonhoeffer T, Klein R, et al. 2002. Mechanism of TrkB-mediated hippocampal long-term potentiation. Neuron 36: 121-137.

Minichiello L, Korte M, Wolfer D, Kuhn R, Unsicker K, et al. 1999. Essential role for TrkB receptors in hippocampus-mediated learning. Neuron 24: 401-414.

Mobley WC, Woo JE, Edwards RH, Riopelle RJ, Longo FM, et al. 1989. Developmental regulation of nerve growth factor and its receptor in the rat caudate-putamen. Neuron 3: 655-664.

Morooka T, Nishida E. 1998. Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. J Biol Chem 273: 24285-24288.

Mu X, Silos-Santiago I, Carroll SL, Snider WD. 1993. Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. J Neurosci 13: 4029-4041.

Mukai J, Hachiya T, Shoji-Hoshino S, Kimura MT, Nadano D, et al. 2000. NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR. J Biol Chem 275: 17566-17570.

Murphy M, Reid K, Brown MA, Bartlett PF. 1993. Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. Development 117: 1173-1182.

Musatov S, Roberts J, Brooks AI, Pena J, Betchen S, et al. 2004. Inhibition of neuronal phenotype by PTEN in PC12 cells. Proc Natl Acad Sci USA 101: 3627-3631.

Mutoh T, Hamano T, Tokuda A, Kuriyama M. 2000. Unglycosylated Trk protein does not co-localize nor associate with ganglioside GM1 in stable clone of PC12 cells overexpressing Trk (PCtrk cells). Glycoconj J 17: 233-237.

Naylor RL, Robertson AG, Allen SJ, Sessions RB, Clarke AR, et al. 2002. A discrete domain of the human TrkB receptor defines the binding sites for BDNF and NT-4. Biochem Biophys Res Commun 291: 501-507.

Neves-Pereira M, Mundo E, Muglia P, King N, Macciardi F, et al. 2002. The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: evidence from a family-based association study. Am J Hum Genet 71: 651-655.

Ng YP, He W, Ip NY. 2003. Leukemia inhibitory factor receptor signaling negatively modulates nerve growth factor-induced neurite outgrowth in PC12 cells and sympathetic neurons. J Biol Chem 278: 38731-38739.

Nilsson AS, Fainzilber M, Falck P, Ibanez CF. 1998. Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. FEBS Lett 424: 285-290.

Ninkina N, Grashchuck M, Buchman VL, Davies AM. 1997. TrkB variants with deletions in the leucine-rich motifs of the extracellular domain. J Biol Chem 272: 13019-13025.

Pap M, Cooper GM. 1998. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem 273: 19929-19932.

Pease ME, McKinnon SJ, Quigley HA, Kerrigan-Baumrind LA, Zack DJ. 2000. Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma. Invest Ophthalmol Vis Sci 41: 764-774.

Peinado-Ramon P, Wallen A, Hallbook. F. 1998. MAP kinase phosphatase-1 mRNA is expressed in embryonic sympathetic neurons and is upregulated after NGF stimulation. Brain Res Mol Brain Res 56: 256-267.

Perez P, Coll PM, Hempstead BL, Martin-Zanca D, Chao MV. 1995. NGF binding to the trk tyrosine kinase receptor requires the extracellular immunoglobulin-like domains. Mol Cell Neurosci 6: 97-105.

Qian X, Ginty DD. 2001. SH2-B and APS are multimeric adapters that augment TrkA signaling. Mol Cell Biol 21: 1613-1620.

Qian X, Riccio A, Zhang Y, Ginty DD. 1998. Identification and characterization of novel substrates of Trk receptors in developing neurons. Neuron 21: 1017-1029.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325: 593-597.

Rajagopal R, Chen ZY, Lee FS, Chao MV. 2004. Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. J Neurosci 24: 6650-6658.

Rakhit S, Pyne S, Pyne NJ. 2001. Nerve growth factor stimulation of p42/p44 mitogen-activated protein kinase in PC12 cells: role of G(i/o), G protein-coupled receptor kinase 2, beta-arrestin I, endocytic processing. Mol Pharmacol 60: 63-70.

Rattiner LM, Davis M, French CT, Ressler KJ. 2004. Brain-Derived Neurotrophic factor and tyrosine kinase receptor B involvement in amygdale-dependent fear conditioning. J Neurosci 24: 4796-4806.

Rodriguez-Tebar A, Dechant G, Götz R, Barde YA. 1992. Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBO J 11: 917-922.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, et al. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370: 527-532.

Rong R, Ahn JY, Chen P, Suh PG, Ye K. 2003. Phospholipase activity of phospholipase C-gamma1 is required for nerve growth factor-regulated MAP kinase signaling cascade in PC12 cells. J Biol Chem 278: 52497-52503.

Rosini P, De Chiara G, Bonini P, Lucibello M, Marcocci ME, et al. 2004. Nerve growth factor-dependent survival of CESS B cell line is mediated by increased expression and decreased degradation of MAPK phosphatase 1. J Biol Chem 279: 14016-14023.

Ross GM, Shamovsky IL, Lawrance G, Solc M, Dostaler SM, et al. 1998. Reciprocal modulation of TrkA and p75NTR affinity states is mediated by direct receptor interactions. Eur J Neurosci 10: 890-898.

Roux PP, Barker PA. 2002. Neurotrophin signaling through the p75 neurotrophin receptor. Prog Neurobiol 67: 203-233.

Rudy B, Kirschenbaum B, Rukenstein A, Greene LA. 1987. Nerve growth factor increases the number of functional Na channels and induces TTX-resistant Na channels in PC12 pheochromocytoma cells. J Neurosci 7: 1613-1625.

Ryden M, Ibanez CF. 1996. Binding of neurotrophin-3 to p75LNGFR, TrkA, and TrkB mediated by a single functional epitope distinct from that recognized by trkC. J Biol Chem 271: 5623-5627.

Ryden M, Murray-Rust J, Glass D, Ilag LL, Trupp M, et al. 1995. Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75LNGFR in NT-4 signalling. EMBO J 14: 1979-1990.

Sachdev P, Zeng L, Wang LH. 2002. Distinct role of phosphatidylinositol 3-kinase and Rho family GTPases in Vav3-induced cell transformation, cell motility, and morphological changes. J Biol Chem 277: 17638-17648.

Salehi AH, Roux PP, Kubu CJ, Zeindler C, Bhakar A, et al. 2000. NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis. Neuron 27: 279-288.

Salehi AH, Xanthoudakis S, Barker PA. 2002. NRAGE, a p75 neurotrophin receptor-interacting protein, induces caspase activation and cell death through a JNK-dependent mitochondrial pathway. J Biol Chem 277: 48043-48050.

Samuels IS, Seibenhener ML, Neidigh KB, Wooten MW. 2001. Nerve growth factor stimulates the interaction of ZIP/p62 with atypical protein kinase C and targets endosomal localization: evidence for regulation of nerve growth factor-induced differentiation. J Cell Biochem 82: 452-466.

Savitz SI, Kessler JA. 2000. Leukemia inhibitory factor requires concurrent p75LNTR signaling to induce apoptosis of cultured sympathetic neurons. J Neurosci 20: 4198-4205.

Schecterson LC, Bothwell M. 1992. Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 9: 449-463.

Schinder AF, Poo M. 2000. The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci 23: 639-645.

Schneider R, Schweiger M. 1991. A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic trk and trkB tyrosine kinase receptors. Oncogene 6: 1807-1811.

Segal RA. 2003. Selectivity in neurotrophin signaling: theme and variations. Annu Rev Neurosci 26: 299-330.

Segal RA, Goumnerova LC, Kwon YK, Stiles CD, Pomeroy SL. 1994. Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. Proc Natl Acad Sci USA 91: 12867-12871.

Semkova I, Krieglstein J. 1999. Ciliary neurotrophic factor enhances the expression of NGF and p75 low-affinity NGF receptor in astrocytes. Brain Res 838: 184-192.

Shao Y, Akmentin W, Toledo-Aral JJ, Rosenbaum J, Valdez G, et al. 2002. Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. J Cell Biol 157: 679-691.

Shatzky S, Moses S, Levy J, Pinsk V, Hershkovitz E, et al. 2000. Congenital insensitivity to pain with anhidrosis (CIPA) in Israeli-Bedouins: genetic heterogeneity, novel mutations in the TrkA/NGF receptor gene, clinical findings, and results of nerve conduction studies. Am J Med Genet 92: 353-360.

Sklar P, Gabriel SB, McInnis MG, Bennett P, Lim YM, et al. 2002. Family-based association study of 76 candidate genes

in bipolar disorder: BDNF is a potential risk locus. Brain-derived neutrophic factor. Mol Psychiatry 7: 579-593.

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, et al. 1994. Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368: 246-249.

Sommerfeld MT, Schweigreiter R, Barde YA, Hoppe E. 2000. Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. J Biol Chem 275: 8982-8990.

Song HJ, Poo MM. 1999. Signal transduction underlying growth cone guidance by diffusible factors. Curr Opin Neurobiol 9: 355-363.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, et al. 1991. The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell 65: 895-903.

Sorkin A, Waters CM. 1993. Endocytosis of growth factor receptors. Bioessays 15: 375-382.

Spiegel S, Milstien S. 2002. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. Biochem Soc Trans 31 (Pt 6): 1216-1219.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, et al. 1991. trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell 65: 885-893.

Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, et al. 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-gamma 1 to mediate NGF responses. Neuron 12: 691-705.

Sutter A, Riopelle RJ, Harris-Warrick RM, Shooter EM. 1979. Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J Biol Chem 254: 5972-5982.

Takai S, Yamada M, Araki T, Koshimizu H, Nawa H, et al. 2002. Shp-2 positively regulates brain-derived neurotrophic factor-promoted survival of cultured ventral mesencephalic dopaminergic neurons through a brain immunoglobulin-like molecule with tyrosine-based activation motifs/Shp substrate-1. J Neurochem 82: 353-364.

Takeda K, Hatai T, Hamazaki TS, Nishitoh H, Saitoh M, et al. 2000. Apoptosis signal-regulating kinase 1 (ASK1) induces neuronal differentiation and survival of PC12 cells. J Biol Chem 275: 9805-9813.

Tessarollo L, Tsoulfas P, Martin-Zanca D, Gilbert DJ, Jenkins NA, et al. 1993. trkC, a receptor for neurotrophin-3, is widely expressed in the developing nervous system and in non-neuronal tissues. Development 118: 463-475.

Tolwani RJ, Cosgaya JM, Varma S, Jacob R, Kuo LE, et al. 2004. BDNF overexpression produces a long-term increase in myelin formation in the peripheral nervous system. J Neurosci Res 77: 662-669.

Toman RE, Payne SG, Watterson KR, Maceyka M, Lee NH, et al. 2004. Differential transactivation of sphingosine-1-phosphate receptors modulates NGF-induced neurite extension. J Cell Biol 166: 381-392.

Tsoulfas P, Soppet D, Escandon E, Tessarollo L, Mendoza-Ramirez JL, et al. 1993. The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. Neuron 10: 975-990.

Ultsch MH, Wiesmann C, Simmons LC, Henrich J, Yang M, et al. 1999. Crystal structures of the neurotrophin-binding domain of TrkA, TrkB and TrkC. J Mol Biol 290: 49-59.

Urfer R, Tsoulfas P, O'Connell L, Shelton DL, Parada LF, et al. 1995. An immunoglobulin-like domain determines the specificity of neurotrophin receptors. EMBO J 14: 2795-2805.

Vaillancourt RR, Gardner AM, Johnson GL. 1994. B-Raf-dependent regulation of the MEK-1/mitogen-activated protein kinase pathway in PC12 cells and regulation by cyclic AMP. Mol Cell Biol 14: 6522-6530.

Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, et al. 1999. Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol 146: 955-966.

Valenzuela DM, Maisonpierre PC, Glass DJ, Rojas E, Nunez L, et al. 1993. Alternative forms of rat TrkC with different functional capabilities. Neuron 10: 963-974.

van Weeren PC, de Bruyn KM, de Vries-Smits AM, van Lint J, Burgering BM. 1998. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol Chem 273: 13150-13156.

Vanhaesebroeck B, Waterfield MD. 1999. Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res 253: 239-254.

von Bartheld CS, Williams R, Lefcort F, Clary DO, Reichardt LF, et al. 1996. Retrograde transport of neurotrophins from the eye to the brain in chick embryos: roles of the p75NTR and trkB receptors. J Neurosci 16: 2995-3008.

von Schack D, Casademunt E, Schweigreiter R, Meyer M, Bibel M, et al. 2001. Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. Nat Neurosci 4: 977-978.

Wang X, Bauer JH, Li Y, Shao Z, Zetoune FS, et al. 2001. Characterization of a p75(NTR) apoptotic signaling pathway using a novel cellular model. J Biol Chem 276: 33812-33820.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z. 2002. P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420: 74-78.

Watson FL, Porcionatto MA, Bhattacharyya A, Stiles CD, Segal RA. 1999. TrkA glycosylation regulates receptor localization and activity. J Neurobiol 39: 323-336.

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, et al. 2001. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci 4: 981-988.

Weiner OD, Neilsen PO, Prestwich GD, Kirschner MW, Cantley LC, et al. 2002. A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. Nat Cell Biol 4: 509-513.

Wiesmann C, Ultsch MH, Bass SH, de Vos AM. 1999. Crystal structure of nerve growth factor in complex with the ligand-binding domain of the TrkA receptor. Nature 401: 184-188.

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, et al. 2002. A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 5: 1302-1308.

Wooten MW, Seibenhener ML, Mamidipudi V, Diaz-Meco MT, Barker PA, et al. 2001. The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. J Biol Chem 276: 7709-7712.

Wu C, Lai CF, Mobley WC. 2001. Nerve growth factor activates persistent Rap1 signaling in endosomes. J Neurosci 21: 5406-5416.

Xing J, Kornhauser JM, Xia Z, Thiele EA, Greenberg ME. 1998. Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation. Mol Cell Biol 18: 1946-1955.

Xu B, Goulding EH, Zang K, Cepoi D, Cone RD, et al. 2003. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat Neurosci 6: 736-742.

Yacoubian TA, Lo DC. 2000. Truncated and full-length TrkB receptors regulate distinct modes of dendritic growth. Nat Neurosci 3: 342-349.

Yamashita H, Avraham S, Jiang S, Dikic I, Avraham H. 1999. The Csk homologous kinase associates with TrkA receptors and is involved in neurite outgrowth of PC12 cells. J Biol Chem 274 (21): 15059-15065.

Yamashita T, Tohyama M. 2003. The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. Nat Neurosci 6: 461-467.

Yamauchi J, Chan JR, Shooter EM. 2003. Neurotrophin 3 activation of TrkC induces Schwann cell migration through the c-Jun N-terminal kinase pathway. Proc Natl Acad Sci USA 100: 14421-14426.

Yamauchi J, Chan JR, Shooter EM. 2004. Neurotrophins regulate Schwann cell migration by activating divergent signaling pathways dependent on Rho GTPases. Proc Natl Acad Sci USA 101: 8774-8779.

Yan Q, Radeke MJ, Matheson CR, Talvenheimo J, Welcher AA, et al. 1997. Immunocytochemical localization of TrkB in the central nervous system of the adult rat. J Comp Neurol 378: 135-157.

Yano H, Cong F, Birge RB, Goff SP, Chao MV. 2000. Association of the Abl tyrosine kinase with the Trk nerve growth factor receptor. J Neurosci Res 59: 356-364.

York RD, Molliver DC, Grewal SS, Stenberg PE, McCleskey EW, et al. 2000. Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. Mol Cell Biol 20: 8069-8083.

York RD, Yao H, Dillon T, Ellig CL, Eckert SP, et al. 1998. Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392: 622-626.

Zampieri N, Chao MV. 2004. Structural biology. The p75 NGF receptor exposed. Science 304: 833-834.

Zanellato A, Comelli MC, Dal Toso R, Carmignoto G. 1993. Developing rat retinal ganglion cells express the functional NGF receptor p140trkA. Dev Biol 159: 105-113.

Zapf-Colby A, Olefsky JM. 1998. Nerve growth factor processing and trafficking events following TrkA-mediated endocytosis. Endocrinology 139: 3232-3240.

Zupan AA, Osborne PA, Smith CE, Siegel NR, Leimgruber RM, et al. 1989. Identification, purification, and characterization of truncated forms of the human nerve growth factor receptor. J Biol Chem 264: 11714-11720.

# 3 CNTF and Related Neurokines

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**Abstract:** Ciliary neurotrophic factor (CNTF) is a member of the neurokine family of cytokines with actions on multiple cell types of the nervous system. CNTF shares a common gp130 receptor subunit with the other neurokines, leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11 (IL-11), cardiotrophin-1 (CT-1), oncostatin-M (OSM), cardiotrophin-like cytokine (CLC), and neuropoietin. Neurokine signaling is mediated principally through the Janus tyrosine kinase-signal transducer and activator of transcription (Jak/STAT) pathway. The biological actions of the various neurokines are overlapping in many instances, but can also be unique. While none of the neurokines appear to be essential individually, they are critical for life. Gene knockout studies reveal that loss of each of the individual receptor subunits results in embryonic or neonatal death. The actions of the neurokines vary depending on cell type and developmental stage. They may enhance the differentiation of glia while inhibiting differentiation of some neurons, promote neuronal survival while also inducing apoptosis of other neurons, promote neuronal repair while also promoting inflammatory responses, or induce early adrenergic differentiation in sympathetic neurons while later inducing adrenergic to cholinergic switching of phenotype. The basis for these varied effects is still not well understood. This chapter will highlight the actions of the neurokines and the current state of our understanding of the signaling pathway, with emphasis on activation and inactivation processes, and discuss some potential roles in neurodegenerative diseases and their treatment.

**List of Abbreviations:** ALS, amyotrophic lateral sclerosis; apoE, apolipoprotein E; ChAT, choline acetyltransferase; CLC, cardiotrophin-like cytokine; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin-1; CyRE, cytokine response element; EGFP, enhanced green fluorescent protein; FGF, fibroblast growth factor; GFAP, glia fibrillary acidic protein; HD, Huntington's disease; HGF, hepatocyte growth factor; IL-6, interleukin-6; IL-11, interleukin-11; Jak, Janus kinase; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; NGF, nerve growth factor; OSM, oncostatin-M; PIAS, protein inhibitor of activated STATs; SHP-1/2, src homology-2-domain-containing protein tyrosine phosphatase-1/2; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; VIP, vasoactive intestinal peptide

## 1 Introduction

Ciliary neurotrophic factor (CNTF) is a member of the neuropoietic cytokine family of proteins, also referred to as neurokines. This family includes avian and mammalian CNTF, interleukin-6 (IL-6), interleukin-11 (IL-11), leukemia inhibitory factor (LIF), oncostatin-M (OSM), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), and neuropoietin. These proteins share a common structural motif and a receptor-signal transduction system (Bazan, 1991). Their biological actions are diverse but very prominent in the hematopoietic system as cytokines and in the nervous system as neurotrophic factors. In this chapter we treat CNTF as a prototype neurokine and highlight key aspects of the other neurokines, reviewing their physiological actions, distribution, regulation, and mechanisms of action in nerve cells and muscle.

## 2 The Discovery and Characterization of CNTF

CNTF was originally described as a ciliary ganglion trophic activity isolated from the embryonic chick eye (Adler et al., 1979; Manthorpe et al., 1980; Nishi and Berg, 1981). It was hypothesized that it functions as a target-derived factor to support neuronal survival during the period of programmed cell death that coincides with cholinergic innervation of the eye by parasympathetic neurons of the chick ciliary ganglion (Landmesser and Pilar, 1974; Adler et al., 1979). CNTF was the second neuronal growth factor discovered after the nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951). The term ciliary neuronotrophic factor describing the survival activity first appeared in a 1984 paper by the Varon group (Barbin

et al., 1984). Nishi and Berg (1981) initially used the term "growth promoting activity," to describe this eye-derived component(s) that stimulated the growth of nerve cells. The activity was prominent in the ciliary neuronal target tissues of the embryonic eye, including the iris, choroid, ciliary body, and pigmented epithelium (Manthorpe et al., 1980; Barbin et al., 1984). Eventually the term was shortened to the term now used: CNTF (Hughes et al., 1988).

The CNTF moiety isolated from the chick eye has three distinguishing biological actions on chick ciliary ganglion neurons. It supports the in vitro survival of chick ciliary ganglion neurons in cell culture (Adler et al., 1979). It stimulates the growth of cell size, without increasing cell numbers (Nishi and Berg, 1981; Halvorsen and Berg, 1989). And finally, it downregulates the levels of alpha-7-subunit-containing acetylcholine receptors on peripheral neurons (Halvorsen and Berg, 1989). While the factor in chick eye resisted molecular purification and identity, all of these activities were shared with a mammalian CNTF-like activity isolated from rat sciatic nerve. The sciatic nerve proved to be a particularly rich source of CNTF activity and permitted its purification and molecular characterization (Manthorpe et al., 1986; Stöckli et al., 1989). However, as we will describe later, other key differences between the factors have emerged and it is likely that this rat CNTF may not be the true mammalian homolog for the original avian CNTF (Finn and Nishi, 1996b).

## 3 The Family of Neurokines

### 3.1 Chemical and Structural Aspects

The first CNTF genes cloned were from rabbit (Lin et al., 1989) and rat (Stöckli et al., 1989), and helped define a new class of neurotrophic factors that was distinct from the NGF and fibroblast growth factor (FGF) families. The human CNTF gene was found to be about 85% identical with rat and rabbit CNTF at the amino acid level (Lam et al., 1991). A puzzling finding from these first CNTF cloning and expression studies was the lack of predominant expression during embryonic development and the lack of a discernible secretory mechanism. It was questioned how this factor could be a significant developmental neurotrophic factor if it was not significantly expressed or secreted during embryogenesis. Later, when avian CNTF was cloned, it was found to be highly expressed during development and released from cells (Leung et al., 1992). Avian CNTF is secreted by a nonclassical pathway that is not used by mammalian CNTF (Reiness et al., 2001). These represent two key differences between mammalian and avian CNTFs (Finn and Nishi, 1996b).

The CNTFs fall into the greater family of proteins containing a four-alpha-helical core structure (Bazan, 1991). These cytokines are all composed of exchangeable functional modules and the crystal structures of LIF, CNTF, IL-6, growth hormone, and leptin all share this common design, as recently reviewed (Kallen et al., 1999; Auernhammer and Melmed, 2000). Several neurokines were first identified as factors active in the immune or hematopoietic systems. LIF and OSM were cloned and found to be glycoproteins active as tumor growth inhibitory factors (Gearing et al., 1987; Rose and Bruce, 1991). LIF was identified as a cholinergic differentiation factor, a glycoprotein from target tissues that induces a switch from adrenergic to cholinergic phenotype in some developing sympathetic neurons (Weber, 1981; Fukada, 1985). LIF is expressed in early embryos and along with CNTF induces stem cells to maintain pluripoteniality (Conover et al., 1993; Cheng et al., 1994; Kiger et al., 2001; Metcalf, 2003). IL-6 and IL-11 were first identified as cytokines important in the hematopoietic system as proinflammatory factors and were later found to be expressed in and functioning in the nervous system (Kishimoto et al., 1992; Yang, 1993; Marz et al., 1999). CT-1 was first described as a neurokine that induces cardiac myocyte hypertrophy and also as a motor neuron survival factor that binds to the LIF receptor complex (Pennica et al., 1995a, 1996). The newest members of the CNTF family are CLC and neuropoietin. CLC is a 22-kDa protein most homologous with CT-1 (Shi et al., 1999). Neuropoietin is a 22-kDa protein exhibiting a 16% identity with CNTF and an 11–27% identity with the other CNTF family members (Derouet et al., 2004). Two properties distinguish neuropoietin most from CNTF: it is expressed at early developmental stages and it possesses a potential hydrophobic signal peptide. These are properties previously associated with avian CNTF.

## 3.2 Distribution in Developing and Mature Nervous Systems

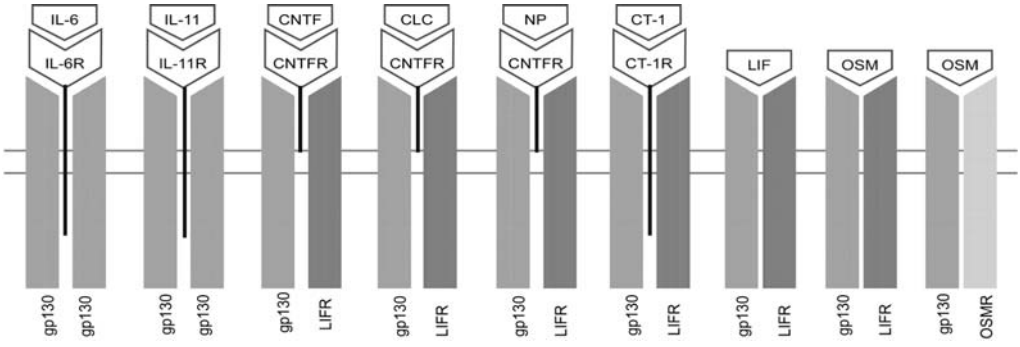
The distribution patterns of the neurokines in the nervous system are varied and too diverse to be adequately described here. Some key sites of expression are described in the following sections, but the reader is referred to other sources for more details (e.g., Leung et al., 1992; Murphy et al., 1997; Qiu et al., 1997; Mufson et al., 1999). Briefly though, the distribution of rat CNTF messenger RNA (mRNA) and protein is highest in optic nerve and olfactory bulb, but is also prominent in postnatal type I astrocytes and Schwann cells (Stöckli et al., 1991). Whereas CNTF is not heavily expressed in the embryo, both CLC and neuropoietin are expressed in embryonic tissues (Uemura et al., 2002; Derouet et al., 2004).

## 3.3 Receptors and Signaling Pathways in Brief

The responses of the neurokines are pleiotropic due to the utilization of receptors with a common set of subunits and signaling pathways (Boulanger et al., 2003a). The first CNTF receptor α subunit was cloned from human SH-SY5Y neuroblastoma cells and found to be an extracellular protein attached to the cell membrane through a glycosylphosphatidyl inositide linkage (Davis et al., 1991). The α subunit binds selectively with CNTF and combines with the transmembrane subunits gp130 and LIF receptor β for signaling (Ip et al., 1992b; Stahl et al., 1993). The known neurokine–receptor combinations with ligand-binding subunits are summarized in ❯ *Figure 3-1*. LIF does not require the α subunit for activity

**Figure 3-1**

**Receptor subunit composition for neurokines. The receptor subunits for the indicated neurokines are shown. The vertical lines indicate the interaction of the alpha subunits with the plasma membrane, either as an integral membrane protein or as a glycophosphatidyl inositol linkage. *CLC* cardiotrophin-like cytokine, *CNTF* ciliary neurotrophic factor, *CT-1* cardiotrophin-1, *IL-6* interleukin-6, *IL-11* interleukin-11, *LIF* leukemia inhibitory factor, *OSM* oncostatin-M, *NP* neuropoietin, *CNTFR* CNTF receptor α subunit, *CT-1R* CT-1 receptor subunit, *IL-6R* IL-6 receptor α subunit, *IL-11R* IL-11 receptor α subunit, *LIFR* LIF receptor β subunit, *OSMR* OSM β receptor subunit**




but competes with CNTF for binding, perhaps through competition between the CNTF-receptor α complex and LIF with the gp130–LIF receptor β complex (Robledo et al., 1996). Structural models derived from studies of the IL-6 and IL-11 receptor complexes suggest that the overall complex is hexameric with two copies each of the ligand, binding subunits, and gp130 (Ward et al., 1994; Barton et al., 2000; Boulanger et al., 2003b). Evidence suggests that CNTF may also form a hexameric receptor complex (De Serio et al., 1995).

The CNTF receptor complex is used by a subclass of neurokines: CNTF, CLC, and neuropoietin (Derouet et al., 2004). CLC is indistinguishable in action from CNTF (Senaldi et al., 1999). It is likely that CNTF receptor α is used by different neurokines during development, for differentiation, and for

maintenance/repair functions. This mechanism for varied function contrasts with that of neurotransmitter receptors. Whereas an individual transmitter uses a variety of different receptors to achieve selectivity in responses, the CNTF receptor has several different ligands to regulate its activity.

Although the signaling pathway for these neurokines is often referred to as the Janus tyrosine kinase-signal transducer and activator of transcription or the "Jak/STAT" pathway, various downstream mediators other than STATs can also be activated. STAT, or signal transducer and activator of transcription, is the principal signaling molecule for all neurokines. gp130 and LIF receptor β each have an associated Jak-binding site (usually Jak1 or Jak2) located near the plasma membrane (Stahl et al., 1994). Upon ligand binding, receptor conformational changes result in the transphosphorylation and activation of these Jaks. Substrates for the Jaks include several docking sites on the gp130 and LIF receptor β (Davis et al., 1993) that when phosphorylated can bind to src homology 2 (SH2) domains of STATs, usually STAT1 and/or STAT3 (Baumann et al., 1994). The STATs are then phosphorylated and activated by Jak, resulting in their release, dimerization, and translocation into the nucleus to regulate gene expression. STAT regulatory elements are present on a number of different genes. The final transcriptional complexes can vary but are likely to include components in addition to the STATs. Although much is known about the specific structural requirements for these events on the Jaks, STATs, and receptors, the mechanisms and other protein participants are still only broadly defined (Pellegrini and Dusanter-Fourt, 1997; Horvath, 2000; Rane and Reddy, 2000; Kisseleva et al., 2002).

Activation of signaling pathways other than STATs can also be initiated by Jak kinases. Jak can activate an SH2-binding site on the receptor and stimulate SHP-1 or SHP-2 tyrosine phosphatase and mitogen-activated protein kinase (MAPK) activity through Ras binding, and Akt can be activated through phosphatidyl inositol-3 kinase. There is evidence from selected systems that MAPK and STAT activation have opposing actions (Wu and Bradshaw, 1996). These alternative Jak signaling pathways have been discussed in a number of recent reviews (Stahl and Yancopoulos, 1994; Inoue et al., 1996; Auernhammer and Melmed, 2000; Decker and Kovarik, 2000).

## 3.4 Gene Knockout Studies

The importance of the CNTF receptor signaling system in development is clearly illuminated by the mouse phenotype of the α subunit knockout, which is lethal perinatally and displays severe motor deficiencies (DeChiara et al., 1995). Null mutants of LIF receptor β subunit also die shortly after birth with multiple problems including reduced astrocyte numbers in the spinal cord and brain stem (Li et al., 1995; Ware et al., 1995). In both cases the receptor mutants result in much more dramatic phenotypes than do null mutants of the factors themselves. CNTF or LIF gene knockouts result in a relatively small amount of motor neuron defects (Masu et al., 1993; Kwon et al., 1995). LIF or CNTF gene knockout, though, does reveal deficits in maintenance of motor neurons and in response following neural trauma. Mice with a LIF/CNTF double gene knockout or LIF/CNTF/CT-1 triple gene knockout show potentiated motor neuron losses (Sendtner et al., 1996; Holtmann et al., 2005). These results strongly discount an absolute requirement for these individual growth factors in nervous system development. Further, the human phenotype for null CNTF appears to have little or no impact on occurrence of several neurodegenerative diseases (Orrell et al., 1995).

# 4 Actions in the Peripheral Nervous System

## 4.1 Autonomic Nervous System

### 4.1.1 Parasympathetic Nervous System

The importance of avian CNTF in the development of chick ciliary ganglion neurons supports the role of CNTF as a target-derived neurotrophic factor. CNTF supports the complete survival of ciliary ganglion

neurons in culture and promotes their growth in size and regulates the expression of a subclass of acetylcholine receptors on the cells (Nishi and Berg, 1981; Halvorsen and Berg, 1989). Ciliary neurons in culture bind to $^{125}$I-CNTF with high affinity and CNTF induces a persistent activation of Jak/STAT signaling (Koshlukova et al., 1996; Wishingrad et al., 1997). Cloning of the chick CNTF gene indicates that the avian form of CNTF differs in significant ways from mammalian CNTF in being expressed during early development and in being secreted as a functional protein from target cells (Leung et al., 1992). Further, CNTF is expressed in eye targets of ciliary ganglion neuron during development where it is the only trophic activity present (Finn and Nishi, 1996a). Retroviral overexpression of CNTF in ovo results in rescue of about 30%, on average, of ciliary neurons that normally die during programmed cell death, and under-expression results in increased cell death (Finn et al., 1998). Any role for other CNTF family members in parasympathetic neuron activity has yet to be identified.

### 4.1.2 Sympathetic Nervous System

CNTF and LIF have significant effects on sympathetic neuron development, both in vivo and in culture. CNTF binds to chick sympathetic neurons in culture and in freshly isolated cells with high affinity (Huber et al., 1993), and in rat cells both LIF and CNTF induce the activation of Jak/STAT signaling (Guo et al., 1999; Kaur et al., 2003). Sympathetic neurons also make and respond to IL-6 (März et al., 1998). CNTF increases vasoactive intestinal peptide (VIP), somatostatin, and substance P expression in sympathetic neurons (Ernsberger et al., 1989; Rao et al., 1992) and decreases neuropeptide Y and muscarinic and nicotinic acetylcholine receptors (Halvorsen and Berg, 1989; Ludlam and Kessler, 1993); LIF-receptor-null mutations result in decreased numbers of VIP-positive neurons (Duong et al., 2002). CNTF promotes the terminal differentiation of cultured progenitor MAH cells into sympathetic neurons (Ip et al., 1994). Chick sympathetic neurons change their sensitivity to CNTF during development. At embryonic day 7, CNTF does not affect the relative expression of choline acetyltransferase (ChAT) and tyrosine hydroxylase but does increase VIP, while at day 12 it increases both VIP and ChAT and decreases tyrosine hydroxylase (Ernsberger et al., 1997). In the rat, gp130 neurokines act as cholinergic differentiation factors for sympathetic neurons. LIF was the first factor identified from heart with the ability to convert sympathetic neurons from an adrenergic to a cholinergic phenotype (Yamamori et al., 1989). The role of gp130 neurokines in the sympathetic cholinergic phenotype has been previously reviewed (Landis, 1996). At higher concentrations, LIF and CNTF cause cell death in a subpopulation of neonatal rat sympathetic neurons in culture (Kessler et al., 1993; Savitz and Kessler, 2000) while later in development CNTF acts as a survival factor for these same neurons (Kotzbauer et al., 1994). Both LIF and CNTF block bone morphogenetic protein-induced dendritic outgrowth in rat sympathetic neurons in culture (Guo et al., 1997, 1999). Neurokines have varied effects on sympathetic neurons, and the regulatory mechanisms for this variability are not yet clear.

### 4.1.3 Sensory Neurons

Subpopulations of sensory neurons respond to neurokines similarly to that of sympathetic neurons. In chick dorsal root ganglion there is early expression of CNTF receptor followed by a survival response protecting cells from programmed cell death (Tolosano et al., 1996; von Holst et al., 1997). Transfection of sensory neurons with antisense gp130 leads to a decreased number of VIP-positive cells (Geissen et al., 1998). CNTF in vivo increases substance P and calcitonin-gene-related peptide (CGRP) expression in sensory neurons (Apfel et al., 1993). Cranial nodose and trigeminal ganglion neurons develop sensitivity to the survival effects of neurokines differently, and sensory neuron populations display different developmental patterns of neurokine responsiveness to CNTF, LIF, OSM, CT-1, and IL-6 (Horton et al., 1998).

# 5 Actions in the Central Nervous System

## 5.1 Motor Neurons

### 5.1.1 Survival

Motor neurons are an especially neurokine-sensitive population of cells. Survival of chick motor neurons was enhanced in ovo by CNTF while sympathetic and sensory neurons were not enhanced (Oppenheim et al., 1991). In other studies, a role for CNTF-related neurokines is implicated during developmental programmed cell death as LIF-receptor-null mutant mice show a 35–50% decline of motor neuron numbers at birth (Li et al., 1995). Both IL-6 and LIF promote survival of cholinesterase-positive rat spinal cord neurons in culture (Kushima and Hatanaka, 1992). Data supporting the possible role for CNTF and LIF as trauma-related survival factors come from axotomy studies where LIF, CNTF, and IL-6 are each able to protect spinal cord motor neurons from cell death after axotomy (Sendtner et al., 1990; Helgren et al., 1994; Li et al., 1994; Ikeda et al., 1996). The mechanism of CNTF-mediated motor neuron survival may be through stimulation of PAP-1 and Reg-2 activity (Liou et al., 1997; Nishimune et al., 2000).

### 5.1.2 Motor Neuron Diseases

Several animal models of motor neuron diseases are responsive to exogenous neurokines. The Wobbler mouse shows delayed onset and less severe symptoms following treatment with CNTF (Mitsumoto et al., 1994). In the *pmn* mouse model of progressive motor neuronopathy, endogenous CNTF released upon axotomy results in enhanced motor neuron survival (Sendtner et al., 1992, 1997). These and other results have provided an impetus for pursuing this class of neurokines as potential therapeutic agents in neurodegenerative motor neuron disease such as amyotrophic lateral sclerosis (ALS).

## 5.2 Brain Neurons

### 5.2.1 Differentiation and Development

CNTF and LIF each show early effects in the central nervous system (CNS) by maintaining neural stem cells as pluripotent (Conover et al., 1993; Niwa et al., 1998; Moon et al., 2002). Neurokines also have later developmental effects on CNS neurons, especially in the retina. CNTF is expressed in pigmented epithelium and macroglia of retina (Finn and Nishi, 1996a; Walsh et al., 2001). CNTF treatment of rat retinal explants redirects rod photoreceptors to other cell types including bipolar, amacrine, and Müller glia cells (Ezzeddine et al., 1997). In culture, CNTF and LIF delay rod photoreceptor cell development in rats (Kirsch et al., 1998b), and CNTF inhibits the development of photoreceptor-like cells in the postnatal pineal gland (Hata et al., 2003). In the chick, CNTF increases ChAT levels in cultured retina (Hofmann, 1988). In the olfactory system, LIF inhibits olfactory receptor neurons' maturation by activating the STAT3 signaling pathway, maintaining a population of olfactory receptor neurons in an immature state (Pain et al., 1996). To generalize, the neurokines appear to delay progression of neurons early in development but often promote to specific phenotypes later.

### 5.2.2 Cell Survival and Regeneration

Survival effects of neurokines are widespread in the CNS. CNTF exhibits survival effects on a variety of CNS neurons including rat hippocampal GABAergic and cholinergic neurons (Ip et al., 1991). CNTF protects against lesioning-induced cell death in thalamic nuclei (Clatterbuck et al., 1993) and lateral geniculate

nuclei (Agarwala and Kalil, 1998) and promotes neurite outgrowth in acoustic neurons (Hartnick et al., 1996). IL-6 also acts as a survival factor for postnatal and fetal tyrosine-hydroxylase-positive cells in the rat midbrain (Kushima et al., 1992).

## 5.3 Glia

### 5.3.1 Development

One of the earliest descriptions of CNTF action in the CNS is that of induction of differentiation of oligodendrocytes O-2A into type 2 astrocytes (Hughes et al., 1988). CNTF and LIF each promote the differentiation of oligodendroglial progenitors (Marmur et al., 1998) and CNTF-null mice exhibit defects in glial maturation (Martin et al., 2003). The CNTF stimulation of cortical precursor cells to differentiate to a glial rather than a neuronal lineage is via the Jak/STAT pathway as opposed to the MAPK signaling pathway (Bonni et al., 1997). LIF receptor and gp130 subunits are critical for glia development, as knockouts of either decrease astrocytic glia fibrillary acidic protein (GFAP) expression (Ware et al., 1995; Nakashima et al., 1999).

### 5.3.2 Expression

Using immunocytochemistry and in situ hybridization, CNTF and its receptor were shown to be constitutively expressed in different astrocyte populations in the mouse brain (Dallner et al., 2002). There have been few details reported of regulation of CNTF expression in glia of the CNS (however, see below for effects of trauma), but expression of CNTF in Schwann cells is decreased by retinoic acid and by inhibition of the ras-Erk pathway (Abe et al., 2001; Johann et al., 2003). Determining the cellular and molecular mechanisms of regulating neurokine expression during glia development is an important ongoing endeavor.

### 5.3.3 Role in Response to Injury and Trauma

Neural injury and trauma are interrelated with neurokines in different ways. An injury may induce neurokine expression, or these neurokines may promote an injury response, or they may help the injured cells survive the insult. In the hippocampus, lesioning and ischemia each lead to increased expression of CNTF by astrocytes (Ip et al., 1992a; Park et al., 2000). There is also an increase in glial CNTF and receptor expression after entorrhinal cortical lesion (Lee et al., 1997b). In adult rat neocortex, CNTF injection or overexpression causes glial cell hypertrophy or a reactive gliosis (Winter et al., 1995; Hudgins and Levison, 1998) and induces astrocytes to increase FGF-2 during remyelination in the spinal cord (Albrecht et al., 2002, 2003). CNTF knockout mice show impaired recovery from sciatic nerve crush (Yao et al., 1999) while IL-6 mRNA production increases in Schwann cells following sciatic nerve injury (distal to the crush), indicative of an inflammatory reaction (Bolin et al., 1995). Effects on the CNS may be different from those in the periphery as optic nerve lesioning results in increased CNTF receptor mRNA in remaining astrocytes at the site as compared with a decrease in receptor mRNA seen in Schwann cells of sciatic nerve (Kirsch et al., 1998a).

# 6 Neurokines in Nonnerve Tissues

## 6.1 Skeletal Muscle

Skeletal muscle is a major nonnerve target and source of neurokines (Jordan, 1996a). Denervation of rat skeletal muscle leads to increased CNTF receptor expression and treatment with CNTF attenuates the

denervation-induced atrophy and increases twitch tension (Helgren et al., 1994). Even without axonal sprouting denervation CNTF treatment maintains twitch tension with age and thus may function as a regulator of muscle strength (Guillet et al., 1999). CNTF induces motor neuron at termini and increases the number of nerve–muscle contacts (Jordan, 1996b; Oyesiku and Wigston, 1996; Siegel et al., 2000). CNTF treatment during neuromuscular junction formation causes muscle to maintain a polyneural innervation (English and Schwartz, 1995). Neurotransmitter release from Xenopus motor neurons in culture is enhanced by CNTF, suggesting a role in enhancing or maintaining synaptic strength (Stoop and Poo, 1995). In immature muscle these neurokines may have a different role as LIF activation of STAT3 inhibits MyoD activity, promoting cell cycle progression and inhibiting differentiation of myoblasts (Kataoka et al., 2003).

## 6.2 Cardiac Muscle

The cloning and expression of CT-1 provided evidence for possible pathological effects of neurokines in cardiac muscle (Pennica et al., 1995b). CT-1, LIF, and other neurokines induce muscle hypertrophy in vitro and in vivo (Kodama et al., 1997; Wang and Halvorsen, 1998b). Both CT-1 and LIF were found to promote neonatal mouse cardiac myocyte proliferation and survival (Sheng et al., 1996). Evidence for a developmental role of CNTF is found in chick heart as well, where treatment of cultured cardiomyocytes with the differentiation factor retinoic acid results in increased expression of CNTF receptor α mRNA and enhanced CNTF responses (Wang and Halvorsen, 1998a). CNTF and its receptor are expressed in embryonic chick heart at their highest levels during parasympathetic synapse formation in the atria. CNTF receptor mRNA levels are increased by stimulating muscarinic acetylcholine receptors in ovo by carbamylcholine and are inhibited by blocking cholinergic receptors with atropine. Further, CNTF treatment of cultured atrial myocytes increased levels of muscarinic receptors. Thus, CNTF enhances cholinergic development and cholinergic activity increases CNTF responsiveness of chick atrium, suggesting a postsynaptic role for CNTF in the ontogenesis of parasympathetic function in the heart (Wang and Halvorsen, 1998b).

## 6.3 Other Tissues

Examination of LIF receptor β subunit knockout mice reveals malfunctions in placental, skeletal, neural, and metabolic systems, suggesting important effects of CNTF-related neurokines in many different organ systems (Ware et al., 1995; Aubert et al., 1999). CNTF and IL-6 induce an acute phase response in liver and inhibit IL-1-beta-mediated insulin release in rat pancreatic islets (Nesbitt et al., 1993; Wadt et al., 1998) and LIF has effects on hepatic lipid metabolism (Nonogaki et al., 1996; Auernhammer and Melmed, 2000). A number of studies have implicated roles of IL-6, IL-11, OSM, LIF, and CNTF in osteoblasts, either in bone metabolism, in development of osteoblasts, or in osteogenesis (Allan et al., 1990; Ishimi et al., 1992; Bellido et al., 1996; Grimaud et al., 2002). LIF has been proposed as an important neuroimmune modulator of endocrine function (Auernhammer and Melmed, 2000). The widespread systemic effects of neurokines are important factors when considering their use as therapeutic agents.

# 7 The gp130 Receptor Family

## 7.1 The Receptor Complex

### 7.1.1 Structure–Function Relationships

The common structure of the CNTF neurokine receptors is responsible for both the pluripotent nature of the neurokines and their redundancy in function. The receptor complexes present in two fundamental groups: either with two copies of gp130 signaling subunits or as one copy each of a gp130 and a LIF receptor β subunit (*Figure 3-1*). The LIF receptor β structure is related to gp130, the signal transducer of IL-6

(Gearing et al., 1991). The exception to these groupings is a type of OSM receptor that uses an OSM receptor β component in lieu of the LIF receptor β subunit (Mosley et al., 1996). The only clearly demonstrated function of the α receptor components is to specify ligand binding.

The redundant biological activities observed for the neurokines is a result of the shared gp130 receptor signaling component, whereas the unique actions are a result, at least in part, of the varied β receptor components. Since there is little difference in the overall types of signaling possible by the neurokines, the molecular bases for these differences in signaling of the receptor subunits is an area of intense interest. The relative roles of receptor signaling subunits in signal transduction have been studied using chimeric receptors composed of a homodimer of extracellular domains of non-CNTF family cytokine receptors coupled with the transmembrane and cytoplasmic regions of either OSM receptor, LIF receptor, or gp130 as intracellular signaling domains (Baumann et al., 1994). Examination of specific responses reveals some cell-type-specific effects. Thus cell proliferation, induction of differentiation, and inhibited differentiation are seen only in selected cell types (Starr et al., 1997; Hermanns et al., 1999). The receptor composition, as well as the environment of the targeted cell, therefore dictates the final cellular response from a specific neurokine.

To determine the roles of the signaling domains within LIF and OSM receptors, OSM receptor and LIF receptor progressive c-terminal truncations were generated. After reconstitution of receptor function in receptor-negative Hep3B hepatoma cells it was found that the distal sequence motif of the OSM receptor was required for signal transduction by the OSM-specific receptor but that LIF signaling was not strictly dependent on the same elements (Kuropatwinski et al., 1997). Further, Hermanns et al. (2000) found that OSM is better than LIF or IL-6 at activating extracellular signal-regulated kinase 1/2 because of Tyr861 on the OSM receptor. Examination of the six carboxy-terminal tyrosine motifs implicated in recruiting STATs to gp130 of the IL-6 receptor reveals they are not equivalent in their capacity for activating STAT factors and genes (Baumann et al., 1994; Schmitz et al., 2000b; Boulanger et al., 2003a).

### 7.1.2 Expression

Although all neurokines share receptor subunits, it is the CNTF receptor α subunit that provides selectivity for CNTF, neuropoietin, and CLC action. Yancopoulos and coworkers first cloned mammalian CNTF receptor α genes and reported their expression in rats and humans (Ip et al., 1993). Several groups have reported in depth on the distribution and variability of the expression of the various receptor subunits in the nervous system; here we will provide just a brief overview. The distribution of CNTF receptor α is widely expressed in embryonic and neonatal rat brain (Kirsch and Hofmann, 1994; Seniuk-Tatton et al., 1995; Maclennan et al., 1996; Lee et al., 1997a). The chick CNTF receptor α is 70% identical to the human CNTF receptor α protein (Heller et al., 1995; Ip et al., 1995). In the chick, expression of the CNTF receptor α subunit is principally localized to the nervous system (Fuhrmann et al., 2003), including the neurons of the peripheral nervous system (PNS); parasympathetic, sympathetic, and sensory neurons; and CNS neurons including motor neurons, retinal ganglion cells, and amacrine cells, all neurons that are known to respond to CNTF by increased survival or differentiation. It is also expressed in cardiac and skeletal muscle (Jordan, 1996b; Wang and Halvorsen, 1998b). Expression of IL-11 receptor α subunit in mouse brain has also been described (Hilton et al., 1994). gp130 expression in rat brain by using immunocytochemistry shows a distribution more widespread than that of the CNTF receptor α or the LIF receptor (Watanabe et al., 1996). LIF-binding sites in rat cranial motor neurons remain mostly steady between embryonic day 18 and postnatal day 21 while levels in other CNS locations begin low and rise between postnatal day 7 and 21 and in the rat PNS, levels start higher and then decline over this period (Qiu et al., 1997). The results support varied roles for these neurokines in different neural populations and at different times in development.

### 7.1.3 Regulation

The ability of a cell to respond selectively to a specific neurokine depends on the appropriate expression of receptor components. A thorough understanding of the mechanisms that regulate the expression during

development and after injury will be key to understanding tissue responsiveness. Agents that promote neural differentiation also affect CNTF responsiveness. Activation of protein kinase C in SH-SY5Y neuroblastoma cells via exposure to phorbol ester causes adrenergic differentiation and a downregulation of CNTF receptor α subunit mRNA and CNTF sensitivity. Treatment of cells with the cholinergic differentiation agent retinoic acid causes an upregulation of CNTF receptor α and gp130 mRNA and an increase in sensitivity to CNTF (Malek and Halvorsen, 1997). In the CNS, axotomy induces an increase in CNTF receptor α in rat medial septal neurons (Lee et al., 1997c). While CNTF receptor α expression was not expressed in regions of the medial septal nucleus of control animals, upregulation was observed in neurons after fimbria–fornix transection. Studies of the LIF receptor β subunit using protein kinase inhibitors and point mutations indicate that downregulation depends on activation of extracellular signal-regulated kinase 1/2 and serine phosphorylation of the cytoplasmic domain of the LIF receptor (Blanchard et al., 2000).

Young et al. (1997, 1998) identified a conserved hormone response element in the α component of the CNTF receptor gene for human TR4 and TR2 orphan steroid receptors. They further showed that CNTF could increase TR4 expression and enhance the DNA-binding capacity of TR4. In situ hybridization results showed TR4 transcripts expressed in a pattern similar to that of CNTF receptor α in the developing and postnatal nervous systems. Their data suggest an interaction between TR2/TR4 and the CNTF signaling pathway during neurogenesis. There is also evidence for an interaction between CNTF receptor expression and androgens in skeletal muscle. Androgen expression in mice lacking a functional CNTF receptor α results in fewer than half as many spinal bulbocavernosus motoneurons than did wild-type male mice, suggesting that the expression of CNTF receptor is androgen-regulated in rat spinal motoneurons (Forger et al., 1997, 1998). So CNTF sensitivity of cells is modulated by a variety of regulatory agents.

## 7.2 The Jak/STAT Signaling Pathway

### 7.2.1 The Activation Cascade: from Plasma Membrane to the Nucleus

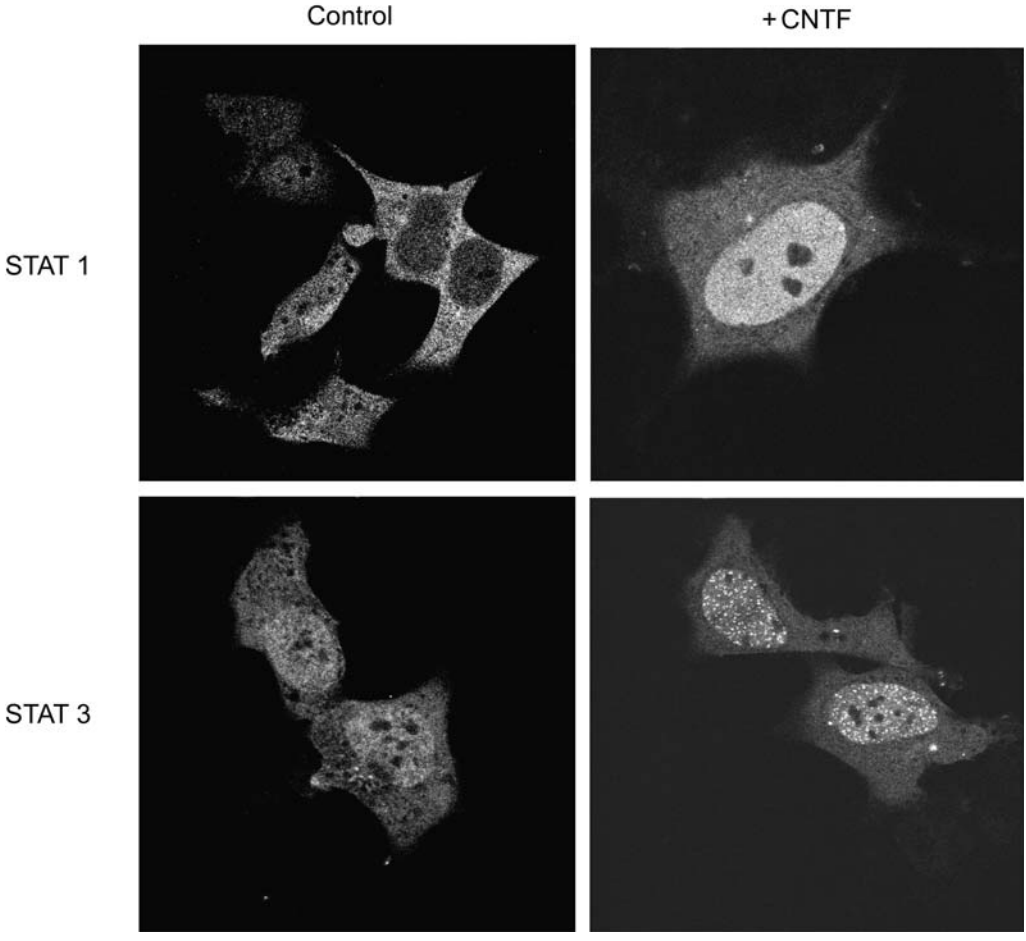
CNTF binds to the CNTF receptor α subunit, which is coupled to a heterodimer consisting of gp130 and the LIF receptor beta subunit (Davis et al., 1991; Stahl et al., 1993, 1994). LIF receptor beta and gp130 are associated with a cytosolic tyrosine kinase, either Jak1 or Jak2, that can induce tyrosine phosphorylation of STAT1 and STAT3 docking sites on the receptor. Once associated with the activated receptor, tyrosine phosphorylation of STAT results in its dimerization and subsequent binding to specific DNA sequences in the nucleus to regulate gene transcription (❯ *Figure 3-2*) (Bonni et al., 1993; Boulton et al., 1994; Taga, 1996; Darnell, 1997; Malek and Halvorsen, 1997; Wishingrad et al., 1997). CNTF induces the phosphorylation by Jak on tyrosine residues and by another kinase(s) on serine/threonine residues, preferentially of STAT3, and to a lesser extent, STAT1. There have been a number of recent reviews of the Jak/STAT pathway dealing with their biological roles (Leonard and O'Shea, 1998; Rane and Reddy, 2000), the structure–function relationships of Jaks and STATs (Pellegrini and Dusanter-Fourt, 1997; Grotzinger, 2002), serine phosphorylation of STAT (Decker and Kovarik, 2000), and results from knockout mice (Akira, 1999; Kisseleva et al., 2002). Evidence for an inhibitory tyrosine phosphorylation site on Jak2 has also been presented (Feener et al., 2004). Treatment of cells with a protein phosphatase inhibitor prevents subsequent neurokine-mediated activation of Jak and terminates nuclear signaling by STATs (Jiao et al., 2003). The identity of this hypothesized phosphatase and whether its site of action is on an inhibitory Jak phosphotyrosine is still unknown.

It appears that both Jak1 and Jak2 are utilized in gp130-containing receptors. Jak1 knockout mice are small at birth, die perinatally, and fail to respond to gp130 neurokines while Jak2 knockout mice exhibit only partial LIF and IL-6 signaling (Neubauer et al., 1998; Parganas et al., 1998; Rodig et al., 1998). These results suggest that Jak1 is absolutely required for neurokine signaling while Jak2 is not an absolute requirement. However, it remains to be determined whether these requirements vary among cell types.

CNTF-related factors that act as target-derived neurotrophic factors have an important barrier to overcome in transmitting their signal to the cell nucleus from the nerve terminal. An unresolved question is whether STATs are activated at the nerve terminal or site of axonal injury and then transported to the

**Figure 3-2**

**Nuclear translocation of STATs following CNTF treatment. BE(2)-C neuroblastoma cells were transfected with either STAT1-EGFP (STAT1) or STAT3-EGFP (STAT3) DNA. Cells were treated with CNTF 1 nM for 30 min or left untreated (control) as indicated and then fixed and observed by confocal microscopy as previously described (Kaur et al., 2005)**




nucleus in order to affect gene expression or if an activated receptor–Jak complex is transported to the cell body where STATs are activated near the nucleus. The nature of this transported signaling complex has not yet been defined. In two studies, radiolabeled CNTF was not shown to undergo detectable retrograde transport in peripheral nerves (Smet et al., 1991; Gupta et al., 1992). However, in other studies there was evidence for retrograde LIF transport (Hendry et al., 1992; Curtis et al., 1994; Ure and Campenot, 1994). Biotinylated LIF was also shown to be retrogradely transported in a subpopulation of sensory neurons in vivo (Thompson et al., 1997). In one study, retrograde transport of CNTF from nerve terminals in sensory neurons was shown to increase after neural injury (Curtis et al., 1993). Whether retrogradely transported LIF or CNTF detected in these studies functions as part of a signaling complex or is simply part of a protein degradation process has not been clarified.

The pathways and genes that are regulated to produce the physiological effects of CNTF neurokines are still being defined. For example, in a rat oligodendroglial progenitor cell line, CNTF activates the GFAP

gene and several putative CNTF-response elements were identified in regions of the GFAP gene promoter (Kahn et al., 1997). CNTF stimulates differentiation of cerebral cortical precursor cells into astrocytes in preference to a neuronal phenotype. Even though CNTF stimulates both the Jak/STAT and the Ras-MAPK signaling pathways in these cells, it is the Jak/STAT pathway that enhances gliogenesis (Bonni et al., 1997). CNTF, LIF, IL-6, and CT-1 mediate survival in developing rat sensory neurons by STAT-mediated activation of NF-κB (Middleton et al., 2000).

Some DNA elements that bind to the STAT transcription complexes have been described and these are of intense interest in order to identify the genes regulated by the neurokines (Decker et al., 1997). In the nonbreakable filament length (NBFL) neuroblastoma cell line and in sympathetic neurons, CNTF, LIF, and OSM each activate VIP gene transcription through a 180-bp cytokine response element (CyRE). Deletion analysis of the VIP CyRE reveals multiple regions are important for CNTF-mediated transcriptional activity but that the STAT element is absolutely required for VIP gene activation (Symes et al., 1994). CNTF causes a rapid and transient increase in expression of the immediate early gene, c-fos, in human SH-SY5Y neuroblastoma cells and in NBFL cells; CNTF increases c-fos, JunB, and JunD levels leading to AP-1 site activity within the CyRE of the VIP promoter in addition to its STAT-binding activity (Halvorsen et al., 1996; Symes et al., 1997a). In embryonic rat striatal neurons, LIF and CNTF lead to increased expression of the gene encoding the opioid-like neuropeptide, nociceptin/orphanin FQ, which binds to ORL-1 (Buzas et al., 1999). Nociceptin/orphanin FQ mRNA levels were increased severalfold by CNTF in striatal and cortical neurons and in primary astrocytes consistent with a mechanism requiring activation of the Jak/STAT pathway. Activation of STAT signaling is clearly an important mediator of neurokine action in the nervous system. As such, it is clear that activation of STAT signaling represents an important mediator of neurokine action in the nervous system.

The persistence of the neurokine activation signal varies among cell types. In most cell lines, neurokine activation is transient, even in the continued presence of the cytokine (Symes et al., 1994; Halvorsen et al., 1996). For example, CNTF or LIF stimulation of phospho-STAT in neuroblastoma cells is maximal after 15–30 min and then decays to near-background levels by 2 h (Kaur et al., 2002). This time course appears inconsistent with the observation that the survival effects of neurokines on primary neurons require continuous exposure; thus withdrawal of CNTF from ciliary ganglion neuron cultures results in cell death within 24 h (Wishingrad et al., 1997). However, the signal is much longer-lived in primary neurons, as continuous CNTF stimulation of cultured ciliary ganglion neurons causes persistent phospho-STAT3 activation for at least 5 days (Wishingrad et al., 1997; Kaur et al., 2002), and similar long-term activation of STAT signaling is seen in neurokine-stimulated sympathetic neurons (Symes et al., 1994; Guo et al., 1999; Kaur et al., 2003). A full understanding of the regulatory mechanisms controlling inactivation of neurokine signaling in nerve cells is clearly needed.

### 7.2.2 Mechanisms of Inactivation

Inactivation of neurokine signaling can be realized at multiple points in the signaling pathway. Some processes may be stimulated by the neurokine while others may be constitutively active. In order to reset the receptor signaling pathway a series of dephosphorylation events must ensue, requiring a set of tyrosine phosphatases that have not yet been fully identified. A few tyrosine phosphatases have been implicated in negative regulation of gp130 receptor signaling. The SH2-domain-containing protein tyrosine phosphatase SHP-1 and SHP-2 are tyrosine-phosphorylated and recruited to gp130 upon neurokine stimulation and decrease Jak activity and inhibit STAT pathways (Symes et al., 1997b; Neel et al., 2003). Inhibition of SHP-2 either by mutating the binding site on gp130 or by expression of dominant-negative SHP-2 results in an increased STAT-dependent VIP gene expression in NBFL and sympathetic neurons (Servidei et al., 1998). However, other results demonstrate that neurokines acting through LIF receptor and gp130 interact with SHP-2 as a linker protein to stimulate MAPK activity (Schiemann et al., 1997). Thus the effects mediated through inhibition of SHP-2 could be from lack of phosphatase activity or by inhibition of the MAPK pathway. Ultimately though, termination of the effects of neurokines require removal of activated, phosphorylated STATs from the nucleus (McBride et al., 2000). The nuclear tyrosine phosphatases, PTP1b, and

the nuclear isoform of TC-PTP have been implicated in the dephosphorylation of phospho-STATs in the nucleus (Myers et al., 2001; ten Hoeve et al., 2002).

The suppressors of cytokine signaling 1 and 3 (SOCS1 and SOCS3, respectively) are neurokine-induced genes that can function in a negative feedback loop to antagonize STAT activity. The current thinking is that SOCS1 binds to Jak and inhibits the kinase activity whereas SOCS3 binds to gp130 at the SHP-2 site and inhibits STAT phosphorylation (Yasukawa et al., 1999; Schmitz et al., 2000a; Inagaki-Ohara et al., 2003; Wormald and Hilton, 2004). Protein inhibitors of activated STATs (PIAS) are proteins that bind to activated STAT dimers and prevent DNA binding (Hilton, 1999; Kisseleva et al., 2002; Wormald and Hilton, 2004). Thus neurons have several mechanisms to regulate this important signaling pathway available to them.

### 7.3 Interactions with Other Cytokines and Growth Factors

Cells are constantly exposed to an array of different signaling molecules in their environment; therefore there is a great deal of interest in understanding the results and mechanisms of these potential interactions. Just a few of such interactions will be explored here. For example, neurokines and interferons signal through independent receptor systems but all activate Jak/STAT signaling. Nerve cells typically respond to interferon-γ by activating STAT1, with little or no activation of STAT3, but following pretreatment of neurons with CNTF, LIF, or IL-6, interferon-γ elicits a pronounced STAT3 response, providing an additional component to the final cellular response to interferon-γ (Kaur et al., 2003). In the CNS, expression of both apolipoprotein E (apoE) and CNTF can increase in response to injury. CNTF survival activity on cultured hippocampal neurons is potentiated by apoE, supporting the idea that apoE secreted at sites of injury can interact with growth factors such as CNTF to facilitate neural repair (Gutman et al., 1997). Hepatocyte growth factor (HGF) also acts synergistically with CNTF. Both are survival factors for motor neurons but neither HGF nor CNTF is effective against vincristine-induced motor neuron toxicity alone, while together they are neuroprotective against this neurotoxicity (Wong et al., 1997). HGF itself is not a survival factor for parasympathetic ciliary ganglion neurons or trigeminal mesencephalic sensory neurons, but it does enhance survival when combined with CNTF and also increases the length and branching of neurite arbors in both types of neurons (Davey et al., 2000). TGF-β isoforms, while not directly supportive of ciliary ganglion neuronal survival, enhance CNTF-mediated survival of these neurons (Krieglstein et al., 1998). Further, blocking the action of TGF-βs released in response to CNTF inhibits the survival response of CNTF in ciliary neurons. CNTF and activin (a TGF-β-type factor) each use the CyRE to increase VIP in NBFL cells, but when added together they promote a synergistic increase in VIP expression (Symes et al., 2000). While CNTF uses STAT and AP-1 proteins at the VIP CyRE, TGF-β uses Smad3 and Smad4 proteins to increase VIP expression (Pitts et al., 2001).

## 8 Clinical Implications

### 8.1 Neurodegenerative Disorders

The finding that CNTF and related factors promote survival of motor neurons has led to intense interest in development of CNTF as a potential treatment for neurodegenerative diseases, especially those associated with a loss of motor function. One of the first reports of a possible therapeutic role for these neurokines was the finding that CNTF treatment in vivo reduced the neuron loss in the progressive motor neuron disorder expressed in *pmn* mice (Sendtner et al., 1992). In another model of motor neuron disease, CNTF and brain-derived neurotrophic factor given together attenuated disease progression as tested by grip strength, motor neuron numbers, myelinated fibers, and muscle atrophy in the Wobbler mouse (Mitsumoto et al., 1994). Although CNTF is a potent agent for motor neuron survival, there is little evidence for the lack of either CNTF or CNTF receptor in the etiology of motor neuron disease. There are reports of decreased CNTF expression in spinal cord motor neurons of patients suffering from ALS (Duberley et al., 1995; Lee et al., 1996; Ono et al., 1999), but overall CNTF expression appears maintained in spinal cords of those with ALS

(Schorr et al., 1996; Takahashi et al., 1996). Further, the CNTF genotype does not appear to affect the clinical phenotype since ALS subjects show no difference in age of onset, disease severity, or duration among patients lacking functional genes for CNTF (Al-Chalabi et al., 2003), although further studies in ALS patients may yet show regional differences in CNTF receptor α mRNA expression. In phase II and III clinical trials of ALS, CNTF was not found to be of benefit (ALS CNTF Treatment Study Group, 1996) and LIF treatment was found to be of only limited benefit in the SOD mutant mouse model of ALS (Azari et al., 2003; Feeney et al., 2003).

Huntington's disease (HD) is a neurodegenerative condition where CNTF has shown promise as a therapeutic agent. Both CNTF and brain-derived neurotrophic factor completely protect striatal neurons against mutant huntingtin-induced apoptosis in a cell model (Saudou et al., 1998). In a primate model of HD, CNTF provided via transfected macroencapsulated baby hamster kidney (BHK) cells prevented neuronal death and restored neostriatal function (Emerich et al., 1997; Mittoux et al., 2000). There are several mutations causing photoreceptor degeneration in retina. The *rds*/*rds* mouse expresses a null mutation in the rds/peripherin gene that may be linked with retinal degeneration in humans. Intraocular adenovirus-mediated gene transfer of CNTF prevents photoreceptor degeneration in rds/perpherin mice, reducing photoreceptor loss and significantly improving the electroretinogram (Cayouette et al., 1998).

Oxidative stress has been associated with stimulation of Jak/STAT signaling and inflammatory responses in nonnerve cells and also with the pathophysiology of neurons in several neurodegenerative diseases (Ames et al., 1993; Beal, 2002). Since neurokines promote neuronal survival through Jak/STAT signaling this raises an apparent contradiction. An explanation for this discrepancy may be that mediators of oxidative stress have different effects in nerve and nonnerve cells. Thus, in nerve cells, unlike that in nonnerve cells, agents that increase oxidative stress block CNTF and other cytokine activation of Jak/STAT signaling (Kaur et al., 2005). These findings suggest that disruption of neurokine signaling is a possible mechanism of oxidative stress-induced cell death and neural disease.

## 8.2 Neural Trauma

The early discovery that in mammals CNTF is predominantly expressed in the adult and without a detectable secretory mechanism led to the hypothesis that it may function as an injury response factor. For example, axotomy of sensory neurons induces IL-6 and the receptor subunits for neurokines, sympathetic axotomy leads to increase LIF release, and there is increased expression of CNTF and α receptor subunit mRNA after spinal cord hemisection (Murphy et al., 1995; Rajan et al., 1995; Oyesiku et al., 1997; Sugiura et al., 2000; Gardiner et al., 2002). Following spinal cord injury CNTF increases the regeneration of neurons (Ye and Houle, 1997). The induction of both the proinflammatory IL-6 and the pro-survival LIF and CNTF neurokines suggests that an injury response may both promote normal inflammatory responses and provide survival and regeneration. The sciatic nerve is a prominent source of CNTF where it is expressed in Schwann cells. Following sciatic nerve transection, CNTF is released and LIF and IL-6 production is stimulated (Kurek et al., 1996). Providing additional CNTF after sciatic transection, using CNTF-linked collagen tubules, improves recovery (Ho et al., 1998). Infiltration of inflammatory cells into crushed sciatic nerve is retarded in LIF knockout mice compared with wild-type mice (Sugiura et al., 2000). It appears that a positive role for neurokines following nerve trauma may require a precise temporal and spatial regulation of neurokine expression.

A number of studies suggest that neurokines may provide protective or regenerative functions after CNS trauma. Brain injury models lead to increased expression of CNTF factors and their receptor subunits. Kainic-acid-induced seizures cause increased IL-6, LIF, IL-6 α receptor, LIF receptor, and gp130 mRNA as detected by Northern blotting and in situ hybridization (Lehtimaki et al., 2003), and lipopolysaccharide-induced brain reactions result in increased IL-6, its α receptor subunit, and gp130 (Vallieres and Rivest, 1997). In the auditory system CNTF infused via osmotic pump enhances the survival of spiral ganglion neurons and enhances auditory responses after cochlear implants (Shinohara et al., 2002). During transient focal ischemia CNTF enhances CNS neuron survival (Hermann et al., 2001). Thus targeted application of neurokines may be of benefit in CNS injuries.

### 8.3 Appetite Control

One of the side effects noted after systemic administration of CNTF in rats and humans is a decrease of appetite and weight loss (Henderson et al., 1994, 1996; ALS CNTF Treatment Study Group, 1996). CNTF corrects the obesity and the diabetes associated with the leptin-receptor-deficient *ob*/*ob* mouse (Gloaguen et al., 1997). The mechanism of action for CNTF appears to be a decrease in the hypothalamic peptide neuropeptide Y, leading to a decrease in appetite (Kalra et al., 1998; Xu et al., 1998). This has led to studies on the utility of CNTF as a long-term appetite suppressant drug (Lambert et al., 2001).

## 9 Concluding Remarks

We have tried to summarize the extent of our understanding of the actions and mechanisms of the neurokines. It should be apparent that we are still in need of a fuller molecular description of the neurokine–receptor–Jak signaling complex. This will provide a better basis for discerning the dynamics of the mechanisms controlling signal activation and inactivation. Revealing these regulatory mechanisms will help our understanding of the physiological roles of the different neurokine signaling pathways, which is key to uncovering the breadth and nature of interactions among neurokines and between these neurokines and other classes of factors and cytokines. Ultimately, identification of the genes regulated by the neurokine signaling pathways and their roles in the final physiological outcomes will be needed in determining the roles neurokines may have as potential therapeutic agents in treatment of degenerative neuropathologies.

## References


Abe K, Namikawa K, Honma M, Iwata T, Matsuoka I, et al. 2001. Inhibition of Ras extracellular-signal-regulated kinase (ERK) mediated signaling promotes ciliary neurotrophic factor (CNTF) expression in Schwann cells. J Neurochem 77: 700-703.

Adler R, Landa K, Manthorpe M, Varon S. 1979. Cholinergic neurotrophic factors: intraocular distribution of soluble trophic activity for ciliary neurons. Science 204: 1434-1436.

Agarwala S, Kalil RE. 1998. Long-term protection of axotomized neurons in the dorsal lateral geniculate nucleus in the rat following a single administration of basic fibroblast growth factor or ciliary neurotrophic factor. J Comp Neurol 392: 264-272.

Akira S. 1999. Functional roles of STAT family proteins: lessons from knockout mice. Stem Cells 17: 138-146.

Albrecht PJ, Dahl JP, Stoltzfus OK, Levenson R, Levison SW. 2002. Ciliary neurotrophic factor activates spinal cord astrocytes, stimulating their production and release of fibroblast growth factor-2, to increase motor neuron survival. Exp Neurol 173: 46-62.

Albrecht PJ, Murtie JC, Ness JK, Redwine JM, Enterline JR, et al. 2003. Astrocytes produce CNTF during the remyelination phase of viral-induced spinal cord demyelination to stimulate FGF-2 production. Neurobiol Dis 13: 89-101.

Al-Chalabi A, Scheffler MD, Smith BN, Parton MJ, Cudkowicz ME, et al. 2003. Ciliary neurotrophic factor genotype does not influence clinical phenotype in amyotrophic lateral sclerosis. Ann Neurol 54: 130-134.

Allan EH, Hilton DJ, Brown MA, Evely RS, Yumita S, et al. 1990. Osteoblasts display receptors for and responses to leukemia inhibitory factor. J Cell Physiol 145: 110-119.

ALS CNTF Treatment Study Group. 1996. A double-blind placebo-controlled clinical trial of subcutaneous recombinant human ciliary neurotrophic factor (rHCNTF) in amyotrophic lateral sclerosis. Neurology 46: 1244-1249.

Ames BN, Shigenaga MK, Hagen TM. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 90: 7915-7922.

Apfel S, Arezzo J, Moran M, Kessler J. 1993. Effects of administration of ciliary neurotrophic factor on normal motor and sensory peripheral nerves in vivo. Brain Res 604: 1-6.

Aubert J, Dessolin S, Belmonte N, Li M, McKenzie FR, et al. 1999. Leukemia inhibitory factor and its receptor promote adipocyte differentiation via the mitogen-activated protein kinase cascade. J Biol Chem 274: 24965-24972.

Auernhammer CJ, Melmed S. 2000. Leukemia-inhibitory factor-neuroimmune modulator of endocrine function. Endocr Rev 21 (3): 313-345.

Azari MF, Lopes EC, Stubna C, Turner BJ, Zang D, et al. 2003. Behavioural and anatomical effects of systemically administered leukemia inhibitory factor in the SOD1(G93A G1H)

mouse model of familial amyotrophic lateral sclerosis. Brain Res 982: 92-97.

Barbin G, Manthorpe M, Varon S. 1984. Purification of the chick eye ciliary neuronotrophic factor. J Neurochem 43: 1468-1478.

Barton VA, Hall MA, Hudson KR, Heath JK. 2000. Interleukin-11 signals through the formation of a hexameric receptor complex. J Biol Chem 275: 36197-36203.

Baumann H, Symes A, Comeau M, Morella K, Wang Y, et al. 1994. Multiple regions within the cytoplasmic domains of the LIF receptor and gp130 cooperate in signal transduction in hepatic and neuronal cells. Mol Cell Biol 14: 138-146.

Bazan FJ. 1991. Neuropoietic cytokines in the hematopoietic fold. Neuron 7: 197-208.

Beal MF. 2002. Oxidatively modified proteins in aging and disease. Free Radic Biol Med 32: 797-803.

Bellido T, Stahl N, Farruggella TJ, Borba V, Yancopoulos GD, et al. 1996. Detection of receptors for interleukin-6, interleukin-11, leukemia inhibitory factor, oncostatin-M, and ciliary neurotrophic factor in bone marrow stromal osteoblastic cells. J Clin Invest 97: 431-437.

Blanchard F, Duplomb L, Wang YP, Robledo O, Kinzie E, et al. 2000. Stimulation of leukemia inhibitory factor receptor degradation by extracellular signal-regulated kinase. J Biol Chem 275: 28793-28801.

Bolin LM, Verity AN, Silver JE, Shooter EM, Abrams JS. 1995. Interleukin-6 production by Schwann cells and induction in sciatic nerve injury. J Neurochem 64: 850-858.

Bonni A, Frank D, Schindler C, Greenberg M. 1993. Characterization of a pathway for ciliary neurotrophic factor signaling to the nucleus. Science 262: 1575-1579.

Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank D, et al. 1997. Regulation of gliogenesis in the central nervous system by the JAK/STAT signaling pathway. Science 278: 477-483.

Boulanger MJ, Bankovich AJ, Kortemme T, Baker D, Garcia KC. 2003a. Convergent mechanisms for recognition of divergent cytokines by the shared signaling receptor gp130. Mol Cell 12: 577-589.

Boulanger MJ, Chow DC, Brevnova EE, Garcia KC. 2003b. Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. Science 300: 2101-2104.

Boulton TG, Stahl N, Yancopoulos GD. 1994. Ciliary neurotrophic factor, leukemia inhibitory factor, interleukin-6, oncostatin-M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. J Biol Chem 269: 11648-11655.

Buzas B, Symes AJ, Cox BM. 1999. Regulation of nociceptin orphanin FQ gene expression by neuropoietic cytokines and neurotrophic factors in neurons and astrocytes. J Neurochem 72: 1882-1889.

Cayouette M, Behn D, Sendtner M, Lachapelle P, Gravel C. 1998. Intraocular gene transfer of ciliary neurotrophic factor prevents death and increases responsiveness of rod photoreceptors in the retinal degeneration slow mouse. J Neurosci 18: 9282-9293.

Cheng LZ, Gearing DP, White LS, Compton DL, Schooley K, et al. 1994. Role of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth. Development 120: 3145-3153.

Clatterbuck RE, Price DL, Koliatsos VE. 1993. CNTF prevents retrograde neuronal death in the adult CNS. Proc Natl Acad Sci USA 90: 2222-2226.

Conover JC, Ip N, Poueymirou W, Bates B, Goldfarb M, et al. 1993. Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. Development 119: 559-565.

Curtis R, Adryan KM, Zhu Y, Harkness PJ, Lindsay RM, et al. 1993. Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. Nature 365: 253-255.

Curtis R, Scherer S, Somogyi R, Adryan K, Ip N, et al. 1994. Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. Neuron 12: 191-204.

Dallner C, Woods AG, Deller T, Kirsch M, Hofmann HD. 2002. CNTF and CNTF receptor alpha are constitutively expressed by astrocytes in the mouse brain. Glia 37: 374-378.

Darnell JE. 1997. STATs and gene regulation. Science 277: 1630-1635.

Davey F, Hilton M, Davies AM. 2000. Cooperation between HGF and CNTF in promoting the survival and growth of sensory and parasympathetic neurons. Mol Cell Neurosci 15: 79-87.

Davis S, Aldrich T, Stahl N, Ip NY, Yancopoulos G. 1993. LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. Science 260: 1805-1808.

Davis S, Aldrich T, Valenzuela D, Wong V, Furth M, et al. 1991. The receptor for ciliary neurotrophic factor. Science 253: 59-63.

De Serio A, Graziani R, Laufer R, Ciliberto G, Paonessa G. 1995. In vitro binding of ciliary neurotrophic factor to its receptors: evidence for the formation of an IL-6-type hexameric complex. J Mol Biol 254: 795-800.

DeChiara T, Vejsada R, Poueymirou W, Acheson A, Suri C, et al. 1995. Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. Cell 83: 313-322.

Decker T, Kovarik P. 2000. Serine phosphorylation of STATs. Oncogene 19: 2628-2637.

Decker T, Kovarik P, Meinke A. 1997. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res 17: 121-134.

Derouet D, Rousseau F, Alfonsi F, Froger J, Hermann J, et al. 2004. Neuropoietin, a new IL-6-related cytokine signaling through the ciliary neurotrophic factor receptor. Proc Natl Acad Sci USA 101: 4827-4832.

Duberley RM, Johnson IP, Anand P, Swash M, Martin J, et al. 1995. Ciliary neurotrophic factor receptor expression in spinal cord and motor cortex in amyotrophic lateral sclerosis. J Neurol Sci 129: 109-113.

Duong CV, Geissen M, Rohrer H. 2002. The developmental expression of vasoactive intestinal peptide (VIP) in cholinergic sympathetic neurons depends on cytokines signaling through LIFR beta-containing receptors. Development 129: 1387-1396.

Emerich DF, Winn SR, Hantraye PM, Peschanski M, Chen EY, et al. 1997. Protective effect of encapsulated cells producing neurotrophic factor CNTF in a monkey model of Huntington's disease. Nature 386: 395-399.

English AW, Schwartz G. 1995. Both basic fibroblast growth factor and ciliary neurotrophic factor promote the retention of polyneuronal innervation of developing skeletal muscle fibers. Dev Biol 169: 57-64.

Ernsberger U, Patzke H, Rohrer H. 1997. The developmental expression of choline acetyltransferase (ChAT) and the neuropeptide VIP in chick sympathetic neurons: evidence for different regulatory events in cholinergic differentiation. Mech Dev 68: 115-126.

Ernsberger U, Sendtner M, Rohrer H. 1989. Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. Neuron 2: 1275-1284.

Ezzeddine ZD, Yang XJ, Dechiara T, Yancopoulos G, Cepko CL. 1997. Postmitotic cells fated to become rod photoreceptors can be respecified by CNTF treatment of the retina. Development 124: 1055-1067.

Feener EP, Rosario F, Dunn SL, Stancheva Z, Myers Jr. MG 2004. Tyrosine phosphorylation of Jak2 in the JH2 domain inhibits cytokine signaling. Mol Cell Biol 24: 4968-4978.

Feeney SJ, Austin L, Bennett TM, Kurek JB, Jean-Francois MJB, et al. 2003. The effect of leukaemia inhibitory factor on SOD1 G93A murine amyotrophic lateral sclerosis. Cytokine 23: 108-118.

Finn TP, Kim S, Nishi R. 1998. Overexpression of ciliary neurotrophic factor in vivo rescues chick ciliary ganglion neurons from cell death. J Neurobiol 34: 283-293.

Finn TP, Nishi R. 1996a. Expression of ciliary neurotrophic factor in targets of ciliary ganglion neurons during and after the cell death phase. J Comp Neurol 366: 559-571.

Finn TP, Nishi R. 1996b. Does ciliary neurotrophic factor serve a different function in the rat versus the chicken? Perspect Dev Neurobiol 4: 91-99.

Forger NG, Howell ML, Bengston L, Mac Kenzie L, De Chiara TM, et al. 1997. Sexual dimorphism in the spinal cord is absent in mice lacking the CNTF receptor. J Neurosci 17: 9605-9612.

Forger NG, Wagner CK, Contois M, Bengston L, Mac Lennan AJ. 1998. Ciliary neurotrophic factor receptor alpha in spinal motoneurons is regulated by gonadal hormones. J Neurosci 18: 8720-8729.

Fuhrmann S, Grabosch K, Kirsch M, Hofmann HD. 2003. Distribution of CNTF receptor alpha protein in the central nervous system of the chick embryo. J Comp Neurol 461: 111-122.

Fukada K. 1985. Purification and partial characterization of a cholinergic neuronal differentiation factor. Proc Natl Acad Sci USA 82: 8795-8799.

Gardiner NJ, Cafferty WBJ, Slack SE, Thompson SWN. 2002. Expression of gp130 and leukaemia inhibitory factor receptor subunits in adult rat sensory neurones: regulation by nerve injury. J Neurochem 83: 100-109.

Gearing D, Gough N, King J, Hilton D, Nicola N, et al. 1987. Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). EMBO J 6: 3995-4002.

Gearing DP, Thut CJ, Vanderbos T, Gimpel SD, Delaney PB, et al. 1991. Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J 10: 2839-2848.

Geissen M, Heller S, Pennica D, Ernsberger U, Rohrer H. 1998. The specification of sympathetic neurotransmitter phenotype depends on gp130 cytokine receptor signaling. Development 125: 4791-4802.

Gloaguen I, Costa P, Demartis A, Lazzaro D, Dimarco A, et al. 1997. Ciliary neurotrophic factor corrects obesity and diabetes associated with leptin deficiency and resistance. Proc Natl Acad Sci USA 94: 6456-6461.

Grimaud E, Blanchard F, Charrier C, Gouin F, Redini F, et al. 2002. Leukaemia inhibitory factor (LIF) is expressed in hypertrophic chondrocytes and vascular sprouts during osteogenesis. Cytokine 20: 224-230.

Grotzinger J. 2002. Molecular mechanisms of cytokine receptor activation. Biochem Biophys Acta 1592: 215-223.

Guillet C, Auguste P, Mayo W, Kreher P, Gascan H. 1999. Ciliary neurotrophic factor is a regulator of muscular strength in aging. J Neurosci 19: 1257-1262.

Guo X, Chandrasekaran V, Lein P, Kaplan PL, Higgins D. 1999. Leukemia inhibitory factor and ciliary neurotrophic factor cause dendritic retraction in cultured rat sympathetic neurons. J Neurosci 19: 2113-2121.

Guo X, Metzler-Northrup J, Lein P, Rueger D, Higgins D. 1997. LIF and CNTF regulate dendritic growth in cultures of rat sympathetic neurons. Dev Brain Res 104: 101-110.

Gupta SK, Altares M, Benoit R, Riopelle RJ, Dunn RJ, et al. 1992. Preparation and biological properties of native and

recombinant ciliary neurotrophic factor. J Neurobiol 23: 481-490.

Gutman CR, Strittmatter WJ, Weisgraber KH, Matthew WD. 1997. Apolipoprotein E binds to and potentiates the biological activity of ciliary neurotrophic factor. J Neurosci 17: 6114-6121.

Halvorsen SW, Berg DK. 1989. Specific down-regulation of the alpha-bungarotoxin binding component on chick autonomic neurons by ciliary neurotrophic factor. J Neurosci 9: 3673-3680.

Halvorsen SW, Malek RL, Wang X, Jiang N. 1996. Ciliary neurotrophic factor regulates nicotinic acetylcholine receptors on human neuroblastoma cells. Neuropharmacology 35: 257-265.

Hartnick CJ, Staecker H, Malgrange B, Lefebvre PP, Liu W, et al. 1996. Neurotrophic effects of BDNF and CNTF, alone and in combination, on postnatal day 5 rat acoustic ganglion neurons. J Neurobiol 30: 246-254.

Hata K, Araki M, Yamamori T. 2003. Ciliary neurotrophic factor inhibits differentiation of photoreceptor-like cells in rat pineal glands in vitro. Dev Brain Res 143: 179-187.

Helgren M, Squinto S, Davis H, Parry D, Boulton T, et al. 1994. Trophic effect of ciliary neurotrophic factor on denervated skeletal muscle. Cell 76: 493-504.

Heller S, Finn TP, Huber J, Nishi R, Geiben M, et al. 1995. Analysis of function and expression of the chick GPA receptor (GPAR alpha) suggests multiple roles in neuronal development. Development 121: 2681-2693.

Henderson JT, Mullen BJM, Roder JC. 1996. Physiological effects of CNTF-induced wasting. Cytokine 8: 784-793.

Henderson JT, Seniuk NA, Richardson PM, Gauldie J, Roder JC. 1994. Systemic administration of CNTF induces cachexia in rodents. J Clin Invest 93: 2632-2638.

Hendry A, Murphy M, Hilton DJ, Nicola NA, Bartlett PF. 1992. Binding and retrograde transport of leukemia inhibitory factor by the sensory nervous system. J Neurosci 12: 3427-3434.

Hermann DM, Kilic E, Kugler S, Isenmann S, Bahr M. 2001. Adenovirus-mediated GDNF and CNTF pretreatment protects against striatal injury following transient middle cerebral artery occlusion in mice. Neurobiol Dis 8: 655-666.

Hermanns HM, Radtke S, Haan C, Schmitz-Van de Leur H, Tavernier J, et al. 1999. Contributions of leukemia inhibitory factor receptor and oncostatin M receptor to signal transduction in heterodimeric complexes with glycoprotein 130. J Immunol 163: 6651-6658.

Hermanns HM, Radtke S, Schaper F, Heinrich PC, Behrmann I. 2000. Non-redundant signal transduction of interleukin-6-type cytokines. The adapter protein Shc is specifically recruited to rhe oncostatin M receptor. J Biol Chem 275: 40742-40748.

Hilton DJ. 1999. Negative regulators of cytokine signal transduction. Cell Mol Life Sci 55: 1568-1577.

Hilton DJ, Hilton AA, Raicevic A, Rakar S, Harrisonsmith M, et al. 1994. Cloning of a murine IL-11 receptor alpha-chain; requirement for gp130 for high affinity binding and signal transduction. EMBO J 13: 4765-4775.

Ho PR, Coan GM, Cheng ET, Niell C, Tarn DM, et al. 1998. Repair with collagen tubules linked with brain-derived neurotrophic factor and ciliary neurotrophic factor in a rat sciatic nerve injury model. Arch Otolaryngol Head Neck Surg 124: 761-766.

Hofmann H-D. 1988. Ciliary neuronotrophic factor stimulates choline acetyltransferase activity in cultured chicken retina neurons. J Neurochem 51: 109-113.

Holtmann B, Wiese S, Samsam M, Grohmann K, Pennica D, et al. 2005. Triple knock-out of CNTF, LIF, and CT-1 defines cooperative and distinct roles of these neurotrophic factors for motoneuron maintenance and function. J Neurosci 25: 1778-1787.

Horton AR, Bartlett PF, Pennica D, Davies AM. 1998. Cytokines promote the survival of mouse cranial sensory neurones at different developmental stages. Eur J Neurosci 10: 673-679.

Horvath CM. 2000. STAT proteins and transcriptional responses to extracellular signals Trends Biochem Sci 25: 496-502.

Huber J, Dittrich F, Phelan P. 1993. Characterization of high-affinity and low-affinity receptors for ciliary neurotrophic factor. Eur J Biochem 218: 1031-1039.

Hudgins SN, Levison SW. 1998. Ciliary neurotrophic factor stimulates astroglial hypertrophy in vivo and in vitro. Exp Neurol 150: 171-182.

Hughes S, Lillien L, Raff M, Rohrer H, Sendtner M. 1988. Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. Nature 335: 70-72.

Ikeda K, Iwasaki Y, Shiojima T, Kinoshita M. 1996. Neuroprotective effect of various cytokines on developing spinal motoneurons following axotomy. J Neurol Sci 135: 109-113.

Inagaki-Ohara K, Hanada T, Yoshimura A. 2003. Negative regulation of cytokine signaling and inflammatory diseases. Curr Opin Pharmacol 3: 435-442.

Inoue M, Nakayama C, Noguchi H. 1996. Activating mechanism of CNTF and related cytokines. Mol Neurobiol 12: 195-209.

Ip NY, Boulton T, Li Y, Verdi J, Birren S, et al. 1994. CNTF, FGF, NGF collaborate to drive the terminal differentiation of MAH cells into postmitotic neurons. Neuron 13: 443-455.

Ip FC, Fu AK, Tsim KW, Ip NY. 1995. Cloning of the alpha component of the chick ciliary neurotrophic factor receptor: developmental expression and down-regulation in denervated skeletal muscle. J Neurochem 65: 2393-2400.

Ip NY, Li YP, van de Stadt I, Panayotatos N, Alderson RF, et al. 1991. CNTF enhances neuronal survival in embryonic rat hippocampal cultures. J Neurosci 11: 3124-3134.

Ip NY, McClain J, Barrezueta N, Aldrich T, Pan L, et al. 1993. The alpha component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. Neuron 10: 89-102.

Ip NY, Nye S, Boulton T, Davis S, Taga T, et al. 1992b. CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell 69: 1121-1132.

Ip NY, Wiegand S, Morse J, Rudge J. 1992a. Injury-induced regulation of ciliary neurotrophic factor mRNA in the adult rat brain. Eur J Neurosci 5: 25-33.

Ishimi Y, Abe E, Jin CH, Miyaura C, Hong MH, et al. 1992. Leukemia inhibitory factor/differentiation-stimulating factor (LIF/D-factor): regulation of its production and possible roles in bone metabolism. J Cell Physiol 152: 71-78.

Jiao J, Kaur N, Lu B, Reeves SA, Halvorsen SW. 2003. Initiation and maintenance of CNTF-Jak/STAT signaling in neurons is blocked by protein tyrosine phosphatase inhibitors. Mol Brain Res 116: 135-146.

Johann V, Jeliaznik N, Schrage K, Mey J. 2003. Retinoic acid downregulates the expression of ciliary neurotrophic factor in rat Schwann cells. Neurosci Lett 339: 13-16.

Jordan CL. 1996a. Morphological effects of CNTF treatment during neuromuscular synapse elimination. J Neurobiol 31: 29-40.

Jordan CL. 1996b. Ciliary neurotrophic factor may act in target musculature to regulate developmental synapse elimination. Dev Neurosci 18: 185-198.

Kahn MA, Huang CJ, Caruso A, Barresi V, Nazarian R, et al. 1997. Ciliary neurotrophic factor activates Jak/STAT signal transduction cascade and induces transcriptional expression of glial fibrillary acidic protein in glial cells. J Neurochem 68: 1413-1423.

Kallen KJ, Grotzinger J, Lelievre E, Vollmer P, Aasland D, et al. 1999. Receptor recognition sites of cytokines are organized as exchangeable modules. Transfer of the leukemia inhibitory factor receptor-binding site from ciliary neurotrophic factor to interleukin-6. J Biol Chem 274: 11859-11867.

Kalra SP, Xu B, Dube MG, Moldewer LL, Martin D, et al. 1998. Leptin and ciliary neurotropic factor (CNTF) inhibit fasting-inducing suppression of luteinizing hormone release in rats: role of neuropeptide Y. Neusosci Lett 240: 45-49.

Kataoka Y, Matsumura I, Ezoe S, Nakata S, Takigawa E, et al. 2003. Reciprocal inhibition between MyoD and STAT3 in the regulation of growth and differentiation of myoblasts. J Biol Chem 278: 44178-44187.

Kaur N, Kim I, Higgins D, Halvorsen SW. 2003. Induction of an interferon-gamma-mediated STAT3 response in nerve cells by pretreatment with gp130 cytokines. J Neurochem 87: 437-447.

Kaur N, Lu B, Monroe RK, Ward SM, Halvorsen SW. 2005. Inducers of oxidative stress block ciliary neurotrophic factor activation of Jak/STAT signaling in neurons. J Neurochem 92: 1521-1530.

Kaur N, Wohlhueter AL, Halvorsen SW. 2002. Activation and inactivation of signal transducers and activators of transcription by ciliary neurotrophic factor in neuroblastoma cells. Cell Signal 14: 419-429.

Kessler J, Ludlam W, Freidin M, Hall D, Michaelson M, et al. 1993. Cytokine-induced programmed death of cultured sympathetic neurons. Neuron 11: 1123-1132.

Kiger AA, Jones DL, Schulz C, Rogers MB, Fuller MT. 2001. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science 294: 2542-2545.

Kirsch M, Hofmann HD. 1994. Expression of ciliary neurotrophic factor receptor mRNA and protein in the early postnatal and adult rat nervous system. Neurosci Lett 180: 163-166.

Kirsch M, Schneider T, Lee MY, Hofmann HD. 1998a. Lesion-induced changes in the expression of ciliary neurotrophic factor and its receptor in rat optic nerve. Glia 23: 239-248.

Kirsch M, Schulz-Key S, Wiese A, Fuhrmann S, Hofmann H. 1998b. Ciliary neurotrophic factor blocks rod photoreceptor differentiation from postmitotic precursor cells in vitro. Cell Tissue Res 291: 207-216.

Kishimoto T, Akira S, Taga T. 1992. Interleukin-6 and its receptor: a paradigm for cytokines. Science 258: 593-597.

Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene 285: 1-24.

Kodama H, Fukuda K, Pan J, Makino S, Baba A, et al. 1997. Leukemia inhibitory factor, a potent cardiac hypertrophic cytokine, activates the JAK/STAT pathway in rat cardiomyocytes. Circ Res 81: 656-663.

Koshlukova S, Finn TP, Nishi R, Halvorsen SW. 1996. Identification of functional receptors for ciliary neurotrophic factor on chick ciliary ganglion neurons. Neuroscience 72: 821-832.

Kotzbauer P, Lampe P, Estus S, Milbrandt J, Johnson E. 1994. Postnatal development of survival responsiveness in rat sympathetic neurons to leukemia inhibitory factor and ciliary neurotrophic factor. Neuron 12: 763-773.

Krieglstein K, Farkas L, Unsicker K. 1998. TGF-beta regulates the survival of ciliary ganglionic neurons synergistically with ciliary neurotrophic factor and neurotrophins. J Neurobiol 37: 563-572.

Kurek JB, Austin L, Cheema SS, Bartlett PF, Murphy M. 1996. Up-regulation of leukaemia inhibitory factor and interleukin-6 in transected sciatic nerve and muscle following denervation. Neuromuscul Disord 6: 105-114.

Kuropatwinski KK, Deimus C, Gearing D, Baumann H, Mosley B. 1997. Influence of subunit combinations on signaling by receptors for oncostatin M, leukemia inhibitory factor, and interleukin-6. J Biol Chem 272: 15135-15144.

Kushima Y, Hama T, Hatanaka H. 1992. Interleukin-6 as a neurotrophic factor for promoting the survival of cultured catecholaminergic neurons in a chemically defined medium from fetal and postnatal rat midbrains. Neurosci Res 13: 267-280.

Kushima Y, Hatanaka H. 1992. Interleukin-6 and leukemia inhibitory factor promote the survival of acetylcholinesterase-positive neurons in culture from embryonic rat spinal cord. Neurosci Lett 143: 110-114.

Kwon YW, Abbondanzo SJ, Stewart CL, Gurney ME. 1995. Leukemia inhibitory factor influences the timing of programmed synapse withdrawal from neonatal muscles. J Neurobiol 28: 35-50.

Lam A, Fuller F, Miller J, Kloss J, Manthorpe M, et al. 1991. Sequence and structural organization of the human gene encoding ciliary neurotrophic factor. Gene 102: 271-276.

Lambert PD, Anderson KD, Sleeman MW, Wong V, Tan J, et al. 2001. Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity. Proc Natl Acad Sci USA 98: 4652-4657.

Landis SC. 1996. The development of cholinergic sympathetic neurons: a role for neuropoietic cytokines. Persp Dev Neurobiol 4: 53-63.

Landmesser L, Pilar G. 1974. Synaptic transmission and cell death during normal ganglionic development. J Physiol 241: 737-749.

Lee MY, Deller T, Kirsch M, Frotscher M, Hofmann HD. 1997b. Differential regulation of ciliary neurotrophic factor (CNTF) and CNTF receptor alpha expression in astrocytes and neurons of the fascia dentata after entorhinal cortex lesion. J Neurosci 17: 11370-1146.

Lee DA, Gross L, Wittrock DA, Windebank AJ. 1996. Localization and expression of ciliary neurotrophic factor (CNTF) in postmortem sciatic nerve from patients with motor neuron disease and diabetic neuropathy. J Neuropathol Exp Neurol 55: 915-923.

Lee MY, Hofmann HD, Kirsch M. 1997a. Expression of ciliary neurotrophic factor receptor-alpha messenger RNA in neonatal and adult rat brain: an in situ hybridization study. Neuroscience 77: 233-246.

Lee MY, Naumann T, Kirsch M, Frotscher M, Hofmann HD. 1997c. Transient up-regulation of ciliary neurotrophic factor receptor-alpha mRNA in axotomized rat septal neurons. Eur J Neurosci 9: 622-626.

Lehtimaki KA, Peltola J, Koskikallio E, Keranen T, Honkaniemi J. 2003. Expression of cytokines and cytokine receptors in the rat brain after kainic acid-induced seizures. Mol Brain Res 110: 253-260.

Leonard WJ, O'Shea JJ. 1998. Jaks and STATs: biological implications. Annu Rev Immunol 16: 293-322.

Leung DW, Parent AS, Cachianes G, Esch F, Coulombe JN, et al. 1992. Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. Neuron 8: 1045-1053.

Levi-Montalcini R, Hamburger V. 1951. Selective growth stimulating effects of mouse sarcoma on sensory and sympathetic nervous system of the chick embryo. J Exp Zoolog 116: 321-362.

Li LX, Oppenheim RW, Lei M, Houenou J. 1994. Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. J Neurobiol 25: 759-766.

Li M, Sendtner M, Smith A. 1995. Essential function of LIF receptor in motor neurons. Nature 378: 724-727.

Lin L-F, Mismer D, Lile J, Armes L, Butler E, et al. 1989. Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science 246: 1023-1025.

Liou JC, Yang RS, Fu WM. 1997. Regulation of quantal secretion by neurotrophic factors at developing motoneurons in Xenopus cell cultures. J Physiol 503: 129-139.

Ludlam W, Kessler J. 1993. Leukemia inhibitory factor and ciliary neurotrophic factor regulate expression of muscarinic receptors in cultured sympathetic neurons. Dev Biol 155: 497-506.

Maclennan AJ, Vinson EN, Marks L, McLaurin DL, Pfeifer M, et al. 1996. Immunohistochemical localization of ciliary neurotrophic factor receptor alpha expression in the rat nervous system. J Neurosci 16: 621-630.

Malek RL, Halvorsen SW. 1997. Opposing regulation of ciliary neurotrophic factor receptors on neuroblastoma cells by distinct differentiating agents. J Neurobiol 32: 81-94.

Manthorpe M, Skaper S, Adler R, Landa K, Varon S. 1980. Cholinergic neuronotrophic factors: fractionation properties of an extract from selected chick embryonic eye tissues. J Neurochem 34: 69-75.

Manthorpe M, Skaper S, Williams L, Varon S. 1986. Purification of adult rat sciatic nerve ciliary neuronotrophic factor. Brain Res 367: 283-286.

Marmur R, Kessler JA, Zhu G, Gokhan S, Mehler MF. 1998. Differentiation of oligodendroglial progenitors derived from cortical multipotent cells requires extrinsic signals including activation of gp130/LIFbeta receptors. J Neurosci 18: 9800-9811.

Martin A, Hofmann HD, Kirsch M. 2003. Glial reactivity in ciliary neurotrophic factor-deficient mice after optic nerve lesion. J Neurosci 23: 5416-5424.

März P, Cheng JG, Gadient RA, Patterson PH, Stoyan T, et al. 1998. Sympathetic neurons can produce and respond to interleukin 6. Proc Natl Acad Sci USA 95: 3251-3256.

März P, Otten U, Rose-John S. 1999. Neural activities of IL-6-type cytokines often depend on soluble cytokine receptors. Eur J Neurosci 11: 2995-3004.

Masu Y, Wolf E, Holtmann B, Sendtner M, Brem G, et al. 1993. Disruption of the CNTF gene results in motor neuron degeneration. Nature 365: 27-32.

McBride KM, McDonald C, Reich NC. 2000. Nuclear export signal located within the DNA-binding domain of the STAT1 transcription factor. EMBO J 19: 6196-6206.

Metcalf D. 2003. The unsolved enigmas of leukemia inhibitory factor. Stem Cells 21: 5-14.

Middleton G, Hamanoue M, Enokido Y, Wyatt S, Pennica D, et al. 2000. Cytokine-induced nuclear factor kappa B activation promotes the survival of developing neurons. J Cell Biol 148: 325-332.

Mitsumoto H, Ikeda K, Klinkosz B, Cedarbaum J, Wong V, et al. 1994. Arrest of motor neuron disease in wobbler mice cotreated with CNTF and BDNF. Science 265: 1107-1110.

Mittoux V, Joseph JM, Conde F, Palfi S, Dautry C, et al. 2000. Restoration of cognitive and motor functions by ciliary neurotrophic factor in a primate model of Huntington's disease. Hum Gene Ther 11: 1177-1187.

Moon C, Yoo JY, Matarazzo V, Sung YK, Kim EJ, et al. 2002. Leukemia inhibitory factor inhibits neuronal terminal differentiation through STAT3 activation. Proc Natl Acad Sci USA 99: 9015-9020.

Mosley B, De Imus C, Friend D, Boiani N, Thoma B, et al. 1996. Dual oncostatin M (OSM) receptors. Cloning and characterization of an alternative signaling subunit conferring OSM-specific receptor activation. J Biol Chem 271: 32635-32643.

Mufson EJ, Kroin JS, Sendera TJ, Sobreviela T. 1999. Distribution and retrograde transport of trophic factors in the central nervous system: functional implications for the treatment of neurodegenerative diseases. Prog Neurobiol 57: 451-484.

Murphy M, Dutton R, Koblar S, Cheema S, Bartlett P. 1997. Cytokines which signal through the lif receptor and their actions in the nervous system. Prog Neurobiol 52: 355-378.

Murphy P, Grondin J, Altares M, Richardson PM. 1995. Induction of interleukin-6 in axotomized sensory neurons. J Neurosci 15: 5130-5138.

Myers MP, Andersen JN, Cheng A, Tremblay ML, Horvath CM, et al. 2001. TYK2 and JAK2 are substrates of protein-tyrosine phosphatase 1B. J Biol Chem 276: 47771-47774.

Nakashima K, Wiese S, Yanagisawa M, Arakawa H, Kimura N, et al. 1999. Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. J Neurosci 19: 5429-5434.

Neel BG, Gu H, Pao L. 2003. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci 28: 284-293.

Nesbitt J, Fuentes N, Fuller G. 1993. Ciliary neurotrophic factor regulates fibrinogen gene expression in hepatocytes by binding to the interleukin-6 receptor. Biochem Biophys Res Commun 190: 544-550.

Neubauer H, Cumano A, Muller M, Wu H, Huffstadt U, et al. 1998. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell 93: 397-409.

Nishi R, Berg DK. 1981. Two components from eye tissue that differentially stimulate growth and development of ciliary ganglion neurons in culture. J Neurosci 1: 505-513.

Nishimune H, Vasseur S, Wiese S, Birling MC, Holtmann B, et al. 2000. Reg-2 is a motoneuron neurotrophic factor and a signalling intermediate in the CNTF survival pathway. Nat Cell Biol 2: 906-914.

Niwa H, Burdon T, Chambers I, Smith A. 1998. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 12: 2048-2060.

Nonogaki K, Pan XM, Moser AH, Shigenaga J, Staprans I. 1996. LIF and CNTF, which share the gp130 transduction system, stimulate hepatic lipid metabolism in rats. Am J Physiol Endocrinol Metab 34: E 521-E 528.

Ono S, Imai T, Igarashi A, Shimizu N, Nakagawa H, et al. 1999. Decrease in the ciliary neurotrophic factor of the spinal cord in amyotrophic lateral sclerosis. Eur Neurol 42: 163-168.

Oppenheim R, Prevette D, Qin-Wei Y, Collins F, Mac Donald J. 1991. Control of embryonic motoneuron survival in vivo by CNTF. Science 251: 1616-1618.

Orrell RW, King AW, Lane RJM, Debelleroche JS. 1995. Investigation of a null mutation of the CNTF gene in familial amyotrophic lateral sclerosis. J Neurol Sci 132: 126-128.

Oyesiku NM, Wigston DJ. 1996. Ciliary neurotrophic factor stimulates neurite outgrowth from spinal cord neurons. J Comp Neurol 364: 68-77.

Oyesiku NM, Wilcox JN, Wigston DJ. 1997. Changes in expression of ciliary neurotrophic factor (CNTF) and CNTF-receptor alpha after spinal cord injury. J Neurobiol 32: 251-261.

Pain B, Clark M, Shen M, Nakazawa H, Sakurai M, et al. 1996. Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. Development 122: 2339-2348.

Parganas E, Wang D, Stravopodis D, Topham DJ, Marine JC, et al. 1998. Jak2 is essential for signaling through a variety of cytokine receptors. Cell 93: 385-395.

Park CK, Ju WK, Hofmann HD, Kirsch M, Kang JK, et al. 2000. Differential regulation of ciliary neurotrophic factor and its receptor in the rat hippocampus following transient global ischemia. Brain Res 861: 345-353.

Pellegrini S, Dusanter-Fourt I. 1997. The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). Eur J Biochem 248: 615-633.

Pennica D, Arce V, Swanson T, Vejsada R, Pollock R, et al. 1996. Cardiotrophin-1, a cytokine present in embryonic muscle supports long-term survival of spinal motoneurons. Neuron 17: 63-74.

Pennica D, King K, Shaw K, Luis E, Rullamas J, et al. 1995b. Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. Proc Natl Acad Sci USA 92: 1142-1146.

Pennica D, Shaw K, Swanson T, Moore M, Shelton D, et al. 1995a. Cardiotrophin-1, Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. J Biol Chem 270: 10915-10922.

Pitts RL, Wang SB, Jones EA, Symes AJ. 2001. Transforming growth factor-beta and ciliary neurotrophic factor synergistically induce vasoactive intestinal peptide gene expression through the cooperation of Smad, STAT, and AP-1 sites. J Biol Chem 276: 19966-19973.

Qiu LQ, Towle MF, Bernd P, Fukada K. 1997. Distribution of cholinergic neuronal differentiation factor leukemia inhibitory factor binding sites in the developing and adult rat nervous system in vivo. J Neurobiol 32: 163-192.

Rajan P, Stewart CL, Fink JS. 1995. LIF-mediated activation of STAT proteins after neuronal injury in vivo. Neuroreport 6: 2240-2244.

Rane SG, Reddy EP. 2000. Janus kinases: components of multiple signaling pathways. Oncogene 19: 5662-5679.

Rao MS, Tyrrell S, Landis S. C. Patterson PH. 1992. Effects of ciliary neurotrophic factor (CNTF) and depolarization on neuropeptide expression in cultured sympathetic neurons. Dev Biol 150: 281-293.

Reiness CG, Seppa MJ, Dion DM, Sweeney S, Foster DN, et al. 2001. Chick ciliary neurotrophic factor is secreted via a nonclassical pathway. Mol Cell Neurosci 17: 931-944.

Robledo O, Auguste P, Coupey L, Praloran V, Chevalier S, et al. 1996. Binding interactions of leukemia inhibitory factor and ciliary neurotrophic factor with the different subunits of their high affinity receptors. J Neurochem 66: 1391-1399.

Rodig SJ, Meraz MA, White JM, Lampe PA, Riley JK, et al. 1998. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell 93: 373-383.

Rose TM, Bruce AG. 1991. Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6. Proc Natl Acad Sci USA 88: 8641-8645.

Saudou F, Finkbeiner S, Devys D, Greenberg ME. 1998. Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. Cell 95: 55-66.

Savitz SI, Kessler JA. 2000. Leukemia inhibitory factor requires concurrent p75LNTR signaling to induce apoptosis of cultured sympathetic neurons. J Neurosci 20: 4198-4205.

Schiemann WP, Bartoe JL, Nathanson NM. 1997. Box 3-independent signaling mechanisms are involved in leukemia inhibitory factor receptor alpha- and gp130-mediated stimulation of mitogen-activated protein kinase. Evidence for participation of multiple signaling pathways which converge at Ras. J Biol Chem 272: 16631-16636.

Schmitz J, Dahmen H, Grimm C, Gendo C, Muller-Newen G, et al. 2000b. The cytoplasmic tyrosine motifs in full-length glycoprotein 130 have different roles in IL-6 signal transduction. J Immunol 164: 848-854.

Schmitz J, Weissenbach M, Haan S, Heinrich PC, Schaper F. 2000a. SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. J Biol Chem 275: 12848-12856.

Schorr M, Zhou LP, Schwechheimer K. 1996. Expression of ciliary neurotrophic factor is maintained in spinal motor neurons of amyotrophic lateral sclerosis. J Neurol Sci 140: 117-122.

Senaldi G, Varnum BC, Sarmiento U, Starnes C, Lile J, et al. 1999. Novel neurotrophin-1/B cell-stimulating factor-3: a cytokine of the IL-6 family. Proc Natl Acad Sci USA 96: 11458-11463.

Sendtner M, Gotz R, Holtmann B, Escary JL, Masu Y, et al. 1996. Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF. Curr Biol 6: 686-694.

Sendtner M, Gotz R, Holtmann B, Thoenen H. 1997. Endogenous ciliary neurotrophic factor is a lesion factor for axotomized motoneurons in adult mice. J Neurosci 17: 6999-7006.

Sendtner M, Kreutzberg G, Thoenen H. 1990. Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 345: 440-441.

Sendtner M, Schmalbruch H, Stöckli K, Kreutzberg PCG, Thoenen H. 1992. Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. Nature 358: 502-504.

Seniuk-Tatton NA, Henderson JT, Roder JC. 1995. Neurons express ciliary neurotrophic factor mRNA in the early postnatal and adult rat brain. J Neurosci Res 41: 663-676.

Servidei T, Aoki Y, Lewis SE, Symes A, Fink JS, et al. 1998. Coordinate regulation of STAT signaling and C-fos expression by the tyrosine phosphatase SHP-2. J Biol Chem 273: 6233-6241.

Sheng ZL, Pennica D, Wood WI, Chien KR. 1996. Cardiotrophin-1 displays early expression in the murine heart tube

and promotes cardiac myocyte survival. Development 122: 419-428.

Shi Y, Wang W, Yourey PA, Gohari S, Zukauskas D, et al. 1999. Computational EST database analysis identifies a novel member of the neuropoietic cytokine family. Biochem Biophys Res Commun 262: 132-238.

Shinohara T, Bredberg G, Ulfendahl M, Pyykko I, Olivius NP, et al. 2002. Neurotrophic factor intervention restores auditory function in deafened animals. Proc Natl Acad Sci USA 99: 1657-1660.

Siegel SG, Patton B, English AW. 2000. Ciliary neurotrophic factor is required for motoneuron sprouting. Exp Neurol 166: 205-212.

Smet PJ, Abrahamson IK, Ressom RE, Rush RA. 1991. A ciliary neuronotrophic factor from peripheral nerve and smooth muscle which is not retrogradely transported. Neurochem Res 16: 612-620.

Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, et al. 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science 263: 92-95.

Stahl N, Davis S, Taga T, Kishimoto T, Ip NY, et al. 1993. Cross-linking identifies leukemia inhibitory factor-binding protein as a ciliary neurotrophic factor receptor component. J Biol Chem 268: 7628-7631.

Stahl N, Yancopoulos GD. 1994. The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. J Neurobiol 25: 1454-1466.

Starr R, Novak U, Willson TA, Inglese M, Murphy V, et al. 1997. Distinct roles for leukemia inhibitory factor receptor alpha-chain and gp130 in cell type-specific signal transduction. J Biol Chem 272: 19982-19986.

Stöckli KA, Lillien LE, Nahernoe M, Breitfeld G, Hughes RA . 1991. Regional distribution, developmental changes, and cellular localization of CNTF messenger RNA and protein in the rat brain. J Cell Biol 115: 447-459.

Stöckli K, Lottspeich F, Sendter M, Masiakowski P, Carroll P, et al. 1989. Molecular cloning and regional distribution of rat CNTF. Nature 343: 920-923.

Stoop R, Poo M-M. 1995. Potentiation of transmitter release by ciliary neurotrophic factor requires somatic signaling. Science 267: 695-699.

Sugiura S, Lahav R, Han J, Kou SY, Banner LR, et al. 2000. Leukaemia inhibitory factor is required for normal inflammatory responses to injury in the peripheral and central nervous systems in vivo and is chemotactic for macrophages in vitro. Eur J Neurosci 12: 457-466.

Symes A, Gearan T, Eby J, Fink JS. 1997a. Integration of Jak-STAT and AP-1 signaling pathways at the vasoactive intestinal peptide cytokine response element regulates ciliary neurotrophic factor-dependent transcription. J Biol Chem 272: 9648-9654.

Symes A, Lewis S, Corpus L, Rajan P, Hyman SE, et al. 1994. STAT proteins participate in the regulation of the vasoactive intestinal peptide gene by the ciliary neurotrophic factor family of cytokines. Mol Endocrinol 8: 1750-1763.

Symes AJ, Pitts RL, Conover J, Kos K, Coulombe J. 2000. Synergy of activin and ciliary neurotrophic factor signaling pathways in the induction of vasoactive intestinal peptide gene expression. Mol Endocrinol 14: 429-439.

Symes A, Stahl N, Reeves SA, Farruggella T, Servidei T, et al. 1997b. The protein tyrosine phosphatase SHP-2 negatively regulates ciliary neurotrophic factor induction of gene expression. Curr Biol 7: 697-700.

Taga T. 1996. GP130, a shared signal transducing receptor component for hematopoietic and neuropoietic cytokines. J Neurochem 67: 1-10.

Takahashi R, Kawamura K, Hu JG, Hayashi M, Deguchi T. 1996. Ciliary neurotrophic factor (CNTF) genotypes and CNTF contents in human sciatic nerves as measured by a sensitive enzyme-linked immunoassay. J Neurochem 67: 525-529.

ten Hoeve J, de Jesus Ibarra-Sanchez M, Fu Y, Zhu W, Tremblay M, et al. 2002. Identification of a nuclear STAT1 protein tyrosine phosphatase. Mol Cell Biol 22: 5662-5668.

Thompson SW, Vernallis AB, Heath JK, Priestley JV. 1997. Leukaemia inhibitory factor is retrogradely transported by a distinct population of adult rat sensory neurons: colocalization with trkA and other neurochemical markers. Eur J Neurosci 9: 1244-1251.

Tolosano E, Cutufia MA, Hirsch E, Stefanuto G, Voyron S, et al. 1996. Ciliary neurotrophic factor constitutively expressed in the nervous system of transgenic mice projects embryonic dorsal root ganglion neurons from apoptosis. Eur J Neurosci 8: 521-529.

Uemura A, Takizawa T, Ochiai W, Yanagisawa M, Nakashima K, et al. 2002. Cardiotrophin-like cytokine induces astrocyte differentiation of fetal neuroepithelial cells via activation of STAT3. Cytokine 18: 1-7.

Ure DR, Campenot RB. 1994. Leukemia inhibitory factor and nerve growth factor are retrogradely transported and processed by cultured rat sympathetic neurons. Dev Biol 162: 339-347.

Vallieres L, Rivest S. 1997. Regulation of the genes encoding interleukin-6, its receptor, and gp130 in the rat brain in response to the immune activator lipopolysaccharide and the proinflammatory cytokine interleukin-1beta. J Neurochem 69: 1668-1683.

von Holst A, Heller S, Junghans D, Geissen M, Ernsberger U, et al. 1997. Onset of CNTFRalpha expression and signal transduction during neurogenesis in chick sensory dorsal root ganglia. Dev Biol 191: 1-13.

Wadt KA, Larsen CM, Andersen HU, Nielsen K, Karlsen AE, et al. 1998. Ciliary neurotrophic factor potentiates the

beta-cell inhibitory effect of IL-1beta in rat pancreatic islets associated with increased nitric oxide synthesis and increased expression of inducible nitric oxide synthase. Diabetes 47: 1602-1608.

Walsh N, Valter K, Stone J. 2001. Cellular and subcellular patterns of expression of bFGF and CNTF in the normal and light stressed adult rat retina. Exp Eye Res 72: 495-501.

Wang X, Halvorsen SW. 1998a. Retinoic acid upregulates ciliary neurotrophic factor receptors in cultured chick neurons and cardiomyocytes. Neurosci Lett 340: 9-12.

Wang X, Halvorsen SW. 1998b. Reciprocal regulation of ciliary neurotrophic factor receptors and acetylcholine receptors during synaptogenesis in embryonic chick atria. J Neurosci 18: 7372-7380.

Ward LD, Howlett GJ, Discolo G, Yasukawa K, Hammacher A, et al. 1994. High affinity interleukin-6 receptor is a hexameric complex consisting of two molecules each of interleukin-6, interleukin-6 receptor, and gp-130. J Biol Chem 269: 23286-23289.

Ware CB, Horowitz MC, Renshaw BR, Hunt JS, Liggitt D, et al. 1995. Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. Development 121: 1283-1299.

Watanabe D, Yoshimura R, Khalil M, Yoshida K, Kishimoto T, et al. 1996. Characteristic localization of gp130 (the signal-transducing receptor component used in common for IL-6/IL-11/CNTF/LIF/OSM) in the rat brain. Eur J Neurosci 8: 1630-1640.

Weber MJ. 1981. A diffusible factor responsible for the determination of cholinergic functions in cultured sympathetic neurons. J Biol Chem 256: 3447-3453.

Winter CG, Saotome Y, Levison SW, Hirsh D. 1995. A role for ciliary neurotrophic factor as an inducer of reactive gliosis, the glial response to central nervous system injury. Proc Natl Acad Sci USA 92: 5865-5869.

Wishingrad MA, Koshlukova S, Halvorsen SW. 1997. Ciliary neurotrophic factor stimulates the phosphorylation of two forms of STAT3 in chick ciliary ganglion neurons. J Biol Chem 272: 19752-19757.

Wong VV, Glass DJ, Arriaga R, Yancopoulos GD, Lindsay RM, et al. 1997. Hepatocyte growth factor promotes motor neuron survival and synergizes with ciliary neurotrophic factor. J Biol Chem 272: 5187-5191.

Wormald S, Hilton DJ. 2004. Inhibitors of cytokine signal transduction. J Biol Chem 279: 821-824.

Wu YY, Bradshaw RA. 1996. Synergistic induction of neurite outgrowth by NGF or EGF and IL-6 in PC12 cells. J Biol Chem 271: 13033-13039.

Xu B, Dube MG, Kalra PS, Farmerie WG, Kaibara A, et al. 1998. Anorectic effects of the cytokine, CNTF, are mediated by hypothalamic neuropeptide Y: comparison with leptin. Endocrinology 139: 466-473.

Yamamori T, Fukada K, Aebersold R, Korsching S, Fann M, et al. 1989. The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science 246: 1412-1415.

Yang Y-C. 1993. Interleukin 11: an overview. Stem Cells 11: 474-486.

Yao M, Moir MS, Wang MZ, To MP, Terris DJ. 1999. Peripheral nerve regeneration in CNTF knockout mice. Laryngoscope 109: 1263-1268.

Yasukawa H, Misawa H, Sakamoto H, Masuhara M, Sasaki A, et al. 1999. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. EMBO J 18: 1309-1320.

Ye JH, Houle JD. 1997. Treatment of the chronically injured spinal cord with neurotrophic factors can promote axonal regeneration from supraspinal neurons. Exp Neurol 143: 70-81.

Young WJ, Lee YF, Smith SM, Chang C. 1998. A bidirectional regulation between the TR2/TR4 orphan receptors (TR2/TR4) and the ciliary neurotrophic factor (CNTF) signaling pathway. J Biol Chem 273: 20877-20885.

Young WJ, Smith SM, Chang C. 1997. Induction of the intronic enhancer of the human ciliary neurotrophic factor receptor (CNTFRalpha) gene by the TR4 orphan receptor. A member of steroid receptor superfamily. J Biol Chem 272: 3109-3116.

# 4 GDNF and Related Proteins

*H. Peterziel · J. Strelau*

**Abstract:** Members of the glial-cell-line-derived neurotrophic factor (GDNF) family serve important functions in development and maintenance of distinct sets of central and peripheral neurons. All four GDNF family ligands (GFLS), GDNF, neurturin (NRTN), artemin (ARTN), and persephin (PSPN), interact with a multi-subunit receptor complex formed by the c-Ret tyrosine kinase, Ret, and a cysteine-rich glycosyl phosphatidylinositol-anchored receptor (GDNF receptor alpha 1-4). Since their discovery, GFLS have received particular attention because of their therapeutic potential in numerous neurological diseases, such as Parkinson's disease (PD), motor neuron diseases, or sensory regeneration and neuropathic pain. Targeted mutagenesis in transgenic mice has shown that Ret and GFL are required for multiple developmental events including the development of the enteric nervous system (ENS), which is affected in Hirschsprung's disease (HD). This chapter focuses on the molecular mechanisms of the initiation and the contextual dependence of signal transduction by GFL, their neuroprotective and neuroregenerative potential, and their involvement in developmental processes.

**List of Abbreviations:** aa, amino acid; ARTN, artemin; CG, ciliary ganglion; DRG, dorsal root ganglion; ENS, enteric nervous system; ERK, extracellular signal-regulated kinase; EST, expressed sequence tag; FAK, focal adhesion kinase; FMTC, familial medullary thyroid carcinoma; GABA, gamma-aminobutyric acid; GDNF, glial-cell-line-derived neurotrophic factor; GFL, GDNF family ligands; GFRα, GDNF receptor alpha; GPI, glycosyl phosphatidylinositol; HD, Hirschsprung's disease; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEN2, multiple endocrine neoplasia type 2; NCAM, neuronal cell adhesion molecule; NGF, nerve growth factor; NRTN, neurturin; PC12, pheochromocytoma cell line; PCR, polymerase chain reaction; PD, Parkinson's disease; PI3K, phosphatidylinositol-3 kinase; PLCγ, phospholipase C gamma; PSPN, persephin; Ret, rearranged in transformation; Ret/PTC, rearranged in transformation/papillary thyroid carcinomas; RTK, receptor protein tyrosine kinase; SCG, superior cervical ganglion; TGFβ, transforming growth factor beta

## 1 Introduction

In multicellular organisms, soluble peptide growth factors play important roles in intercellular communications. They accomplish their functions by signaling through cell surface membrane receptors, which in turn interact with a multitude of intracellular second messenger systems.

There is one group of growth factors, termed neurotrophins, that profoundly affects survival, development, function, and plasticity of cells in the nervous system (Korsching, 1993; Lewin and Barde, 1996). The importance of these factors is underlined by the fact that at least half of the original cell population of the nervous system is eliminated as a result of apoptosis. This ontogenetic cell death includes nerve cells, glial cells, and neural progenitors (Oppenheim, 1989; Nijhawan et al., 2000).

One hypothesis to explain the mechanism of this massive loss of neurons during development is the neurotrophic factor concept. This concept is based on the observation that distinct target tissues produce trophic factors in limited amounts for their afferent neurons, which compete for these messengers. The factors are bound by selective receptors on the afferent terminals of restricted neuronal types, retrogradely transported to the neuronal cell body, thus selectively limiting neuronal death occurring during development (Purves et al., 1986; Oppenheim, 1989). This is thought to be a means to match neuron and target cell populations. Today, the term neurotrophic is mostly used for substances that enhance neuronal differentiation as well as neuronal survival. Consequently, target-derived neurotrophic factors play a crucial role in the regulation of multiple aspects of development and maintenance of the central nervous system (CNS) and the peripheral nervous system (PNS) (Nijhawan et al., 2000).

The first description of a soluble factor with neurotrophic activity, later named nerve growth factor (NGF), dates back as far as 1948, when Elmer Bueker observed that implanted tissue from mouse sarcoma promoted outgrowth of sensory neurons from dorsal root ganglia (DRGs) in chick embryos (Bueker, 1948).

Four decades later the first member of a novel family of growth factors, namely the glial-cell-line-derived neurotrophic factor (GDNF), appeared on the neurotrophic factor scene. GDNF was isolated from

a rat glial tumor cell line culture in a search for secreted factors that promoted neuronal survival in primary culture. It was identified by virtue of its ability to induce dopamine uptake and cell survival in cultures of embryonic ventral midbrain dopaminergic neurons (Lin et al., 1993). The factor was classified as a distantly related member of the transforming growth factor beta (TGFβ) superfamily of peptidic growth factors as it contains seven highly conserved cysteine residues in the same relative spacing as other members of this family. Although the primary structure of GDNF has only 20% sequence similarity to TGFβ2, the location of cysteine residues, forming the characteristic cysteine knot motif, is highly homologous to that of TGFβ2 (Lin et al., 1993; Haniu et al., 1996). Two groups simultaneously reported the identification of the tyrosine kinase Ret (rearranged in transformation) as a functional GDNF receptor, one group showed that Ret mediated the neurotrophic effects of GDNF on motoneurons and dopaminergic neurons (Trupp et al., 1996) and the other group showed that normal Ret function was necessary for GDNF-mediated signaling, by using explant cultures from Ret-deficient mouse embryos (Durbec et al., 1996). However, shortly thereafter, Jing et al. (1996) reported that GDNF does not or only weakly bind to Ret directly, but uses a receptor complex composed of Ret and a glycosyl phosphatidylinositol (GPI)-linked cell surface protein, which binds GDNF with high affinity and was named GDNF receptor alpha (GDNFRα or GFRα1; Jing et al., 1996; Treanor et al., 1996).

Three other members of the GDNF family have been found so far, termed neurturin (NRTN), artemin (ARTN), and persephin (PSPN). NRTN was isolated from Chinese hamster ovary cell-conditioned media. Like GDNF, it was identified based on its survival-promoting effect on primary, in this case sympathetic, neurons in culture (Kotzbauer et al., 1996). The preferred receptor for NRTN was shown by several groups to be another GPI-linked receptor of the same family and is now termed GFRα2 (Baloh et al., 1997; Buj-Bello et al., 1997; Jing et al., 1997; Klein et al., 1997; Suvanto et al., 1997).

PSPN was cloned by using polymerase chain reaction (PCR) with degenerate primers derived from the known sequences of GDNF and NRTN (Milbrandt et al., 1998), while the last member, ARTN, was identified by searching databases for a potential novel GDNF family ligand (GFL) with the sequence of the mature NRTN as a query (Baloh et al., 1998a). ARTN was characterized by its ability to activate Ret-mediated signaling in the presence of GFRα3, which had been identified as an expressed sequence tag (EST), homologous to GFRα1 and GFRα2, and had been an orphan GFL receptor before (Jing et al., 1997; Baloh et al., 1998b; Naveilhan et al., 1998; Widenfalk et al., 1998; Worby et al., 1998). The high-affinity GPI-linked receptor for PSPN was shown to be GFRα4, which was first identified in the chick (Enokido et al., 1998; Thompson et al., 1998). The mammalian GFRα4 has some peculiarities compared with the other GFRα family members. First, it is structurally divergent due to a lack of the aminoterminal cysteine-rich domain; second, besides the GPI-linked form, a putative secreted and a transmembrane GFRα4 protein may be synthesized from differentially spliced transcripts; and third, it differs in its ability to interact with Ret (Lindahl et al., 2000; Masure et al., 2000; Zhou et al., 2001; Yang et al., 2004).

Thus, as depicted in ❯ *Figure 4-1*, all GFLS favor binding to their cognate GFRα receptors, resulting in recruitment and activation of the transmembrane Ret tyrosine kinase.

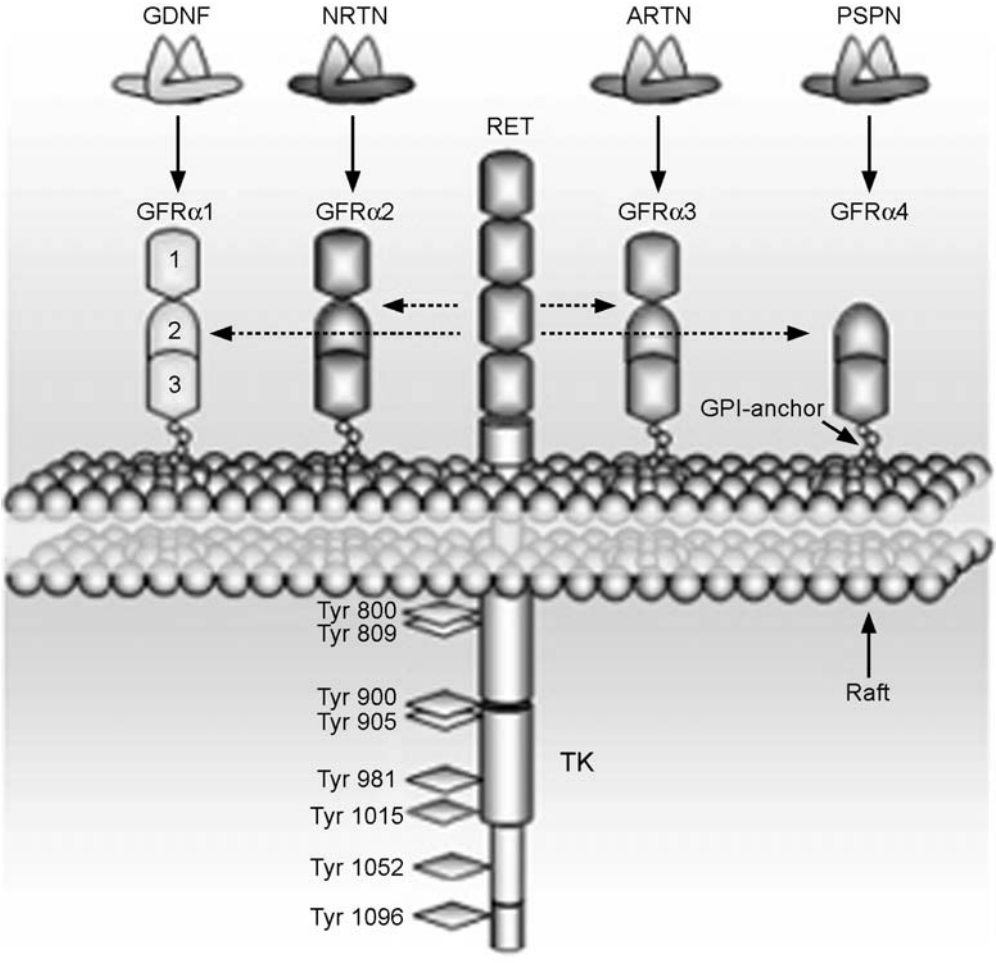
## 2 Protein Structures of GFLS and Their Receptors

### 2.1 GDNF Family Ligands

GFLS are heterogeneously glycosylated homodimers that are synthesized in various tissues as preproproteins. These precursors contain signal sequences, which are released upon secretion by proteolytic cleavage, resulting in an active form. Seven highly conserved cysteine residues form the characteristic cysteine knot fold, in the same relative spacing as the other members of the TGFβ superfamily. Each homodimer consists of a polypeptide chain of about 110 residues. The atomic-level resolution of GDNF has been done by X-ray crystallography (Eigenbrot and Gerber, 1997). Each GDNF monomer has two pairs of antiparallel twisted beta strands tightly joined by three disulfide bonds. The two protomers interact via an additional intermolecular disulfide linkage to form an antiparallel dimer with explicit left-right symmetry.

**Figure 4-1**

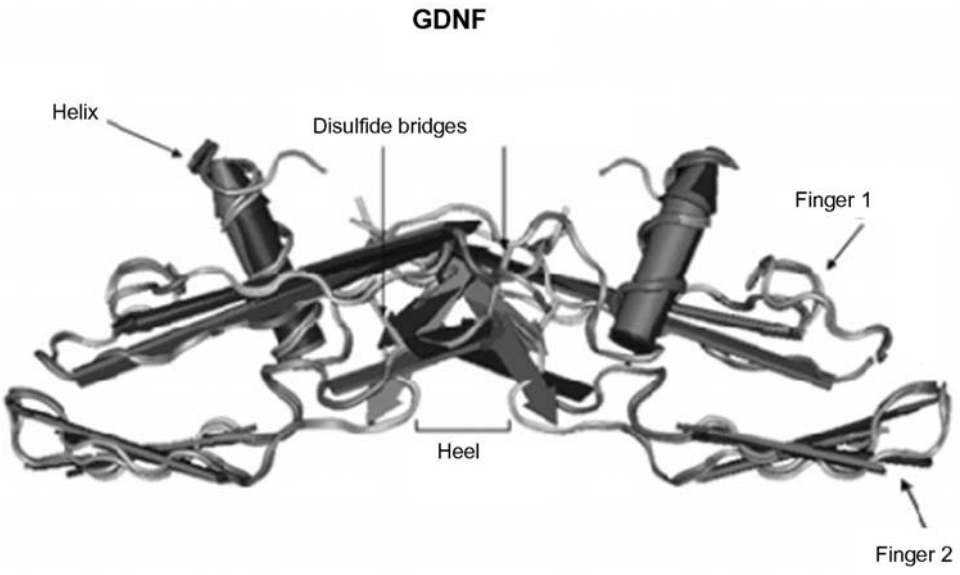
**Interaction of GDNF family ligand (GFL) with receptors. GFL homodimers first bind to their cognate GFRα receptors. Arrows show the preferred ligand–receptor specificity. Heterotetramerization induces Ret autophosphorylation of distinct tyrosine residues in the intracellular catalytic domain**




This symmetry is due to the two "fingers" on each side of the paired ligands, corresponding to the symmetric binding sites for the dimerized receptors (*Figure 4-2*).

The specificity of binding of GDNF to its cognate receptor GFRα1 is mediated by residues along the two fingers, while the center of the dimerized molecule, including the so-called heel region, does not seem to be important for receptor binding (Eketjäll et al., 1999; Baloh et al., 2000). Few amino acid exchanges are able to alter the binding specificity of the ligands; however, the different GFLs have different requirements for binding to their receptors. While insertion of specific amino acid residues from finger 2 of GDNF into the appropriate part of PSPN allows the resultant mutant PSPN to bind and activate a GFRα1/Ret complex, binding of NRTN to GFRα2 and ARTN to GFRα3 is additionally dependent on residues in the heel region

**Figure 4-2**
**Three-dimensional glial-cell-line-derived neurotrophic factor (GDNF) structure. Each GDNF molecule contains two fingers and one helix. Disulfide bridges are forming the characteristic cysteine-knot motif. The amino acids determining the specificity of binding to GFRα1 are predominantly located on the two fingers. The center of the dimer, the so-called heel region, seems to be less important**




(Baloh et al., 2000). These differential structural requirements could contribute to the different degrees of promiscuity of the ligand–receptor interactions: at least in vitro NRTN and ARTN can also bind to GFRα1 and GDNF to GFRα2 and GFRα3, but none of these three ligands has been shown to bind to GFRα4, nor does PSPN bind to any other receptor. However, for each GFL the affinity to a receptor other than the cognate receptor is much lower and the effect of this crosstalk is probably much less important than the favored binding to the specific receptors in vivo.

## 2.2 GDNF Receptors alpha

The GFRαs are cell surface receptors that are bound to the plasma membrane by a GPI anchor. Because of a conserved cysteine pattern GFRα1, 2, and 3 proteins are predicted to have three homologous globular cysteine-rich domains (for a review see Airaksinen et al., 1999), whereas the mammalian GFRα4 has only two, corresponding to the second and the third domain of the other receptors (Masure et al., 2000). These globular subdomains are joined by less conserved adaptor regions (Airaksinen et al., 1999). Domains 2 and 3 form a core, which is connected to the GPI anchor via a C-terminal extension, while the N-terminal domain 1, which is lacking in GFRα4, is separated by a hinge region from this core. Scott and Ibanez (2001) have shown, by creating a panel of chimeric GFRα receptor constructs, that the N terminus is not required for specific ligand binding, while the central domain (equivalent to the core above) contains the major determinants of ligand binding. Specificity of ligand binding is, according to their model, further determined by subcentral domains (Scott and Ibanez, 2001). While these authors proposed a single large central domain, recent crystallization of domain 3 of GFRα1 indicates that domains 2 and 3 are independent structures. Each is made up of a novel protein fold, consisting of a bundle of five alpha helices with five disulfide bridges consistent with the conserved cysteine pattern present in all GFRαs (Leppanen et al., 2004).

## 2.3 Ret Tyrosine Kinase

Ret is a receptor protein tyrosine kinase (RTK) initially identified as an oncogenic product with transformation capability (Takahashi and Cooper, 1987; Takahashi et al., 1988). Ret, like other receptor tyrosine kinases, is activated by dimerization induced by ligand binding. The active receptor dimer exists in a conformation compatible with trans-autophosphorylation on tyrosine residues and stimulation of protein tyrosine kinase activity (for a review see Schlessinger, 2000). In addition to stimulation of tyrosine kinase activation, protein phosphorylation is crucial for recruitment and activation of different downstream effectors (for a review see Pawson, 2002).

The cytoplasmic kinase domain of Ret is highly conserved between all vertebrate and invertebrate species where it has been identified so far. It shares the typical protein tyrosine kinase structure, namely a conserved catalytic core with a smaller amino-terminal subdomain comprising a five-stranded β-sheet and one α-helix, and a larger carboxy-terminal subdomain that is mainly α-helical. ATP binds in the deep cleft between the two lobes, while a tyrosine of the peptide substrate binds to the carboxy-terminal lobe. There are several universally conserved residues in this core domain. Activation is usually by relief of autoinhibition, which is mediated by phosphorylation of key residues leading to distinct conformational changes. Crystal structures of the tyrosine kinase domain of RTKS, with and without bound ligands, have shed remarkable light on how phosphorylation tightly controls the catalytic activity. In unstimulated RTKS the activation loop is in autoinhibitory conformation, either occluding substrate tyrosine binding or ATP binding to the active site. As a result of ligand stimulation, which is the case of Ret is by interaction with the GFL/GFRα complex, one of the tyrosine residues in the activation loop is phosphorylated in trans by the dimeric receptor partner, which is brought closer sterically due to dimerization. This leads to a change in the conformational structure of the activation loop away from the active site, allowing access of substrate and ATP to the active site (for a review see Huse and Kuriyan, 2002).

Unlike other known receptor tyrosine kinases, the extracellular domain of the Ret molecule lacks immunoglobulin- or fibronectin-like domains or leucine repeats. It is formed by a highly conserved pattern of 14 cysteine residues in a 120-amino-acid (aa) stretch in the "hinge region" adjacent to the transmembrane segment, followed by four N-terminal modules, each with a length of about 110 residues. These folds show high similarity to the extracellular domains of the cadherin family of $Ca^{2+}$-dependent cell adhesion molecules (Iwamoto et al., 1993; Kuma et al., 1993; Anders et al., 2001). Indeed, Ret is able to bind $Ca^{2+}$ directly and the presence of calcium ions is a prerequisite for binding of Ret to the GDNF/GFRα1 complex. Ligand binding, together with the GFRα coreceptors, is mediated by specific residues located in the N-terminal first cadherin-like domain. Mutagenesis studies showed that loss of these residues results in a complete loss of ligand binding (Kjaer and Ibanez, 2003a).

Ret exists in at least two isoforms, differing in their C-terminal amino acid (aa) sequence (9 aa in the short (Ret9), 150-kDa form; 51 aa in the long (Ret51), 170-kDa form), which are generated by differential 3′ polyadenylation and splicing of a single transcript. Interestingly, they are not able to interact with each other, indicating that the interaction of the Ret monomers in the dimer is mediated by these C-terminal stretches. Furthermore, the two isoforms are functionally different in that they interact in part with different adaptor molecules to mediate intracellular signaling or the ubiquitin system, leading to different turnovers of Ret9 and Ret51 (Borrello et al., 2002; Tsui-Pierchalla et al., 2002). The in vivo importance of these differences is reflected by the fact that a lack of Ret9 leads to kidney agenesis and loss of enteric innervation in mice, whereas Ret51-deficient mice do not show any defects during development (de Graaff et al., 2001). Moreover, the kidney defect in Ret9 knockout mice cannot be rescued by substitution with Ret51. However, Ret51-mediated signaling might be related to differentiation events in later kidney organogenesis and/or in the adult kidney (Lee et al., 2002).

The most likely stoichiometry of the multimeric GDNF/GFRα/Ret complex is presumed to be $(GDNF)_1(GFR\ \alpha)_2(Ret)_2$ (Jing et al., 1996). So far there are two models that try to explain the receptor complex formed by Ret and GFRαs. The first one hypothesizes that the dimeric ligand binds to a monomeric or dimeric GFRα and this complex then interacts with Ret and induces its dimerization (Jing et al., 1996). Alternatively, Ret might be preassociated with GFRα before binding to the ligand (Ibanez, 1998; Trupp et al., 1998; Eketjäll et al., 1999).

## 3 Expression of GFLS and Their Receptors: Knockout Phenotypes

### 3.1 GDNF and GFRα1

A detailed analysis of GDNF expression in the developing mouse brain has been performed by Hellmich et al. (1996). GDNF messenger RNA (mRNA) was detectable in the anterior neuroectoderm during early stages of neurogenesis. In organs outside the nervous system that develop through inductive epithelial–mesenchymal interactions, GDNF expression is strictly confined to mesenchymal tissues. A similar expression pattern has been described in rats (Choi-Lundberg and Bohn, 1995; Suvanto et al., 1996). Studies on GDNF expression in later stages of development and in early postnatal stages revealed a region-specific temporally defined expression pattern in neuronal and nonneuronal cells in the CNS (Nosrat et al., 1996). Clearly, neuronal expression is present in Clarke's column of the spinal cord, and in the Purkinje cell layer of the cerebellum. All brain areas that are innervated by dopaminergic neurons and the noradrenergic locus coeruleus are positive for GDNF; however, expression in the striatum appears to be rather low. In the adult rat brain, GDNF levels generally seem to decrease; by in situ hybridization neuronal expression can be detected in the striatum and the substantia nigra, where it is mainly found in dopaminergic neurons. Moreover, GDNF has been found in cortex, hippocampus, thalamus, cerebellum, olfactory bulb, and spinal cord (Pochon et al., 1997; Trupp et al., 1997, for review see Unsicker et al., 1999).

In humans, GDNF expression shifts from a mostly superficial glial and ependymal expression in early stages of development (10–15 weeks) to deeper localized neuronal and glial structures of the brain in later development (25–35 weeks), most prominently in cerebellar Purkinje cells (Koo and Choi, 2001). More recently, Serra et al. (2002) reported that GDNF-expressing neurons were also found in neonate and adult human hippocampus, suggesting a role for GDNF in the development and maintenance of neuronal function in human archicortex. Investigations of the dopaminergic system of the adult human brain showed GDNF expression in the striatum, with highest levels in the caudate nucleus, relatively low levels in the putamen, but no detectable expression in the neurons of the substantia nigra, consistent with the role of GDNF as a target-derived factor essential for the survival of the nigral dopaminergic neurons (Schaar et al., 1994). Accordingly, the cell surface GDNF receptor GFRα1 and GDNF are mostly found in segregated regions in the basal ganglia; however, the coexpression of GDNF and GFRα1 in other brain regions like cerebellum, hippocampus, and olfactory bulb indicates that GDNF also exerts a paracrine mode of action (Trupp et al., 1997). A thorough analysis of the transmitter phenotype of GFRα1-positive neurons was performed by Sarabi et al. (2003). They found GFRα1 expressed in gamma-aminobutyric acid (GABA)-containing neurons, i.e., in cortex and thalamus, in cholinergic motoneurons in the spinal cord and few septal cholinergic neurons, in catecholaminergic neurons in the periventricular hypothalamic nucleus, dorsal raphe nucleus, and locus coeruleus, in many serotonergic neurons within the raphe, and in few neurons containing nitric oxide synthase. As expected from the survival-promoting effect of GDNF on dopamine neurons, GFRα1 mRNA is strongly expressed in developing and adult dopamine neurons.

Outside the CNS, GDNF mRNA is found in many mesenchyme and mesenchyme-derived tissues, most prominently in the developing kidney and the smooth muscle layer of the gastrointestinal tract, as well as in developing skin, whisker pad, and testis (Trupp et al., 1995; Nosrat et al., 1996). The superior cervical ganglion (SCG) and DRG of the PNS also express, albeit, low levels of GDNF mRNA. Consistent with the survival-promoting role of GDNF, its expression increases dramatically in lesion paradigms, i.e., after sciatic nerve transection (Trupp et al., 1995). GDNF supports the survival of motoneurons and a subpopulation of sensory neurons in vivo and in vitro; consequently, GDNF is also found in embryonic mouse in limb bud at the time when axons enter the limb (Wright and Snider, 1996). Recently, it has been shown that GDNF acts as a peripheral signal to induce PEA3 expression in specific motor neuron pools, thereby regulating both cell body position and muscle innervation by promoting axon arborization (Haase et al., 2002).

In the testis, GDNF is normally expressed in Sertoli cells, while GFRα1 and Ret are found on undifferentiated spermatogenic stem cells (Meng et al., 2000). Interestingly, both reduction and overexpression of GDNF lead to abnormal spermatogenesis in mice, the first by depletion of spermatogenic stem cells due to increased differentiation, the second exactly by the opposite effect, namely by inhibiting

differentiation and maturation of these stem cells (Meng et al., 2000, 2001). These observations indicate that the regulation of the amount of GDNF in Sertoli cells of the testis is essential for proper spermatogenesis. Expression has also been demonstrated in the penis of adult rats from where it is retrogradely transported in penile parasympathetic and sensory nerves (Laurikainen et al., 2000).

In the kidney and the gastrointestinal tract, GFRα1 mRNA and Ret mRNA distributions overlap. DRGs, cranial ganglia, and developing peripheral nerves are also positive. GFRα1 was additionally found in sensory areas (inner ear, eye, olfactory epithelium, and vomeronasal organ) and in developing teeth (Nosrat et al., 1997).

Although GDNF is expressed in dopaminergic and noradrenergic neurons and efficiently promotes the survival of these neuron populations in vitro and in vivo, none of the three groups that independently generated GDNF knockout mice in 1996 (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) reported any changes in hindbrain noradrenergic or midbrain dopaminergic neurons. These findings indicated that at least during embryonic development GDNF is not essential for the development of catecholaminergic neurons. As GDNF-deficient mice die shortly after birth the effect of GDNF ablation on later stages of dopaminergic neuron development cannot be investigated directly. To have a means to, nevertheless, study the continued fate of the dopaminergic neuron population in the absence of GDNF, Granholm et al. (2000) transplanted fetal neural tissues of GDNF knockout mice into the midbrain region of adult wild-type mice. The resulting reduction of dopaminergic neuron number and fiber outgrowth indicate that postnatal survival and/or phenotypic maintenance of ventral mesencephalic dopaminergic neurons is dependent on GDNF.

In the PNS, GDNF-deficient mice have deficits in DRG, sympathetic, and nodose neurons. The most severe defects are the complete lack of the enteric nervous system (ENS) distal to the stomach and, hence, proper innervation of the gastrointestinal tract, and renal agenesis or dysgenesis, due to the lack of ureteric bud formation and branching. GFRα1-deficient mice also demonstrate absence of enteric neurons and agenesis of the kidney, and no change in midbrain dopaminergic and motor neurons, characteristics that are reminiscent of both GDNF- and Ret-deficient mice (Cacalano et al., 1998; Enomoto et al., 1998). Unexpectedly, the peripheral ganglia that are severely affected in GDNF knockout mice show only minor or no changes. These observations indicate that although in renal and ENS development, GDNF effects are solely mediated via the GDNF/GFRα1 system, there might be some promiscuity or compensation between GFLS and receptors in the development of the peripheral ganglia.

GDNF- and GFRα1-deficient mice show a 25% loss of motoneurons in the lumbar spinal cord (Moore et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998). Specific subpopulations of motoneurons in the spinal cord are strongly positive for GFRα1 expression. These cells are lost during ontogenetic cell death as a result of increased apoptosis in the absence of GDNF signaling and hence proper neurotrophic support (Garces et al., 2000). ❯ *Figure 4-3* shows an in situ hybridization for GFRα1 and Ret in motoneurons of the cervical spinal cord of the mouse on embryonic day 18.5.

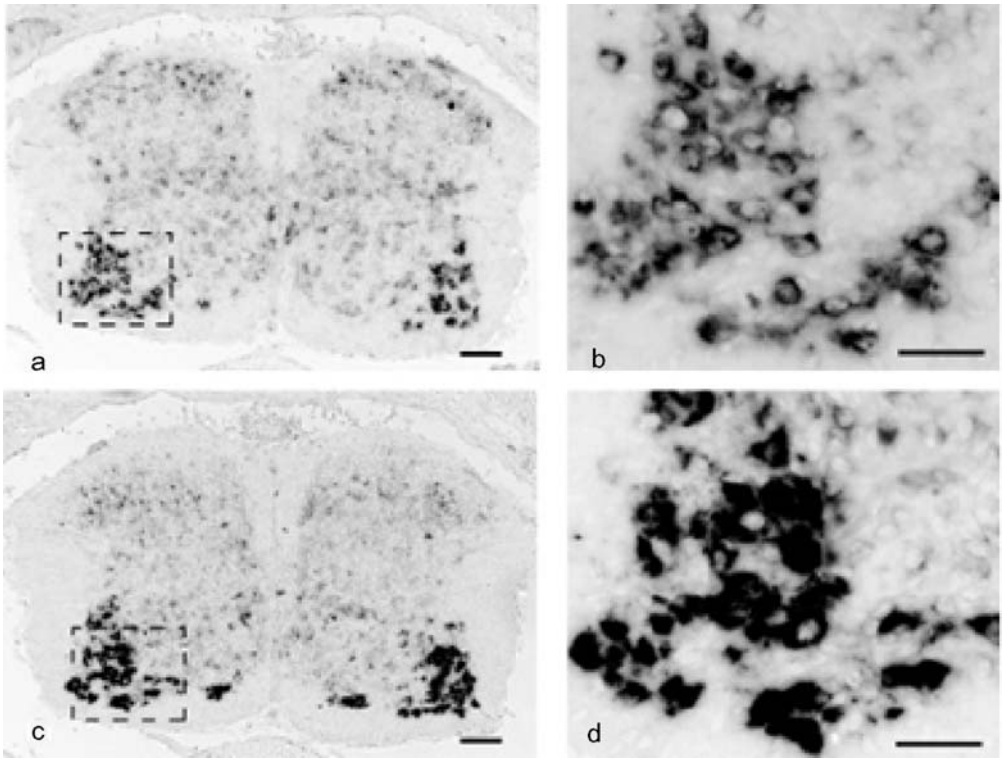
## 3.2 Neurturin and GFRα2

The second GFL, NRTN, had been discovered on the basis of its survival-promoting effect on sympathetic, nodose, and DRG neurons in culture (Kotzbauer et al., 1996). In the CNS, NRTN is expressed in postnatal cerebral cortex, striatum, several brainstem areas, and the pineal gland. Its cognate receptor GFRα2 is more widely expressed in the developing and adult CNS, including cerebral cortex, cerebellum, thalamus, zona incerta, hypothalamus, brainstem, and spinal cord (Widenfalk et al., 1997). Cholinergic neurons carry GFRα2 on their terminals and somata and respond to NRTN support (Wanigasekara et al., 2004).

GFRα2 and Ret are expressed in neurons of the SCG, in subpopulations of sensory neurons, in developing peripheral nerves, and in the myenteric intestinal plexuses (Baloh et al., 1997). In developing salivary glands NRTN has an epithelial expression, whereas GFRα2 is expressed in the surrounding tissue. In the gonads, NRTN is detectable in Sertoli cells in males and in the epithelium of the oviduct in females, while GFRα2 is expressed by the germ cell line, again confirming the complementary expression of ligand and receptor in accordance with the neurotrophic factor concept (Widenfalk et al., 1997). In the adult rat,

**Figure 4-3**

**Motoneurons in the cervical spinal cord express GFRa1 and Ret. In situ hybridization for GFRα1 (a, b) and Ret (c, d) on kryosections of mouse E 18.5 cervical spinal cord. Motoneurons expressing GFRα1 and Ret in the ventral horn are shown in higher magnification in b and d, respectively. Bars in a and c = 100 μm, in b and d = 50 μm. (Photographs from K. Huber)**




GFRα2 expression resembles the distribution of GFRα1, with highest levels in lung, spleen, and brain and lower amounts in kidney and heart, but in contrast to GFRα1, GFRα2 is lacking in the liver (Jing et al., 1997). A detailed review of the expression of GDNF, NRTN, and their receptor systems in neuronal and nonneuronal tissues can be found in Unsicker et al. (1999).

NRTN-deficient mice are viable and fertile. They show moderate defects in the ENS, including reduced myenteric plexus innervation density leading to a reduction in gastrointestinal motility. NRTN seems to be mandatory for proper development of parasympathetic neurons, as a lack of NRTN leads to a major reduction in the innervation of the lacrimal and submandibular salivary glands. Consistent with the expression pattern of GFRα2, neurons in the trigeminal ganglia and DRGs are depleted (Heuckeroth et al., 1999). Studies on mice lacking a functional GFRα2 receptor indicate its responsibility for most NRTN effects, as they also show absence of parasympathetic cholinergic innervation in the lacrimal and salivary glands and a severe reduction in the small bowel, whereas the sympathetic innervation appears normal (Rossi et al., 1999). However, a distinct cholinergic minority of sympathetic neurons, expressing GFRα2 in wild-type mice, fails to properly innervate their target tissues, i.e., sweat glands in the footpads and periosteum, in GFRα2-deficient mice, whereas the noradrenergic sympathetic innervation is not affected. These results indicate that NRTN, acting via GFRα2, functions as a general target-derived innervation factor for cholinergic neurons in the autonomic nervous system (Hiltunen and Airaksinen, 2004).

## 3.3 Artemin and GFRα3

ARTN expression is detectable in various fetal and adult peripheral tissues in humans, in adults with highest levels in pituitary gland, trachea, prostate, placenta, pancreas, heart, and kidney, and in embryos in kidney and lung (Baloh et al., 1998a; Masure et al., 1999). The factor is only marginally expressed in fetal and adult brain, with detectable but low levels in basal ganglia and thalamus. In embryonic rats (E14) ARTN has not been found in the CNS, but it is detectable in immature Schwann cell precursors of developing peripheral nerve roots (Baloh et al., 1998a). The expression of ARTN is in most cases complementary to the expression of its cognate receptor GFRα3, indicating that ARTN acts as a typical target-derived factor (Naveilhan et al., 1998). Compared with GFRα1 and GFRα2, which are widely distributed in the CNS and peripheral organs, the expression of GFRα3 is much more selective and seems to be absent in the CNS, consistent with the lack of ARTN (Baloh et al., 1998b; Yu et al., 1998). High GFRα3 expression is found in nociceptive subpopulations of developing sensory DRGs, in trigeminal and in glossopharyngeal ganglia (Baloh et al., 1998a; Naveilhan et al., 1998; Widenfalk et al., 1998). Additionally, GFRα3 is expressed in the SCGs, and in nonneuronal tissue predominantly in regions in the lower urogenital and digestive tracts (Widenfalk et al., 1998; Worby et al., 1998). Nonneuronal expression is also found in olfactory ensheathing cells, in chromaffin cells in the adrenal gland, and in small clusters of cells in the intestinal epithelium (Widenfalk et al., 1998).

The rostral migration of cells to form the SCG and the extension of axons along blood vessels involve ARTN signaling through Ret and GFRα3 (Honma et al., 2002; Young et al., 2004). Consistently, both ARTN- and GFRα3-deficient mice show abnormalities in the migration and the axonal projection pattern of the entire sympathetic nervous system. ARTN is acting as a guidance factor, as GFRα3-expressing sympathetic fibers grow toward the ARTN source, which is released from vascular smooth muscle cells. A lack of either the factor or its receptor results in abnormal innervation of target tissues. Subsequent cell death is not due to a missing proliferation or survival-promoting effect of ARTN itself, but due to the loss of its chemoattractive property and the resulting deficit in proper innervation (Honma et al., 2002). Although GFRα3 is highly expressed in sensory ganglia, lack of the ARTN/GFRα3 system affects neither their development and phenotypic appearance nor their innervation pattern. This might be due to the complex neurotrophic dependency of sensory neurons, including effects of other GFL, as GFRα3-positive neuron populations also express GFRα1 and/or GFRα2 (Naveilhan et al., 1998; Baudet et al., 2000).

## 3.4 PSPN and GFRα4

The PSPN/GFRα4 system is the most recently discovered of the GFL/receptor complexes in mammals and least is known about its functions. In neonatal rats, PSPN is synthesized throughout the nervous system, i.e., in cortex, hippocampus, striatum, mesencephalon, cerebellum, and spinal cord, as well as in the sympathetic SCG and sensory DRG neurons. Expression can also be detected in peripheral nerves, in purified cortical, striatal, and mesencephalic astroglial cell cultures of newborn rats, but not in oligodendrocytes or their precursors (Jaszai et al., 1998, Strelau and Unsicker, 1999). PSPN promotes the survival of midbrain dopaminergic neurons and spinal cord motor neurons in vitro and in vivo (Milbrandt et al., 1998; Bilak et al., 1999; Soler et al., 1999), but, in contrast to the other GFL, does not support any peripheral neurons (Milbrandt et al., 1998). Consistent with the neurotrophic effect on dopaminergic neurons in culture, the ventral mesencephalon and the striatum express high levels of PSPN, whereas GFRα4 is present on dopaminergic neurons (Akerud et al., 2002). However, deficiency in PSPN does not affect the number of mesencephalic dopaminergic neurons or locus coeruleus neurons in vivo, nor do the PSPN knockout mice show any other developmental or behavioral deficits, indicating that PSPN might not act as a typical neurotrophic factor. Moreover, GFRα4 in the brain seem to be spliced predominantly to a putative intracellular or secreted form, whether this splice form is functional, and if so, what effects this form promotes, remains enigmatic so far (Lindahl et al., 2000). The only indication of an important function of PSPN in the brain is the hypersensitivity of PSPN-deficient mice to cerebral ischemia and the potent neuroprotective effect of recombinant PSPN protein in this disease (Tomac et al., 2002).

Organs outside the nervous system, where the GPI-linked splice variant is expressed, include the juvenile thyroid and parathyroid glands. Consistently, GFRα4-positive thyroid C cells are lost in Ret-deficient mice, indicating the importance of a functional GFRα4/Ret complex for proper development of these cells. Moreover, GFRα4 expression in the thyroid gland might explain the occurrence of tumors upon Ret rearrangement in this organ (Lindahl et al., 2000). Outside the nervous system, PSPN is also expressed in the developing kidney, and promotes ureteric branching in vitro (Sariola and Saarma, 1999).

### 3.5 Ret Tyrosine Kinase

In the mouse, Ret expression in neural crest lineages starts as early as day 8.5, in the rat at day 11.5 of embryogenesis. Consistent with the function of Ret as the common transmembrane tyrosine kinase receptor component for all four GFLs, Ret transcripts can be found in virtually all central and peripheral neuron populations that coexpress one of the GFRαs. These include all cranial ganglia, autonomic ganglia and subsets of DRGs, enteric neuroblasts, and myenteric ganglia of the gut (Pachnis et al., 1993; Schuchardt et al., 1994; Tsuzuki et al., 1995). Additionally, Ret is found in spinal cord motoneurons, basal ganglia, cerebellum, hippocampus, the subthalamic nucleus, and the olfactory bulb (Trupp et al., 1997).

Outside the nervous system Ret is expressed in the excretory system, i.e., the ureteric bud of the developing kidney and the Wolffian duct. Furthermore, Ret expression can be found in the acinar cells of the salivary gland (Tsuzuki et al., 1995).

Analysis of mice lacking the Ret gene revealed the major requirement of this kinase for most of the GFL actions. Ret is obligatory for normal renal organogenesis and enteric neurogenesis; mice homozygous for a targeted mutation in Ret die soon after birth, showing renal agenesis or severe dysgenesis, and lacking enteric neurons throughout the digestive tract (Schuchardt et al., 1994), as is the case for GDNF or GFRα1 knockout mice and partially for lack of NRTN or GFRα2. Both Ret and GFRα2 mutant mice exhibit a reduction in cardiac ganglia and cholinergic innervation of the ventricular conduction system (Hiltunen et al., 2000). It was first described that Ret-deficient mice lack SCGs (Durbec et al., 1996), but later it turned out, that, as in ARTN- or GFRα3-deficient mice, neuronal precursors throughout the entire sympathetic nervous system fail to migrate and project axons properly, leading, among other abnormalities, to a mislocation of the SCG (Enomoto et al., 2001). Furthermore, Ret has been shown to be indispensable for maturation of cholinergic sympathetic neurons (Burau et al., 2004).

However, the temporospatial pattern of expression of GFRα mRNAs does not always match that of Ret mRNA. For instance, GFRα1 mRNA is also found in the developing ventral striatum, the olfactory tubercle, and the hippocampus. These areas seem to be devoid of Ret mRNA, suggesting that GFRα1 might also have functions unrelated to Ret (Nosrat et al., 1997). This lead to the postulation of other coreceptors for the GFRαs, which should be able to mediate intracellular signaling after binding to the ligand-bound GPI-linked receptors. One such alternative coreceptor, the neuronal cell adhesion molecule (NCAM), has recently been described, as will be discussed later.

## 4 Ret-Related Diseases

Ret mutations are responsible for at least five human diseases: Hirschsprung's disease (HD); papillary thyroid carcinoma; and three types of inherited cancer syndromes called multiple endocrine neoplasia type 2 A (MEN2A) and type 2 B (MEN2B), and familial medullary thyroid carcinoma (FMTC). Interestingly, both gain of function and loss of function of Ret lead to pathologic syndromes, highlighting the particular importance of tight regulation of Ret function in vivo (for a review see Pasini et al., 1996; Edery et al., 1997).

Loss of function leads to a defect in the development of the ENS, a congenital malformation known as HD, resulting in the absence of the enteric plexuses in the hindgut and, subsequently, in partial to complete intestinal obstruction during the first years of life. Ret mutations are scattered along the entire coding region of the gene in patients suffering from Hirschsprung's disease (reviewed in Eng and Mulligan, 1997; Martucciello et al., 2000). In many cases, frame shift or missense mutations in the region encoding the

intracellular domain of Ret result in total disruption or a partial change of the structure of the tyrosine kinase domain (Romeo et al., 1994), including mutations that interfere with the docking of adaptor molecules that mediate Ret downstream signaling (Geneste et al., 1999, for a review see Manie et al., 2001). Mutations in the extracellular domain mostly affect processing in the endoplasmic reticulum and result in a reduction of Ret expression at the cell surface (Kjaer and Ibanez, 2003b). Interestingly, although mutations in the genes for GDNF and NRTN have been detected in patients suffering from Hirschsprung's disease, rather than being causative, they might modulate the pathogenesis of the disease (Angrist et al., 1996; Salomon et al., 1996; Doray et al., 1998; Eketjäll and Ibanez, 2002).

Gain-of-function mutations have been found in all four types of Ret-related neoplastic diseases. The most frequent genetic alteration that has been identified in thyroid papillary carcinomas is a rearrangement of the Ret gene, called Ret/PTC (rearranged in transformation/papillary thyroid carcinomas). At least 15 types of Ret/PTC rearrangements have been described, where the tyrosine kinase domain of Ret is fused to the N-terminal encoding region of ten different genes, the most common types being Ret/PTC-1, followed by Ret/PTC-3 (for review see Nikiforov, 2002; Ichihara et al., 2004). In these fusion proteins, Ret tyrosine kinase is constitutively active, which has been attributed to the fact that the fused proteins contain coiled coil domains that mediate protein oligomerization, which is essential for its oncogenic properties.

The multiple endocrine neoplasia type 2 syndromes MEN2A/B and FMTC are attributed to point mutations in Ret resulting in its constitutive activation, yet by different molecular mechanisms. Most MEN2A and FMTC mutations induce disulfide-linked Ret dimerization on the cell surface due to mutations in the cysteine-rich region in the extracellular domain. MEN2B is caused by a methionine to a threonine exchange at position 918 in the tyrosine kinase domain, resulting in Ret activation without dimerization, due to a conformational change in the catalytic core region. The detection of Ret mutations are used in the clinics for diagnosis of medullary thyroid carcinoma and are important parameters included in the decision for the most promising therapeutic approach in this neoplastic diseases (for review see Massoll and Mazzaferri, 2004).

Analyses of the molecular mechanisms activated by these naturally occurring constitutively active Ret mutants have significantly contributed to the characterization of the cellular pathways activated by GFL.

## 5 GFL Signaling

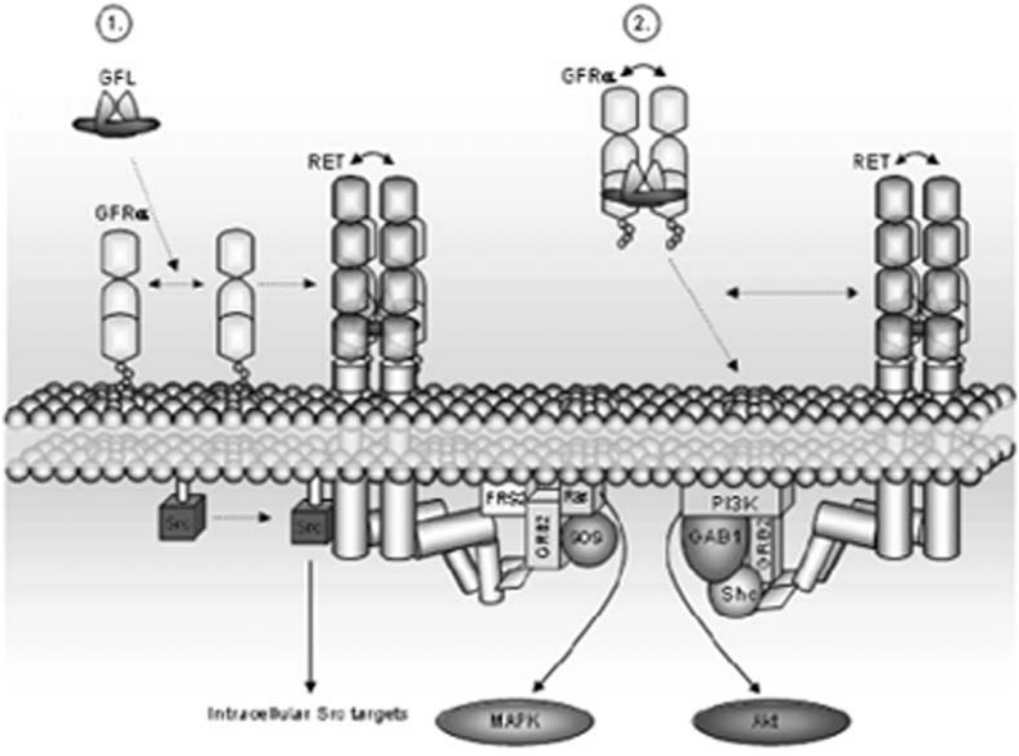
### 5.1 Intracellular Signal Transduction Pathways Activated by GFL

All four GFLs bind preferentially to their cognate GPI-linked alpha receptors GFRα1–4. Because of the lipid anchor the receptors are targeted to detergent-resistant apical membrane microdomains, the so-called lipid rafts, which are characterized by the abundance of sphingolipids and cholesterol. Upon ligand binding, the transmembrane receptor tyrosine kinase Ret, which cannot bind GFLs by itself, is recruited to the site of the GFRαs in the lipid raft microdomain triggering the activation of various intracellular pathways (❯ *Figure 4-4*; Tansey et al., 2000).

Downstream signaling of Ret had been intensely studied even before the connection between GFLs and the activation of Ret had been discovered. Mass spectrometric analysis of recombinant Ret protein revealed that $Tyr^{806}$, $Tyr^{809}$, $Tyr^{900}$, $Tyr^{905}$, $Tyr^{981}$, $Tyr^{1062}$, $Tyr^{1090}$, and $Tyr^{1096}$ are the autophosphorylation sites of this kinase (Kawamoto et al., 2004). The specific tyrosine residues in Ret are associated with docking sites for adaptor proteins like Grb2 (Besset et al., 2000), phospholipase C gamma (PLCγ; Borrello et al., 1996), Frs2 (Kurokawa et al., 2001; Melillo et al., 2001a), and Shc (Asai et al., 1996). The splicing site of Ret is located just downstream of tyrosine 1062. Interestingly, tyrosine residue 1062 represents a binding site for several signaling molecules including Shc, Enigma, SNT/FRS2, the catalytic phosphatidylinositol-3 kinase (PI3K) subunit p85 (Segouffin-Cariou and Billaud, 2000), Dok proteins (Grimm et al., 2001; Murakami et al., 2002), and Irs1 (Melillo et al., 2001b). Binding to this residue initiates the activation of different signaling pathways (Hayashi et al., 2001) and has been the subject of several studies in the last decade. Ret phosphorylation leads, depending on the cell type studied, to the activation of different mitogen-activated protein kinase (MAPK) pathways. The major MAPK pathways that are activated are the extracellular

**Figure 4-4**

**GDNF family ligand (GFL) signaling pathways. 1. GFL bind to their cognate GPI-anchored GFRα receptors. Subsequently Ret is recruited to lipid rafts, resulting in the phosphorylation of several tyrosine residues and intracellular activation of specific signaling pathways. 2. GFL can also bind to soluble GFRα receptors and activate Ret in trans**




signal-regulated kinases 1/2/5 (ERK1/2/5; Worby et al., 1996) or Jun N-terminal kinase (JNK, Chiariello et al., 1998). Additionally, PI3K and PLCγ have been shown to be closely associated with Ret signaling (for review see Airaksinen and Saarma, 2002).

The effects of activation of the different signaling pathways by GFLS are dependent on the cellular context; in most instances activation of PI3K has been associated with neuron survival, whereas ERK seems to be mostly important for differentiation and neurite outgrowth, but there are also indications that ERKs are involved in GFL-mediated survival of specific neuron populations and that PI3K activation triggers neurite outgrowth (Kaplan and Miller, 2000; Airaksinen and Saarma, 2002; Peterziel et al., 2002). Activation of PLCγ increases the level of inositol-1,4,5-trisphosphate and thereby regulates the level of intracellular calcium ions. Integrity of the PLCγ docking site has been linked to the full oncogenic potential of the constitutively active Ret/PTC2 mutation (Borrello et al., 1996; Xing et al., 1998) but the cellular effects of GFL-mediated PLCγ activation have not been clarified up to now. However, it has been shown that GDNF potentiates spontaneous and evoked transmitter release at neuromuscular synapses and facilitates $Ca^{2+}$ influx into the nerve terminals by enhancing $Ca^{2+}$ currents through a mechanism involving N-type $Ca^{2+}$ channels (Wang et al., 2001). Whether this might be an effect of PLCγ activation or a downstream event of Akt phosphorylation has not been investigated so far. Activation of the JNK pathway by GDNF in the rat pheochromocytoma cell line (PC12) is mediated by Cdc42 and Rac1, members of the family of Rho/Rac-related small GTPases, and does not require tyrosine 1062 of Ret (Chiariello et al., 1998). It has been shown that activation of these

small GTPases either affects cytoskeleton organization (lamellipodia formation), which could influence neuritogenesis (Kozma et al., 1997, Brown et al., 2000), or, depending on the cellular context, might be involved in control of cell proliferation as has been demonstrated for NGF (Seo et al., 2003).

Another signaling molecule, ERK5, has originally been connected to brain-derived neurotrophic factor (BDNF)-promoted survival of developing cerebellar granule neurons (Liu et al., 2003; Shalizi et al., 2003), and it seems to be important for the retrograde survival signal of endocytosed neurotrophin receptors (Trks) at the nerve terminals (Watson et al., 2001). GFL can be retrogradely transported in several neuronal populations (Leitner et al., 1999; Laurikainen et al., 2000). In motoneurons this transport depends on the expression of the appropriate GFRα at the neuronal cell body (Leitner et al., 1999). Recently, Coulpier and Ibáñez (2004) have used compartmentalized cultures of sympathetic neurons to investigate the effect of GDNF addition to either the distal axon or the cell body. They have shown that GDNF and GFRα1 are retrogradely transported from distal axon terminals to the cell body, resulting in both neuronal survival and neurite outgrowth. Interestingly, while in the distal axon compartment both Akt and ERK1/2 phosphorylation were observed, only Akt phosphorylation was triggered in the cell body after GDNF application to the axon compartment. However, further studies will be needed to clarify whether activated Ret is also retrogradely transported or whether the differential activation of signaling components in the axon and the cell body might be due to sequential activation of downstream Ret targets. In this context it will be interesting to find out whether ERK5 activation at the nerve terminal might be connected to signal propagation to the nucleus as has been shown for NGF (Watson et al., 2001).

## 5.2 Impact of Lipid Raft Localization of the Receptor System on Downstream GFL Signaling

Recruitment of Ret to the lipid raft microdomain determines the intracellular effects of GFL action. Tansey et al. (2000) have demonstrated that localization of Ret to lipid rafts is essential for effective GDNF-induced downstream signaling and subsequent differentiation/neurite outgrowth, or neuronal survival. Interaction of Ret with members of the Src family leads to a fast activation of PI3K and MAPK only when it is localized to these microdomains. Recent observations indicate that Ret tyrosine 981 constitutes the major binding site of Src, and mutation of this tyrosine interferes with the survival-promoting effect of GDNF-induced Ret downstream signaling in transfected cerebellar granule neurons (Encinas et al., 2004). Other laboratories have studied the interaction of Frs2 with Ret, which results in MAPK activation (Kurokawa et al., 2001; Melillo et al., 2001a). This effect has also been described to depend on the lipid raft localization of Ret, while outside the raft, phosphorylated Ret predominantly interacts with Shc.

Because its constitutive activity is independent of GFRα-interaction, Ret/PTC too does not reside in lipid rafts. Phosphorylation of Tyr 1015 has been shown to be essential for confering full oncogenic potential for Ret/PTC (Durick et al., 1998), indicating that activation of PLCγ activation by Ret might also differ depending on Ret localization inside or outside lipid rafts. Analyses of the cellular and tissue-context-specific determinants of these differential activation properties of Ret in different membrane microdomains will help in elucidating the details and functions of the signaling events that occur upon GFL binding to the variety of responsive cell populations.

## 5.3 Ret-Independent Signaling of GFLS

The incomplete overlap of GFRα and Ret expression in various tissues has evoked discussions about the possibility of GFL signaling in trans for a long time (Trupp et al., 1997; Yu et al., 1998; Golden et al., 1999). Several models have been proposed regarding how GFRαs and Ret expressed on different cells could interact, including the possibility of a secreted form of GFRα that could affect cells at some distance from the cells where it is produced. A schematic representation of the possible mechanisms of these trans-signaling events is included in ❯ *Figure 4-3.* Studies where a soluble GFRα1/immunoglobulin G fusion

protein was added to cells expressing Ret but not the endogenous GPI-anchored GFRα1 demonstrated that this soluble receptor recruits Ret to lipid rafts and enables the activation of Ret downstream signaling in the presence of GDNF (Paratcha et al., 2001). However, the kinetics of this mechanism varies depending on the cell types used (Tansey et al., 2000). The assumption that processes involving the release of soluble GFRα1 are of physiological relevance is confirmed by the finding that GFRα1 release is increased after sciatic nerve lesion, which might mediate axonal regeneration (Paratcha et al., 2001). Another putative role for secreted GFRα1 as a chemoattractant cue for developing peripheral neurons is also possible (Ledda et al., 2002). So far, little is known about the secretion of the other GFRαs. However, it has been proposed that the PSPN receptor GFRα4 can be spliced to three isoforms, i.e., transmembrane, secreted, and GPI-anchored forms, which are distributed in a tissue-specific manner (Lindahl et al., 2000), indicating distinct functions of the different GFRα4 protein isoforms.

While signaling in trans requires the presence of both Ret and GFRαs, although on separate cells, it has become evident in the past few years that not all actions of GFL are mediated through activation of Ret (for a review see Sariola and Saarma, 2003). Efficient Src phosphorylation and subsequent Src downstream signaling has been shown in cell lines lacking Ret tyrosine kinase, in primary DRG neurons isolated from Ret-deficient mice (Poteryaev et al., 1999), as well as in neuronal precursor cells lacking Ret expression (Trupp et al., 1999). One obvious explanation for Ret-independent effects is that GFL–GFRα complexes may interact with other transmembrane receptors. Indeed, it has been shown that GDNF–GFRα1 can activate the Met receptor tyrosine kinase in several Ret-deficient/GFRα1-positive or GFRα1/Ret-coexpressing cell lines. However, the described Met activation was not mediated directly by GDNF–GFRα1; rather, it was activated indirectly via an Src-dependent mechanism (Popsueva et al., 2003). In two Ret-negative cell lines, a glial cell line expressing GFRα1 and a neuronal cell line expressing GFRα1 and GFRα2, GDNF stimulated rapid phosphorylation of cAMP response element-binding protein (CREB). Interestingly, NRTN was not able to evoke CREB phosphorylation in the presence of GFRα2, indicating that the two receptors might differ in their ability to interact with receptors other than Ret (Pezeshki et al., 2001). Most notably, in a recent work, Paratcha et al. (2003) found that in the absence of Ret, GDNF-bound GFRα1 could interact directly with the 140-kDa isoform of NCAM ($p140^{NCAM}$) in an immortalized neuronal precursor cell line and in primary Schwann cells. This interaction leads to a disruption of homophilic $p140^{NCAM}$–interactions and to the activation of intracellular NCAM signaling via the Src-kinase Fyn and focal adhesion kinase (FAK). These processes result in a loss of cell adhesion and subsequent migration of the Schwann cells, and in Ret-independent axon growth in primary neuron cultures. In this context, as shown by coimmunoprecipitation experiments, GFRα2 does not differ from GFRα1 in its ability to interact with NCAM in vitro, but whether this interaction results in a similar loss of adhesion is not known. Further experiments will be needed to clarify whether interaction of GFRα1 and $p140^{NCAM}$ is disrupted upon addition of Ret, or whether signaling via both receptors, Ret and $p140^{NCAM}$, could occur in one cell. Recently, Pozas and Ibáñez (2005) have shown that differentiation and tangential migration of cortical GABAergic neurons, mediated by the GDNF/GFRα1 complex, depends neither on Ret nor on NCAM, indicating that there may exist other alternative receptors interacting with GFRαs.

However, the in vivo significance of these Ret-independent GDNF-signaling pathways has not been clarified so far. To address this question, Enomoto et al. (2004) have reintroduced GFRα1 into the Ret locus of GFRα1 knockout mice, thereby generating mice lacking Ret-independent GFRα1 expression. These mice do not exhibit any obvious phenotype, especially they do not show any defect in migration of the rostral migratory stream and in the size of the olfactory bulb, where the GFRα1/NCAM interaction has been shown to occur. These results may argue against a physiological role of alternative partners interacting with GFRα1. However, as GFRαs can also be secreted from cells and act as soluble factors in trans, further experiments have to be designed to incontrovertibly answer this question.

## 5.4 Modulation of GFL-Mediated Signaling

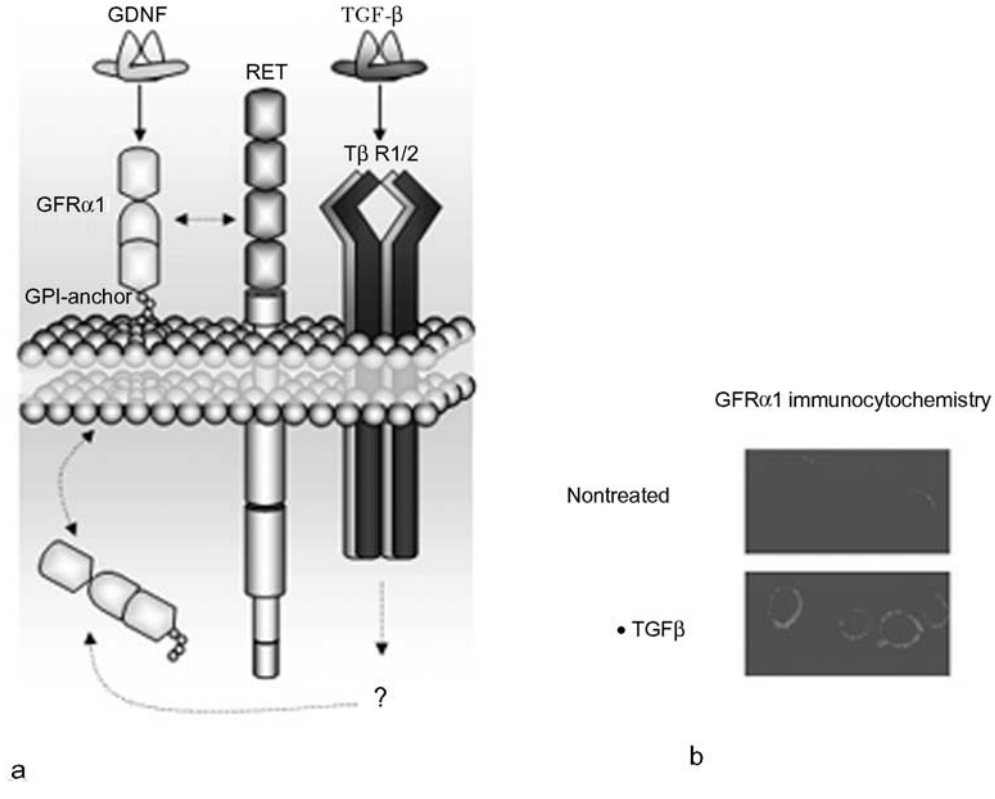
Recent data indicate that modulation of GFL signaling might be still more complex than originally thought (for review see Sariola and Saarma, 2003). Heparan sulfate glycosaminoglycans are able to modulate GDNF

downstream signaling, resulting in elevated phosphorylation of intracellular targets like ERK1/2 or increased expression of immediate early genes in neuroblastoma cells (Tanaka et al., 2002). In the absence of glycosaminoglycans, GDNF-induced Ret phosphorylation is inhibited and GDNF-mediated effects like scattering of epithelial cells or axonal outgrowth of neuronal cells are disturbed (Barnett et al., 2002). Mechanistically, extracellular matrix heparan sulfate proteoglycans might modulate GFL functions by decreasing their diffusion and thus increasing their local concentration at the receptor site on the cell surface.

Integration of different signal transduction pathways may be an important general cellular strategy to tightly regulate GFL-mediated effects. Results from our group indicate that the presence of TGFβ is mandatory for GDNF-mediated survival of various neuronal populations of the CNS and PNS in vitro and in vivo (Krieglstein et al., 1998, 2002; Schober et al., 1999). In serum-free low-density ciliary ganglion (CG) neuron cultures GDNF promotes survival and downstream signaling only if TGFβ is present. In these cells TGFβ induces responsiveness to GDNF by recruiting GFRα1 to its active site at the cell surface by a mechanism involving TGFβ signaling via its specific receptor complex (◉ *Figure 4-5*; Peterziel et al., 2002). Similar cooperative effects have been described on the protection of inner ear hair cells against ototoxicity by adenovirus-mediated overexpression of GDNF and TGFβ1 (Kawamoto et al., 2003). These observations indicate that context-dependent interactions between GFL and other signaling pathways may be important for fine-tuning the response to these neurotrophic factors in vivo.

**◘ Figure 4-5**

**Synergistic effect of TGFβ and GDNF. (a) TGFβ induces the recruitment of GFRα1 to the plasma membrane via a mechanism involving TGFβ-specific receptor types 1 and 2 (TβRI, TβRII). (b) Immunocytochemical staining for GFRα1 on chicken ciliary ganglion (CG) neurons in culture. Treatment with TGFβ results in a higher GFRα1 immunoreactivity on the cell surface**



## 6 Potential Therapeutic Applications of GFLS in Neurodegenerative Diseases

All GFL are able to support the survival of dopaminergic midbrain neurons and spinal and facial motor neurons in vitro, as well as in different lesion models in vivo. GDNF was considered a potential therapeutic agent in the treatment of neurodegenerative diseases from the moment of its discovery. As dopaminergic neurons are the type of cells that typically degenerate in brains of Parkinson's disease (PD) patients, molecules that might exert trophic influences on midbrain dopamine neurons could potentially be of therapeutic value in the treatment of PD. Indeed, in several rodent and nonhuman primate models of PD, GDNF promotes recovery of the injured nigrostriatal dopamine system and exerts both neuroprotective and, more importantly with regard to the human disease, neuroregenerative effects on dopamine neurons resulting in amelioration of motor deficits and reduction of brain damage (Gash et al., 1996; Kordower et al., 2000; Grondin et al., 2002; for reviews see Grondin and Gash, 1998; Bjorklund et al., 2000). As in these preclinical studies GDNF proved to be a powerful trophic factor, clinical trials were started to test the effect of GDNF delivery in PD patients. Initial studies with intermittent intraventricular applications were disappointing, as there was no evidence pointing to a reduction in symptoms of PD, but even in contrast, the occurrence of severe side effects was observed (Kordower et al., 1999; Nutt et al., 2003). However, recently Gill et al. (2003) reported that the chronic delivery of GDNF directly into the putamen of Parkinson's patients markedly reduced the PD symptoms according to the Unified Parkinson's Disease Rating Scale and increased putamen dopamine storage, without any severe side effects. These promising results indicate that GDNF could be successfully used for the treatment of PD if the right strategies for administration are applied.

In addition to PD, the neuroprotective effect of GDNF on spinal cord motoneurons indicates a possible use of the factor for the treatment of motoneuron diseases, i.e., amyotrophic lateral sclerosis (Acsadi et al., 2002; Manabe et al., 2002; Wang et al., 2002). These possible therapeutic applications are boosting the basic research focusing on functions and mechanisms of action of GFL. Vice versa, detailed characterization of GFL-mediated events, especially the interactions with signal transduction pathways induced by other cytokines, or the formation of alternative receptor complexes, i.e., GFRα/NCAM, might have important implications in the development of successful strategies to treat neurogenerative diseases.

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## References

Acsadi G, Anguelov RA, Yang H, Toth G, Thomas R, et al. 2002. Increased survival and function of SOD1 mice after glial cell-derived neurotrophic factor gene therapy. Hum Gene Ther 13: 1047-1059.

Airaksinen MS, Saarma M. 2002. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci 3: 383-394.

Airaksinen MS, Titievsky A, Saarma M. 1999. GDNF family neurotrophic factor signaling: four masters, one servant? Mol Cell Neurosci 13: 313-325.

Akerud P, Holm PC, Castelo-Branco G, Sousa K, Rodriguez FJ, et al. 2002. Persephin-overexpressing neural stem cells regulate the function of nigral dopaminergic neurons and prevent their degeneration in a model of Parkinson's disease. Mol Cell Neurosci 21: 205-222.

Anders J, Kjaer S, Ibanez CF. 2001. Molecular modeling of the extracellular domain of the RET receptor tyrosine kinase reveals multiple cadherin-like domains and a calcium-binding site. J Biol Chem 276: 35808-35817.

Angrist M, Bolk S, Halushka M, Lapchak PA, Chakravarti A. 1996. Germline mutations in glial cell line-derived neurotrophic factor (GDNF) and RET in a Hirschsprung disease patient. Nat Genet 14: 341-344.

Asai N, Murakami H, Iwashita T, Takahashi M. 1996. A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and

association with shc adaptor proteins. J Biol Chem 271: 17644-17649.

Baloh RH, Gorodinsky A, Golden JP, Tansey MG, Keck CL, et al. 1998b. GFRalpha3 is an orphan member of the GDNF/neurturin/persephin receptor family. Proc Natl Acad Sci USA 95: 5801-5806.

Baloh RH, Tansey MG, Golden JP, Creedon DJ, Heuckeroth RO, et al. 1997. TrnR2, a novel receptor that mediates neurturin and GDNF signaling through Ret. Neuron 18: 793-802.

Baloh RH, Tansey MG, Johnson EM Jr, Milbrandt J. 2000. Functional mapping of receptor specificity domains of glial cell line-derived neurotrophic factor (GDNF) family ligands and production of GFRalpha1 Ret-specific agonists. J Biol Chem 275: 3412-3420.

Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, et al. 1998a. Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3–RET receptor complex. Neuron 21: 1291-1302.

Barnett MW, Fisher CE, Perona-Wright G, Davies JA. 2002. Signalling by glial cell line-derived neurotrophic factor (GDNF) requires heparan sulphate glycosaminoglycan. J Cell Sci 115: 4495-4503.

Baudet C, Mikaels A, Westphal H, Johansen J, Johansen TE, et al. 2000. Positive and negative interactions of GDNF, NTN and ART in developing sensory neuron subpopulations, and their collaboration with neurotrophins. Development 127: 4335-4344.

Besset V, Scott RP, Ibanez CF. 2000. Signaling complexes and protein–protein interactions involved in the activation of the Ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase. J Biol Chem 275: 39159-39166.

Bilak MM, Shifrin DA, Corse AM, Bilak SR, Kuncl RW. 1999. Neuroprotective utility and neurotrophic action of neurturin in postnatal motor neurons: comparison with GDNF and persephin. Mol Cell Neurosci 13: 326-336.

Bjorklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, et al. 2000. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res 886: 82-98.

Borrello MG, Alberti L, Arighi E, Bongarzone I, Battistini C, et al. 1996. The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cgamma. Mol Cell Biol 16: 2151-2163.

Borrello MG, Mercalli E, Perego C, Degl'Innocenti D, Ghizzoni S, et al. 2002. Differential interaction of Enigma protein with the two RET isoforms. Biochem Biophys Res Commun 296: 515-522.

Brown MD, Cornejo BJ, Kuhn TB, Bamburg JR. 2000. Cdc42 stimulates neurite outgrowth and formation of growth cone filopodia and lamellipodia. J Neurobiol 43: 352-364.

Bueker ED. 1948. Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbosacral nervous system. Anat Rec 102: 369-390.

Buj-Bello A, Adu J, Pinon LG, Horton A, Thompson J, et al. 1997. Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature 387: 721-724.

Burau K, Stenull I, Huber K, Misawa H, Berse B, et al. 2004. c-ret regulates cholinergic properties in mouse sympathetic neurons: evidence from mutant mice. Eur J Neurosci 20: 353-362.

Cacalano G, Farinas I, Wang LC, Hagler K, Forgie A, et al. 1998. GFRalpha1 is an essential receptor component for GDNF in the developing nervous system and kidney. Neuron 21: 53-62.

Chiariello M, Visconti R, Carlomagno F, Melillo RM, Bucci C, et al. 1998. Signalling of the Ret receptor tyrosine kinase through the c-Jun NH2-terminal protein kinases (JNKS): evidence for a divergence of the ERKs and JNKs pathways induced by Ret. Oncogene 16: 2435-2445.

Choi-Lundberg DL, Bohn MC. 1995. Ontogeny and distribution of glial cell line-derived neurotrophic factor (GDNF) mRNA in rat. Brain Res Dev Brain Res 85: 80-88.

Coulpier M, Ibáñez CF. 2004. Retrograde propagation of GDNF-mediated signals in sympathetic neurons. Mol Cell Neurosci 27: 132-139.

de Graaff E, Srinivas S, Kilkenny C, D'Agati V, Mankoo BS, et al. 2001. Differential activities of the RET tyrosine kinase receptor isoforms during mammalian embryogenesis. Genes Dev 15: 2433-2444.

Doray B, Salomon R, Amiel J, Pelet A, Touraine R, et al. 1998. Mutation of the RET ligand, neurturin, supports multigenic inheritance in Hirschsprung disease. Hum Mol Genet 7: 1449-1452.

Durbec P, Marcos-Gutierrez CV, Kilkenny C, Grigoriou M, Wartiowaara K, et al. 1996. GDNF signalling through the Ret receptor tyrosine kinase. Nature 381: 789-793.

Durick K, Gill GN, Taylor SS. 1998. Shc and Enigma are both required for mitogenic signaling by Ret/ptc2. Mol Cell Biol 18: 2298-2308.

Edery P, Eng C, Munnich A, Lyonnet S. 1997. RET in human development and oncogenesis. Bioessays 19: 389-395.

Eigenbrot C, Gerber N. 1997. X-ray structure of glial cell-derived neurotrophic factor at 1.9 A resolution and implications for receptor binding. Nat Struct Mol Biol 4: 435-438.

Eketjäll S, Ibanez CF. 2002. Functional characterization of mutations in the GDNF gene of patients with Hirschsprung disease. Hum Mol Genet 11: 325-329.

Eketjäll S, Fainzilber M, Murray-Rust J, Ibanez CF. 1999. Distinct structural elements in GDNF mediate binding to GFRalpha1 and activation of the GFRalpha1–c-Ret receptor complex. EMBO J 18: 5901-5910.

Encinas M, Crowder RJ, Milbrandt J, Johnson EM Jr. 2004. Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem 279: 18262-18269.

Eng C, Mulligan LM. 1997. Mutations of the RET proto-oncogene in the multiple endocrine neoplasia type 2 syndromes, related sporadic tumours, and Hirschsprung disease. Hum Mutat 9: 97-109.

Enokido Y, de Sauvage F, Hongo J-A, Ninkina N, Rosenthal A, et al. 1998. GFRα4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr Biol 8: 1019-1022.

Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, et al. 1998. GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron 21: 317-324.

Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth RO, Johnson EM Jr, et al. 2001. RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development 128: 3963-3974.

Enomoto H, Hughes I, Golden J, Baloh RH, Yonemura S, et al. 2004. GFRα1 expression in cells lacking RET is dispensable for organogenesis and nerve regeneration. Neuron 44: 623-636.

Garces A, Haase G, Airaksinen MS, Livet J, Filippi P, et al. 2000. GFRα1 is required for development of distinct subpopulations of motoneuron. J Neurosci 20: 4992-5000.

Gash DM, Zhang Z, Ovadia A, Cass WA, Yi A, et al. 1996. Functional recovery in parkinsonian monkeys treated with GDNF. Nature 380: 252-255.

Geneste O, Bidaud C, De Vita G, Hofstra RM, Tartare-Deckert S, et al. 1999. Two distinct mutations of the RET receptor causing Hirschsprung's disease impair the binding of signalling effectors to a multifunctional docking site. Hum Mol Genet 8: 1989-1999.

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, et al. 2003. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat Med 9: 589-595.

Golden JP, DeMaro JA, Osborne PA, Milbrandt J, Johnson EM Jr. 1999. Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Exp Neurol 158: 504-528.

Granholm AC, Reyland M, Albeck D, Sanders L, Gerhardt G, et al. 2000. Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. J Neurosci 20: 3182-3190.

Grimm J, Sachs M, Britsch S, Di Cesare S, Schwarz-Romond T, et al. 2001. Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation. J Cell Biol 154: 345-354.

Grondin R, Gash DM. 1998. Glial cell line-derived neurotrophic factor (GDNF): a drug candidate for the treatment of Parkinson's disease. J Neurol 245: 35-42.

Grondin R, Zhang Z, Yi A, Cass WA, Masswood N, et al. 2002. Striatal GDNF infusion promotes structral and functional recovery in advanced parkinsonian monkeys. Brain 125: 2190-2200.

Haase G, Dessaud E, Garces A, de Bovis B, Birling M, et al. 2002. GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. Neuron 35: 893-905.

Haniu M, Hui J, Young Y, Le J, Katta V, et al. 1996. Glial cell line-derived neurotrophic factor: selective reduction of the intermolecular disulfide linkage and characterization of its disulfide structure. Biochemistry 35: 16799-16805.

Hayashi Y, Iwashita T, Murakamai H, Kato Y, Kawai K, et al. 2001. Activation of BMK1 via tyrosine 1062 in RET by GDNF and MEN2A mutation. Biochem Biophys Res Commun 281: 682-689.

Hellmich HL, Kos L, Cho ES, Mahon KA, Zimmer A. 1996. Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial–mesenchymal interactions. Mech Dev 54: 95-105.

Heuckeroth RO, Enomoto H, Grider JR, Golden JP, Hanke JA, et al. 1999. Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. Neuron 22: 253-263.

Hiltunen JO, Laurikainen A, Airaksinen MS, Saarma M. 2000. GDNF family receptors in the embryonic and postnatal rat heart and reduced cholinergic innervation in mice hearts lacking ret or GFRalpha2. Dev Dyn 219: 28-39.

Hiltunen PH, Airaksinen MS. 2004. Sympathetic cholinergic target innervation requires GDNF family receptor GFRalpha2. Mol Cell Neurosci 26: 450-457.

Honma Y, Araki T, Gianino S, Bruce A, Heuckeroth R, et al. 2002. Artemin is a vascular-derived neurotrophic factor for developing sympathetic neurons. Neuron 35: 267-282.

Huse M, Kuriyan J. 2002. The conformational plasticity of protein kinases. Cell 109: 275-282.

Ibanez CF. 1998. Emerging themes in structural biology of neurotrophic factors. Trends Neurosci 21: 438-444.

Ichihara M, Murakumo Y, Takahashi M. 2004. RET and neuroendocrine tumors. Cancer Lett 204: 197-211.

Iwamoto T, Taniguchi M, Asai N, Ohkusu K, Nakashima I, et al. 1993. cDNA cloning of mouse ret proto-oncogene and

its sequence similarity to the cadherin superfamily. Oncogene 8: 1087-1091.

Jaszai J, Farkas L, Galter D, Reuss B, Strelau J, et al. 1998. GDNF-related factor persephin is widely distributed throughout the nervous system. J Neurosci Res 53: 494-501.

Jing S, Wen D, Yu Y, Holst PL, Luo Y, et al. 1996. GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell 85: 1113-1124.

Jing S, Yu Y, Fang M, Hu Z, Holst PL, et al. 1997. GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family. J Biol Chem 272: 33111-33117.

Kaplan DR, Miller FD. 2000. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10: 381-391.

Kawamoto K, Yagi M, Stöver T, Kanzaki S, Raphael Y. 2003. Hearing and hair cells are protected by adenoviral gene therapy with TGFβ1 and GDNF. Mol Ther 7: 484-492.

Kawamoto Y, Takeda K, Okuno Y, Yamakawa Y, Ito Y, et al. 2004. Identification of RET autophosphorylation sites by mass spectrometry. J Biol Chem 279: 14213-14224.

Kjaer S, Ibanez CF. 2003a. Identification of a surface for binding to the GDNF–GFR alpha 1 complex in the first cadherin-like domain of RET. J Biol Chem 278: 47898-47904.

Kjaer S, Ibanez CF. 2003b. Intrinsic susceptibility to misfolding of a hot-spot for Hirschsprung disease mutations in the ectodomain of RET. Hum Mol Genet 12: 2133-2144.

Klein RD, Sherman D, Ho WH, Stone D, Bennett GI, et al. 1997. A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature 387: 717-721.

Koo H, Choi BH. 2001. Expression of glial cell line-derived neurotrophic factor (GDNF) in the developing human fetal brain. Int J Dev Neurosci 19: 549-558.

Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, et al. 2000. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290: 767-773.

Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, et al. 1999. Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. Ann Neurol 46: 419-424.

Korsching S. 1993. The neurotrophic factor concept: a reexamination. J Neurosci 13: 2739-2748.

Kotzbauer PT, Lampe PA, Heuckeroth RO, Golden JP, Creedon DJ, et al. 1996. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 384: 467-470.

Kozma R, Sarner S, Ahmed S, Lim L. 1997. Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. Mol Cell Biol 17: 1201-1211.

Krieglstein K, Henheik P, Farkas L, Jaszai J, Galter D, et al. 1998. Glial cell line-derived neurotrophic factor requires transforming growth factor-β for exerting its full neurotrophic potential on peripheral and CNS neurons. J Neurosci 18: 9822-9834.

Krieglstein K, Strelau J, Schober A, Sullivan A, Unsicker K. 2002. TGF-beta and the regulation of neuron survival and death. J Physiol Paris 96: 25-30.

Kuma K, Iwabe N, Miyata T. 1993. Motifs of cadherin- and fibronectin type III-related sequences and evolution of the receptor-type-protein tyrosine kinases: sequence similarity between proto-oncogene ret and cadherin family. Mol Biol Evol 10: 539-551.

Kurokawa K, Iwashita T, Murakami H, Hayashi H, Kawai K, et al. 2001. Identification of SNT/FRS2 docking site on RET receptor tyrosine kinase and its role for signal transduction. Oncogene 20: 1929-1938.

Laurikainen A, Hiltunen JO, Vanhatalo S, Klinge E, Saarma M. 2000. Glial cell line-derived neurotrophic factor is expressed in penis of adult rat and retrogradely transported in penile parasympathetic and sensory nerves. Cell Tissue Res 302: 321-329.

Ledda F, Paratcha G, Ibanez CF. 2002. Target-derived GFRalpha1 as an attractive guidance signal for developing sensory and sympathetic axons via activation of Cdk5. Neuron 36: 387-401.

Lee DC, Chan KW, Chan SY. 2002. RET receptor tyrosine kinase isoforms in kidney function and disease. Oncogene 21: 5582-5592.

Leitner ML, Molliver DC, Osborne PA, Vejsada R, Golden JP, et al. 1999. Analysis of the retrograde transport of glial cell line-derived neurotrophic factor (GDNF), neurturin, and persephin suggests that in vivo signaling for the GDNF family is GFRalpha coreceptor-specific. J Neurosci 19: 9322-9331.

Leppanen VM, Bespalov MM, Runeberg-Roos P, Puurand U, Merits A, et al. 2004. The structure of GFRalpha1 domain 3 reveals new insights into GDNF binding and RET activation. EMBO J 23: 1452-1462.

Lewin GR, Barde YA. 1996. Physiology of the neurotrophins. Annu Rev Neurosci 19: 289-317.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F. 1993. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260: 1130-1132.

Lindahl M, Timmusk T, Rossi J, Saarma M, Airaksinen MS. 2000. Expression and alternative splicing of mouse Gfra4 suggest roles in endocrine cell development. Mol Cell Neurosci 15: 522-533.

Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, et al. 2003. ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but

not mature cortical neurons. Proc Natl Acad Sci USA 100: 8532-8537.

Manabe Y, Nagano I, Gazi MS, Murakami T, Shiote M, et al. 2002. Adenovirus-mediated gene transfer of glial cell line-derived neurotrophic factor prevents motor neuron loss of transgenic model mice for amyotrophic lateral sclerosis. Apoptosis 7: 329-334.

Manie S, Santoro M, Fusco A, Billaud M. 2001. The RET receptor: function in development and dysfunction in congenital malformation. Trends Genet 17: 580-589.

Martucciello G, Ceccherini I, Lerone M, Jasonni V. 2000. Pathogenesis of Hirschsprung's disease. J Pediatr Surg 35: 1017-1025.

Massoll N, Mazzaferri EL. 2004. Diagnosis and management of medullary thyroid carcinomas. Clin Lab Med 24: 49-83.

Masure S, Geerts H, Cik M, Hoefnagel E, Kieboom Van Den G, et al. 1999. Enovin, a member of the glial cell-line-derived neurotrophic factor (GDNF) family with growth promoting activity on neuronal cells. Existence and tissue-specific expression of different splice variants. Eur J Biochem 266: 892-902.

Masure S, Cik M, Hoefnagel E, Nosrat CA, Linden Van der I, et al. 2000. Mammalian GFRalpha-4, a divergent member of the GFRalpha family of coreceptors for glial cell line-derived neurotrophic factor family ligands, is a receptor for the neurotrophic factor persephin. J Biol Chem 275: 39427-39434.

Melillo RM, Carlomagno F, De Vita G, Formisano P, Vecchio G, et al. 2001b. The insulin receptor substrate (IRS)-1 recruits phosphatidylinositol 3-kinase to Ret: evidence for a competition between Shc and IRS-1 for the binding to Ret. Oncogene 20: 209-218.

Melillo RM, Santoro M, Ong SH, Billaud M, Fusco A, et al. 2001a. Docking protein FRS2 links the protein tyrosine kinase RET and its oncogenic forms with the mitogen-activated protein kinase signaling cascade. Mol Cell Biol 21: 4177-4187.

Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, et al. 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287: 1489-1493.

Meng X, de Rooij DG, Westerdahl K, Saarma M, Sariola H. 2001. Promotion of seminomatous tumors by targeted overexpression of glial cell line-derived neurotrophic factor in mouse testis. Cancer Res 61: 3267-3272.

Milbrandt J, de Sauvage FJ, Fahrner TJ, Baloh RH, Leitner ML, et al. 1998. Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 20: 245-253.

Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, et al. 1996. Renal and neuronal abnormalities in mice lacking GDNF. Nature 382: 76-79.

Murakami H, Yamamura Y, Shimono Y, Kawai K, Kurokawa K, et al. 2002. Role of Dok1 in cell signaling mediated by RET tyrosine kinase. J Biol Chem 277: 32781-32790.

Naveilhan P, Baudet C, Mikaels A, Shen L, Westphal H, et al. 1998. Expression and regulation of GFRalpha3, a glial cell line-derived neurotrophic factor family receptor. Proc Natl Acad Sci USA 95: 1295-1300.

Nijhawan D, Honarpour N, Wang X. 2000. Apoptosis in neural development and disease. Annu Rev Neurosci 23: 73-87.

Nikiforov Y E. 2002. RET/PTC rearrangement in thyroid tumors. Endocr Pathol 13: 3-16.

Nosrat CA, Tomac A, Hoffer BJ, Olson L. 1997. Cellular and developmental patterns of expression of Ret and glial cell line-derived neurotrophic factor receptor alpha mRNAs. Exp Brain Res 115: 410-422.

Nosrat CA, Tomac A, Lindqvist E, Lindskog S, Humpel C, et al. 1996. Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. Cell Tissue Res 286: 191-207.

Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, et al. 2003. Implanted intracerebroventricular. Glial cell line-derived neurotrophic factor. Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 60: 69-73.

Oppenheim RW. 1989. The neurotrophic theory and naturally occurring motoneuron death. Trends Neurosci 12: 252-255.

Pachnis V, Mankoo B, Costantini F. 1993. Expression of the c-ret proto-oncogene during mouse embryogenesis. Development 119: 1005-1017.

Paratcha G, Ledda F, Baars L, Coulpier M, Besset V, et al. 2001. Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. Neuron 29: 171-184.

Paratcha G, Ledda F, Ibanez CF. 2003. The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell 113: 867-879.

Pasini B, Ceccherini I, Romeo G. 1996. RET mutations in human disease. Trends Genet 12: 138-144.

Pawson T. 2002. Regulation and targets of receptor tyrosine kinases. Eur J Cancer 38: S3-S10.

Peterziel H, Unsicker K, Krieglstein K. 2002. TGFβ induces GDNF responsiveness in neurons by recruitment of GFRα1 to the plasma membrane. J Cell Biol 159: 157-167.

Pezeshki G, Franke B, Engele J. 2001. Evidence for a ligand-specific signaling through GFRalpha-1, but not GFRalpha-2, in the absence of Ret. J Neurosci Res 66: 390-395.

Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, et al. 1996. Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382: 73-76.

Pochon NA, Menoud A, Tseng JL, Zurn AD, Aebischer P. 1997. Neuronal GDNF expression in the adult rat nervous

system identified by in situ hybridization. Eur J Neurosci 9: 463-471.

Popsueva A, Poteryaev D, Arighi E, Meng X, Angers-Loustau A, et al. 2003. GDNF promotes tubulogenesis of GFRalpha1-expressing MDCK cells by Src-mediated phosphorylation of Met receptor tyrosine kinase. J Cell Biol 161: 119-129.

Poteryaev D, Titievsky A, Sun YF, Thomas-Crusells J, Lindahl M, et al. 1999. GDNF triggers a novel ret-independent Src kinase family-coupled signaling via a GPI-linked GDNF receptor alpha1. FEBS Lett 463: 63-66.

Pozas E, Ibáñez CF. 2005. GDNF and GFRα1 promote differentiation and tangential migration of cortical GABAergic neurons. Neuron 45: 701-713.

Purves D, Rubin E, Snider WD, Lichtman J. 1986. Relation of animal size to convergence, divergence, and neuronal number in peripheral sympathetic pathways. J Neurosci 6: 158-163.

Romeo G, Ronchetto P, Luo Y, Barone V, Seri M, et al. 1994. Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature 367: 377-378.

Rossi J, Luukko K, Poteryaev D, Laurikainen A, Sun YF, et al. 1999. Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR alpha2, a functional neurturin receptor. Neuron 22: 243-252.

Salomon R, Attie T, Pelet A, Bidaud C, Eng C, et al. 1996. Germline mutations of the RET ligand GDNF are not sufficient to cause Hirschsprung disease. Nat Genet 14: 345-347.

Sanchez MP, Silos-Santiago I, Frisen J, He B, Lira SA, et al. 1996. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature 382: 70-73.

Sarabi A, Hoffer BJ, Olson L, Morales M. 2003. Glial cell line neurotrophic factor-family receptor alpha-1 is present in central neurons with distinct phenotypes. Neuroscience 116: 261-273.

Sariola H, Saarma M. 1999. GDNF and its receptors in the regulation of the ureteric branching. Int J Dev Biol 43: 413-418.

Sariola H, Saarma M. 2003. Novel functions and signalling pathways for GDNF. J Cell Sci 116: 3855-3862.

Schaar DG, Sieber BA, Sherwood AC, Dean D, Mendoza G, et al. 1994. Multiple astrocyte transcripts encode nigral trophic factors in rat and human. Exp Neurol 130: 387-393.

Schlessinger J. 2000. Cell signaling by receptor tyrosine kinases. Cell 103: 211-225.

Schober A, Hertel R, Arumae U, Farkas L, Jaszai J, et al. 1999. Glial cell line-derived neurotrophic factor rescues target-deprived sympathetic spinal cord neurons but requires transforming growth factor-beta as cofactor in vivo. J Neurosci 19: 2008-2015.

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V. 1994. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 367: 380-383.

Scott RP, Ibanez CF. 2001. Determinants of ligand binding specificity in the glial cell line-derived neurotrophic factor family receptor alpha S. J Biol Chem 276: 1450-1458.

Segouffin-Cariou C, Billaud M. 2000. Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. J Biol Chem 275: 3568-3576.

Seo JY, Kim JS, Ghang JH, Kang TC, Suh JG, et al. 2003. Nerve growth factor induces proliferation of PC12 cells through Cdc42. Neuroreport 14: 1277-1281.

Serra MP, Quartu M, Lai ML, Follesa P, del Fiacco M. 2002. Expression of glial cell line-derived neurotrophic factor mRNA in the human newborn and adult hippocampal formation. Brain Res 928: 160-164.

Shalizi A, Lehtinen M, Gaudilliere B, Donovan N, Han J, et al. 2003. Characterization of a neurotrophin signaling mechanism that mediates neuron survival in a temporally specific pattern. J Neurosci 23: 7326-7336.

Soler RM, Dolcet X, Encinas M, Egea J, Bayascas JR, et al. 1999. Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. J Neurosci 19: 9160-9169.

Strelau J, Unsicker K. 1999. GDNF family members and their receptors: expression and functions in two oligodendroglial cell lines representing distinct stages of oligodendroglial development. Glia 26: 291-301.

Suvanto P, Hiltunen JO, Arumae U, Moshnyakov M, Sariola H, et al. 1996. Localization of glial cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by in situ hybridization. Eur J Neurosci 8: 816-822.

Suvanto P, Wariovaara K, Lindahl M, Arumae U, Moshnyakov M, et al. 1997. Cloning, mRNA distribution and chromosomal localisation of the gene for glial cell line-derived neurotrophic factor receptor β, a homologue to GDNFR-α. Hum Mol Genet 6: 1267-1273.

Takahashi M, Buma Y, Iwamoto T, Inaguma Y, Ikeda H, et al. 1988. Cloning and expression of the ret proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. Oncogene 3: 571-578.

Takahashi M, Cooper GM. 1987. Ret transforming gene encodes a fusion protein homologous to tyrosine kinases. Mol Cell Biol 7: 1378-1385.

Tanaka M, Xiao H, Kiuchi K. 2002. Heparin facilitates glial cell line-derived neurotrophic factor signal transduction. Neuroreport 13: 1913-1916.

Tansey MG, Baloh RH, Milbrandt J, Johnson EM Jr. 2000. GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. Neuron 25: 611-623.

Thompson J, Doxakis E, Pinon LG, Strachan P, Buj-Bello A, et al. 1998. GFRalpha-4, a new GDNF family receptor. Mol Cell Neurosci 11: 117-126.

Tomac AC, Agulnick AD, Haughey N, Chang CF, Zhang Y, et al. 2002. Effects of cerebral ischemia in mice deficient in persephin. Proc Natl Acad Sci USA 99: 9521-9526.

Treanor JJ, Goodman L, de Sauvage F, Stone DM, Poulsen KT, et al. 1996. Characterization of a multicomponent receptor for GDNF. Nature 382: 80-83.

Trupp M, Arenas E, Fainzilber M, Nilsson AS, Sieber BA, et al. 1996. Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature 381: 785-789.

Trupp M, Belluardo N, Funakoshi H, Ibanez CF. 1997. Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. J Neurosci 17: 3554-3567.

Trupp M, Raynoschek C, Belluuardo N, Ibanez CF. 1998. Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. Mol Cell Neurosci 11: 47-63.

Trupp M, Ryden M, Jornvall H, Funakoshi H, Timmusk T, et al. 1995. Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130: 137-148.

Trupp M, Scott R, Whittemore SR, Ibanez CF. 1999. Ret-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells. J Biol Chem 274: 20885-20894.

Tsui-Pierchalla BA, Ahrens RC, Crowder RJ, Milbrandt J, Johnson EM Jr. 2002. The long and short isoforms of RET function as independent signalling complexes. J Biol Chem 277: 34618-34625.

Tsuzuki T, Takahashi M, Asai N, Iwashita T, Matsuyama M, et al. 1995. Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. Oncogene 10: 191-198.

Unsicker K, Suter-Crazzolara Krieglstein K, 1999. Neurotrophic roles of GDNF and related factors. Handbook of experimental pharmacology. Hefti F, editor. Berlin, Heidelberg, New York: Springer; Vol. 134, pp. 189-224.

Wang CY, Yang F, He X, Chow A, Du J, et al. 2001. Ca(2+) binding protein frequenin mediates GDNF-induced potentiation of Ca(2+) channels and transmitter release. Neuron 32: 99-112.

Wang LJ, Lu YY, Muramatsu S, Ikeguchi K, Fujimoto K, et al. 2002. Neuroprotective effects of glial cell line-derived neurotrophic factor mediated by an adeno-associated virus vector in a transgenic animal model of amyotrophic lateral sclerosis. J Neurosci 22: 6920-6928.

Wanigasekara Y, Airaksinen MS, Heuckeroth RO, Milbrandt J, Keast JR. 2004. Neurturin signalling via GFRalpha2 is essential for innervation of glandular but not muscle targets of sacral parasympathetic ganglion neurons. Mol Cell Neurosci 25: 288-300.

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, et al. 2001. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci 4: 981-988.

Widenfalk J, Nosrat C, Tomac A, Westphal H, Hoffer B, et al. 1997. Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR-beta), novel proteins related to GDNF and GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. J Neurosci 17: 8506-8519.

Widenfalk J, Tomac A, Lindqvist E, Hoffer B, Olson L. 1998. GFRalpha-3, a protein related to GFRalpha-1, is expressed in developing peripheral neurons and ensheathing cells. Eur J Neurosci 10: 1508-1517.

Worby CA, Vega QC, Chao HH, Seasholtz AF, Thompson RC, et al. 1998. Identification and characterization of GFRalpha-3, a novel co-receptor belonging to the glial cell line-derived neurotrophic receptor family. J Biol Chem 273: 3502-3508.

Worby CA, Vega QC, Zhao Y, Chao HH, Seasholtz AF, et al. 1996. Glial cell line-derived neurotrophic factor signals through the RET receptor and activates mitogen-activated protein kinase. J Biol Chem 271: 23619-23622.

Wright DE, Snider WD. 1996. Focal expression of glial cell line-derived neurotrophic factor in developing mouse limb bud. Cell Tissue Res 286: 209-217.

Xing S, Furminger TL, Tong Q, Jhiang SM. 1998. Signal transduction pathways activated by RET oncoproteins in PC12 pheochromocytoma cells. J Biol Chem 273: 4909-4914.

Yang J, Lindahl M, Lindholm P, Virtanen H, Coffey E, et al. 2004. PSPN/GFRalpha4 has a significantly weaker capacity than GDNF/GFRalpha1 to recruit RET to rafts, but promotes neuronal survival and neurite outgrowth. FEBS Lett 569: 267-271.

Young HM, Anderson RB, Anderson CR. 2004. Guidance cues involved in the development of the peripheral autonomic nervous system. Auton Neurosci 112: 1-14.

Yu T, Scully S, Yu Y, Fox GM, Jing S, et al. 1998. Expression of GDNF family receptor components during development: implications in the mechanisms of interaction. J Neurosci 18: 4684-4696.

Zhou B, Bae SK, Malone AC, Levinson BB, Kuo YM, et al. 2001. hGFRalpha-4: a new member of the GDNF receptor family and a candidate for NBIA. Pediatr Neurol 25: 156-161.

# 5 Fibroblast Growth Factors in Brain Functions

*K. Unsicker · B. Reuss · O. von Bohlen und Halbach*

**Abstract:** Fibroblast growth factors (FGFs) constitute a family of growth factors with multiple roles in development, differentiation, maintenance, and repair of many different types of cells and in virtually all tissues and organs. The family comprises 23 members, of which ten have been identified in the nervous system. FGF receptors (FGFRs) are receptor tyrosine kinases and are encoded by four different genes, all of which are expressed in the nervous system. Extracellular domains of the receptor proteins exhibit a large number of variants due to alternative splicing creating a significant diversity with regard to ligand binding and signaling properties. In addition, ligand binding is modified by heparan sulfate proteoglycans, which act as low-affinity receptors. Activation of FGFRs triggers several intracellular signaling cascades. These include phosphorylation of src and phospholipase C γ (PLC γ), leading finally to activation of protein kinase C (PKC), as well as activation of Crk and Shc. SNT/FRS2 serves as an alternative link of FGFRs for the activation of PKC and, in addition, activates the Ras signaling cascade. In the central nervous system (CNS), FGFs are widely expressed; FGF-1, -2, -4, -5, -8, -9, -10, and -15 seem to be the most important ones in relation to neural functions. FGF-2 is predominantly synthesized by astrocytes, whereas other FGF family members, e.g., FGF-1, -5, -8, -9, -10, -15, and -18, are primarily synthesized by neurons. FGFs play important roles in neurogenesis, axon growth, and differentiation. Furthermore, FGFs are major determinants of neuronal survival both in development and in adulthood. FGF-2 is an important factor for neurogenesis in the adult brain. Increasing evidence suggests that FGF-1 and -2 may be involved in the regulation of synaptic plasticity and processes attributed to learning and memory.

**List of Abbreviations:** BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DAG, diacylglycerol; DRG, dorsal root ganglia; EGF, epidermal growth factor; EGL, external granular layer; FGF, fibroblast growth factor; FGFR, FGF receptor; GABA, gamma amino butyric acid; GDNF, glial-cell-line-derived neurotrophic factor; IGF, insulin-like growth factor; IGL, internal granular layer; IP3, inositol trisphosphate; LTP, long-term potentiation; MAP, mitogen-activated protein; MCAO, middle cerebral artery occlusion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NMDA, *N*-methyl-D-aspartate; NT-4, neurotrophin 4; PDGFR, platelet-derived growth factor receptor; PKC, protein kinase C; PLC γ, phospholipase C γ; SH2, src homology 2

## 1 Introduction

Development, functions, plasticity, and repair processes of the central nervous system (CNS) require permanent adaptation of neurons and glial cells to changing demands. To a large extent, such changes depend on cytokine signaling networks, whose complexities are still largely enigmatic. Evidence assembled during the last 20 years suggests that members of the fibroblast growth factor (FGF) family serve as important signals in the developing, adult, and lesioned nervous system. This chapter takes into account the fact that the field has frequently been the subject of excellent reviews. We therefore focus on progress achieved during the past decade (cf. Bieger and Unsicker, 1996).

Basic FGF (FGF-2) was the first FGF discovered and cloned (Abraham et al., 1986a, b). By now the FGF family comprises 23 family members that signal via four receptors, whose gene and protein structures and intracellular signaling cascades have been extensively investigated (Powers et al., 2000; Ornitz and Itoh, 2001). Not all FGF family members are found throughout all vertebrate species. FGF-15 has not been identified in the human genome, and FGF-19 has not been detected in mice, resulting in a total of only 22 FGF family members in each of these species. FGF and FGF receptor (FGFR) knockout animals have significantly contributed to expanding our knowledge of the biological significance of ligand and receptor molecules (Ornitz and Itoh, 2001). Even so, analysis of FGF and FGFR mutant mice with respect to their CNS and peripheral nervous system (PNS) phenotypes progresses slowly, and many assumed functions of FGFs in the nervous system are extrapolations from pharmacological experiments and applications of exogenous FGFs. Important roles of FGFs in neurogenesis, differentiation, axonal branching, neuron survival, neurodegenerative disorders, and cognitive processes have mostly been suggested based on pharmacological experiments and await confirmation from the analysis of FGF mutant mice (Haynes, 1988; Calamandrei and Alleva, 1995; Mocchetti and Wrathall, 1995).

## 2 FGF Signaling

### 2.1 Gene Structure of FGFs

**Coding Regions.** All known FGF genes consist of three coding exons, with exon 1 containing the start codon. However, some FGF genes, e.g., FGF-2 and FGF-3, contain additional 5′ untranscribed regions initiating from upstream CUG codons (Kiefer et al., 1994; Arnaud et al., 1999). The size of the coding region of FGF genes ranges from under 5 kb (FGF-3 and FGF-4) to over 100 kb (FGF-12). In certain FGF subfamilies, exon 1 is subdivided into two or four alternatively spliced subexons (1A–1D for FGF-8) with a single initiation codon residing in exon 1A. Other family members (e.g., FGF-11 to -14) have alternatively spliced amino-terminal regions resulting from the use of alternative 5′ exons.

**Chromosomal Localization.** The chromosomal localization for all human FGF genes, with the exception of FGF-16, is known. Several human FGF genes are clustered on distinct chromosomal regions. For example, genes encoding FGF-3, -4, and -19 are located on chromosome 11q13 and separated by only 40 and 10 kb, respectively. The FGF-6 and -23 genes are located within a small 55-kb fragment of chromosome 12p13. Similarly, the FGF-17 and -20 genes also reside together on chromosome 8p21–8p22 (Kelley et al., 1992). For the mouse, localization of 16 FGF genes has been determined, revealing some similarities with respect to their arrangement on human chromosomes (Ornitz and Itoh, 2001).

**Evolution.** FGFs are relatively old molecules that arose during invertebrate evolution. Although FGF-like genes have been identified in several viral genomes (Li et al., 2002), genomes of unicellular organisms (*Escherichia coli*, *Saccharomyces cerevisiae*) contain no FGF-like genes. One FGF-like sequence (branchless) has been discovered in Drosophila (Sutherland et al., 1996), and two (egl-17 and let-756) have been discovered in *Caenorhabditis elegans* (Burdine et al., 1997; Coulier et al., 1997). FGF proteins are highly conserved throughout vertebrate species and share greater than 90% amino acid sequence homology. Four FGFs have been identified in zebrafish (FGF-3, -8, -17, and -18), six in the clawed toad Xenopus (FGF-3, Fgfi, Fgfii, FGF-8, FGF-9, and FGF-20), and seven in chicken (FGF-2, -4, -8, -12, -14, -18, and -19).

**Subfamilies of Human FGFs.** Human FGFs can be grouped into subfamilies based on different degrees of sequence and functional homologies. One such subfamily includes FGF-8, -17, and -18, which share 70–80% of their amino acid sequences, resemble each other in receptor binding specificity, and share partially overlapping expression sites, e.g., the midbrain–hindbrain boundary.

### 2.2 FGF Protein Structure

Vertebrate FGF proteins range in molecular weight from 17 to 34 kDa, in contrast to the Drosophila FGF homolog branchless, which is an 84-kDa protein. All FGFs have an internal core region that consists of 28 highly conserved and 6 identical amino acid residues (❯ *Figure 5-1*; Ornitz, 2000). Ten of these conserved residues are responsible for interactions with the FGF receptors (Plotnikov et al., 2000). In FGF-1 and -2 the core domain of the protein consists of twelve antiparallel beta strands (❯ *Figure 5-2*; Eriksson et al., 1991; Zhu et al., 1991). Two of these beta strands (❯ *Figure 5-2*: β10 and β11) contain basic amino acid residues forming the heparin-binding site on FGF-2 (Li et al., 1994; Moy et al., 1996).

### 2.3 Subcellular Localization and Secretion of FGFs

Most FGFs (FGF-3, -4, -5, -6, -7, -8, -10, -15, -17, -18, -19, -21, -22, -23) possess amino-terminal signal peptides and may be readily secreted from cells. In contrast, FGF-1, -2, -9, -16, and -20 lack conventional signal peptides, and their modes of secretion are largely enigmatic. Even so, they all seem to be secretable (Miyamoto et al., 1993; Miyake et al., 1998; Ohmachi et al., 2000). One mode of secretion may be related to cell damage (Mignatti et al., 1992; Friesel and Maciag, 1999). FGF-9 possesses a noncleaved amino-terminal

**Figure 5-1**
**Schematic domain alignment of different members of the fibroblast growth factor (FGF) family of protein growth factors. All FGFs consist of two highly conserved core domains (black), separated by a central spacer region of variable length. Also C- and N-terminal regions differ in their length, with some FGFs (FGF-2, -3, and -9) bearing alternative splice sites for their N-terminal regions (modified from Bieger and Unsicker, 1996)**

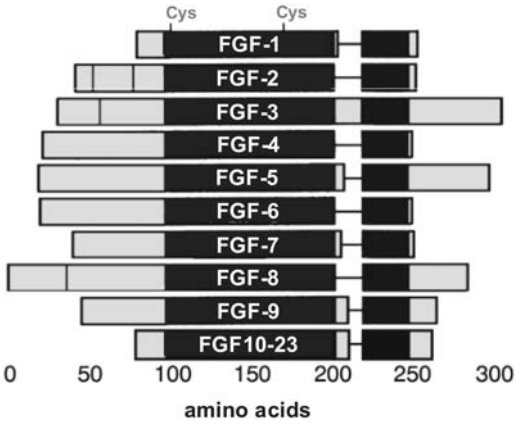
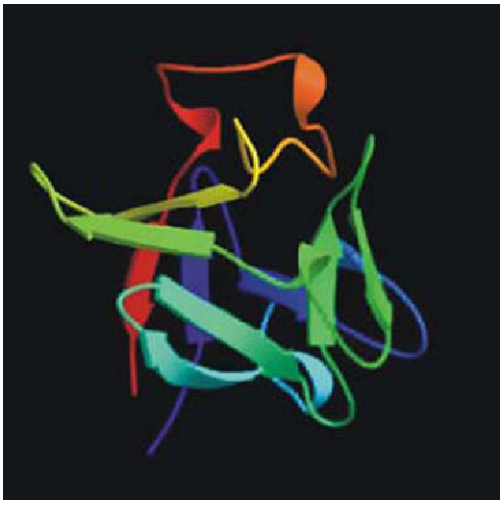



**Figure 5-2**
**Three-dimensional structure of the FGF-2 protein (modified from Zhu et al., 1991)**



hydrophobic sequence responsible for secretion (Miyakawa et al., 1999; Revest et al., 2000). FGF-2 and -3 also come as high-molecular-weight forms with predominant nuclear localization (Antoine et al., 1997; Coulier et al., 1997; Arnaud et al., 1999; Claus et al., 2004a, b). The discovery that FGF-2(23) forms a complex with the survival of motoneuron (SMN) protein, an important component of the splicing machinery, has opened an avenue for understanding the role of FGFs with a nuclear localization (Claus et al., 2004a, b). Distinct functions of the 18-kDa and 21/23-kDa FGF-2 have been suggested based on distinct roles in PC12 cells overexpressing the respective isoforms (Grothe et al., 1998).

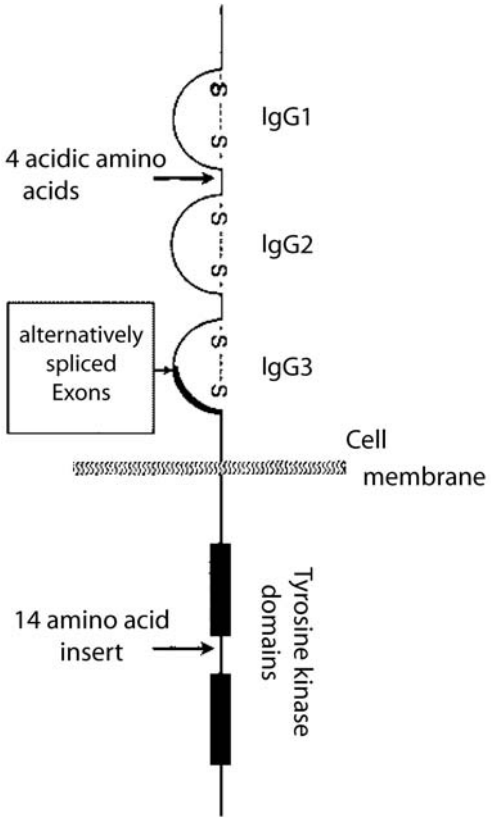
## 2.4 The FGF Receptors

FGFs elicit their diverse effects through activation of cell-surface-bound tyrosine kinase receptors (Coughlin et al., 1988). Binding occurs with an affinity of $K_D = 20$ pmol/L (Moscatelli, 1987); receptor proteins range from 125 to 160 kDa in molecular weight (Neufeld and Gospodarowicz, 1985, 1986; Friesel et al., 1986; Moenner et al., 1986; Blanquet et al., 1989).

Lee et al. (1989) were the first to clone a complementary DNA (cDNA) coding for an FGFR with high affinity for FGF-1 from chicken tissue. Structural characterization of this molecule allowed the identification of the prototypic structural hallmarks of all FGFRs, which are transmembrane proteins with three extracellular Ig-like domains (IgI, IgII, and IgIII), an acidic domain between IgI and IgII, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase domain, respectively (*Figure 5-3*; Johnson et al., 1990).

**Figure 5-3**

**Structure of the fibroblast growth factor receptors (FGFRs). Receptors consist of three extracellular Ig-like domains, a transmembrane domain, and two intracellular tyrosine kinase domains. An acidic domain of four amino acids is important for heparin binding and thus for receptor dimerization (modified from Bieger and Unsicker, 1996)**




Cloning of FGFR-1 and -2 (Dionne et al., 1990) revealed their identity with the previously isolated tyrosine kinase proteins flg and bek, respectively (Kornbluth et al., 1988; Ruta et al., 1989). By now, four different subtypes of FGFRs have been identified (Johnson and Williams, 1993). Affinity of FGFRs for their ligands is highly diverse, with different affinities for each member of the FGF family of growth factors (*Table 5-1*).

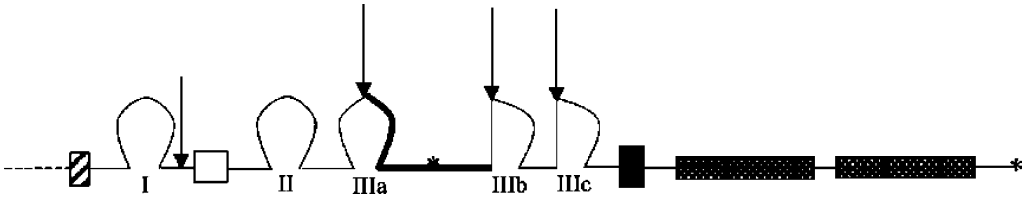
**Table 5-1**
**Receptor binding of FGF family members to the four FGFR subtypes**

| | FGFR-1 | FGFR-2 | FGFR-3 | FGFR-4 |
|---|---|---|---|---|
| FGF-1 | + | + | + | + |
| FGF-2 | + | + | + | + |
| FGF-3 | + | + | − | − |
| FGF-4 | + | + | + | + |
| FGF-5 | + | + | − | − |
| FGF-6 | + | + | − | + |
| FGF-7 | − | + | − | − |
| FGF-8 | + | + | + | + |
| FGF-9 | − | + | + | + |
| FGF-10 | − | + | − | − |
| FGF-11 to -16 | ? | ? | ? | ? |
| FGF-17 | − | + | + | + |
| FGF-18 | − | + | + | + |
| FGF-19 to -23 | ? | ? | ? | ? |

(+) receptor binding, (−) no receptor binding, (?) not investigated
Modified from Ornitz et al. (1996)

The diverse effects elicited by different FGF family members depends, in part, on a significant structural and functional diversity of FGFRs (*Figure 5-4*). Diversity of FGFRs beyond the four receptor subtypes is achieved by the generation of alternative splice variants of a given FGFR gene (Ornitz et al., 1996). The protein region with the highest impact on FGFR binding specificity is a portion of the IgIII domain for which three different splice variants, termed IgIIIa, IgIIIb, and IgIIIc, have been identified so far (for relative binding affinities of different FGFs to the different IgIII splice variants, see *Table 5-2*). All three splice variants exist for FGFR-1 and FGFR-2 (Johnson et al., 1991; Chellaiah et al., 1994); for FGFR-3 only the IgIIIb and IgIIIc variants occur. FGFR-4 exists exclusively as the IgIIIc variant (Vainikka et al., 1992).

**Figure 5-4**
**Structure of the fibroblast growth factor receptor (FGFR) gene. Four possible splice sites exist leading either to molecules with a truncated C-terminal region or to molecules with different variants of the third Ig-like domain (IgIIIa, IgIIIb, and IgIIIc) (modified from Johnson et al., 1991)**




## 2.5 The FGF/FGFR Complex and Intracellular Signaling

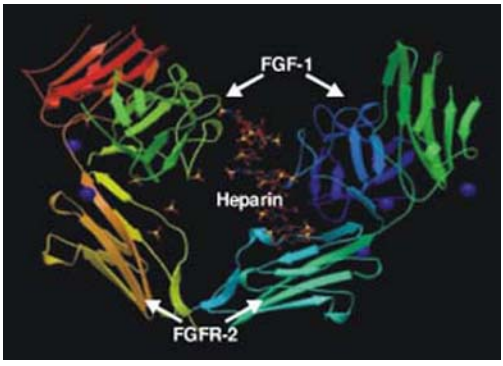
Ligand binding to FGFRs initiates a receptor complex consisting of two FGF molecules bound to a receptor and to a heparan sulfate proteoglycan molecule, e.g., heparin (*Figure 5-5*). The consensus mechanism is

**Table 5-2**
**Activation of different FGFR splice variants by different FGF family members**

| FGFR | FGF-1 | FGF-2 | FGF-3 | FGF-4 | FGF-5 | FGF-6 | FGF-7 | FGF-8 | FGF-9 |
|---|---|---|---|---|---|---|---|---|---|
| 1, IIIb | 100 | 60 | 34 | 16 | 4 | 5 | 6 | 4 | 4 |
| 1, IIIc | 100 | 104 | 0 | 102 | 59 | 55 | 0 | 1 | 21 |
| 2, IIIb | 100 | 9 | 45 | 15 | 5 | 5 | 81 | 4 | 7 |
| 2, IIIc | 100 | 64 | 4 | 94 | 25 | 61 | 2.5 | 16 | 89 |
| 3, IIIb | 100 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 42 |
| 3, IIIc | 100 | 107 | 1 | 69 | 12 | 9 | 1 | 41 | 96 |
| 4 | 100 | 113 | 6 | 108 | 7 | 79 | 2 | 76 | 75 |

Each value represents the percentage of activation caused by FGF-1 with a given splice variant
Modified from Ornitz et al. (1996)

**Figure 5-5**
**Three-dimensional structure of the extracellular portion of a complex consisting of two FGF-1/FGFR-2 heterodimers, linked together by heparin (modified from Pellegrini et al., 2000)**




thought to consist of the formation of two independent FGF/FGFR complexes that are subsequently connected by a heparin-like glycosaminoglycan (Venkataraman et al., 1999; Stauber et al., 2000). Formation of the receptor complex triggers receptor activation by phosphorylation, leading to recruitment and phosphorylation of intracellular signaling molecules.

Important signaling proteins known to bind to the activated FGFR complex include the group of src homology 2 (SH2) domain proteins. The SH2 domain shared by these proteins serves the intracellular interaction with the receptor complex. SH2 proteins may themselves serve as substrates for receptor-mediated phosphorylation, or may function as adaptor proteins to recruit other target proteins.

Because of the high degree of homology at the amino acid level between different FGFRs, their signaling pathways are probably quite similar (Johnson and Williams, 1993). Studies using chimeric receptors (Raffioni et al., 1999) with the cytosolic domain of FGFRs and the extracellular portion of the platelet-derived growth factor receptor (PDGFR) have shown that the principal difference between FGFRs is the strength of tyrosine kinase activity, suggesting that all FGFR subtypes drive the same signaling cascades, but with different strengths.

**Structure of the FGFR Intracellular Domain.** Seven tyrosine residues in the cytoplasmic tail of FGFR-1 can serve as substrates for phosphorylation (Tyr463, Tyr583, Tyr585, Tyr653, Tyr654, Tyr730, and Tyr766). Tyr653 and Tyr654 are important for the catalytic activity of the activated FGFR and essential for signaling (Mohammadi et al., 1996). Tyr766 binds the SH2 domain of PLC $\gamma$ (Mohammadi et al., 1991). The significance of the other tyrosines is unknown; they can be mutated to phenylalanine residues

without loss of mitogen-activated protein (MAP) kinase activity and mitogenic signaling (Mohammadi et al., 1996).

**The PLC γ Signaling Pathway.** PLC γa 150-kDa phosphoprotein that cleaves phosphatidyl-inositol-4,5-bisphosphate to inositol trisphosphate (IP3) and diacylglycerol (DAG) associates with the ligand-activated FGFR through Tyr766 (Burgess et al., 1990; Mohammadi et al., 1991). Tyr766 is essential for phosphatidylinositol hydrolysis (Mohammadi et al., 1992; Peters et al., 1992), but is apparently not involved in FGFR-mediated mitogenesis, neuronal differentiation (Spivak-Kroizman et al., 1994b), or mesoderm induction in a Xenopus animal cap model (Muslin et al., 1994).

**The src Signaling Pathway.** Src is a nonreceptor tyrosine kinase that constitutes a putative link from the FGFR to cortactin (Zhan et al., 1994), a focal adhesion-associated protein that binds filamentous actin (Wu et al., 1991).

**Crk-Mediated Signaling.** Crk, a SH2-/SH3-containing adaptor protein, probably links FGFR to the downstream signaling molecules Shc, C3G, and Cas. Signaling through Crk has no effect on cell motility, yet endothelial cells expressing FGFR-1 with a phenylalanine substitution at Tyr463 do not proliferate. However, the impact of Tyr463 for mitogenesis is controversial (Mohammadi et al., 1996) and may differ depending on the type of cell analyzed.

**The SNT-1/FRS2 Signaling Pathway.** An alternative tyrosine-phosphorylation-independent signaling pathway of FGFRs that involves a novel 90-kDa phosphoprotein, SNT-1 (Wang et al., 1996), or FRS2 (Kouhara et al., 1997), has been shown to exist. SNT-1/FRS2 links the FGFR signaling to the Ras/MAPK signaling pathway, which is important for growth-factor-induced cell cycle progression. Activation of SNT-1/FRS2 recruits Ras to the FGFR complex through the adaptor protein Grb-2/Sos (Kouhara et al., 1997). In addition to associating with Grb-2, activated FRS2 also binds the protein tyrosine phosphatase Shp2 (Ong et al., 2000). SNT-1/FRS2 is localized to the inner leaflet of the cell membrane by myristoylation and interacts with FGFR-1 at amino acid residues 407–433 of the juxtamembrane region (Xu et al., 1998). Interestingly, Yan et al. (2002) have reported that Trk neurotrophin receptors also employ SNT-1/FRS2 in their signaling pathways, but in this case association of FRS2 with Trk receptors depends on receptor activation. As a consequence, FGFR-1 may regulate Trk signaling by sequestering FRS2 from ligand-bound Trk receptors.

Thus, FGFRs mediate signal transduction by at least two independent pathways. First, FGFRs utilize the canonical SH2-linked pathway joining FGFR directly to PLC γ and Crk, and probably indirectly to Src. Second, activation of FGFR is linked to SNT1/FRS2 through an interaction at the juxtamembrane domain. The precise role of the second pathway, which seems to be independent of receptor phosphorylation, is still largely enigmatic, despite its similarity to insulin receptor signaling (Yenush and White, 1997).

### 2.6 FGF Knockout Mice and Transgenic Mouse Models

Mutant mice with disruptions of FGF and FGFR genes constitute important model systems for providing more insights into the biological functions of FGFs (› *Table 5-3*). Several FGF knockout mice have relatively mild phenotypes, possibly due to functional redundancy of different members of the FGF family. For example, FGF-8 and -17 seem to be able to substitute each other with respect to the formation of the midbrain–hindbrain boundary (Xu et al., 2000).

## 3 Neural Functions of FGFs

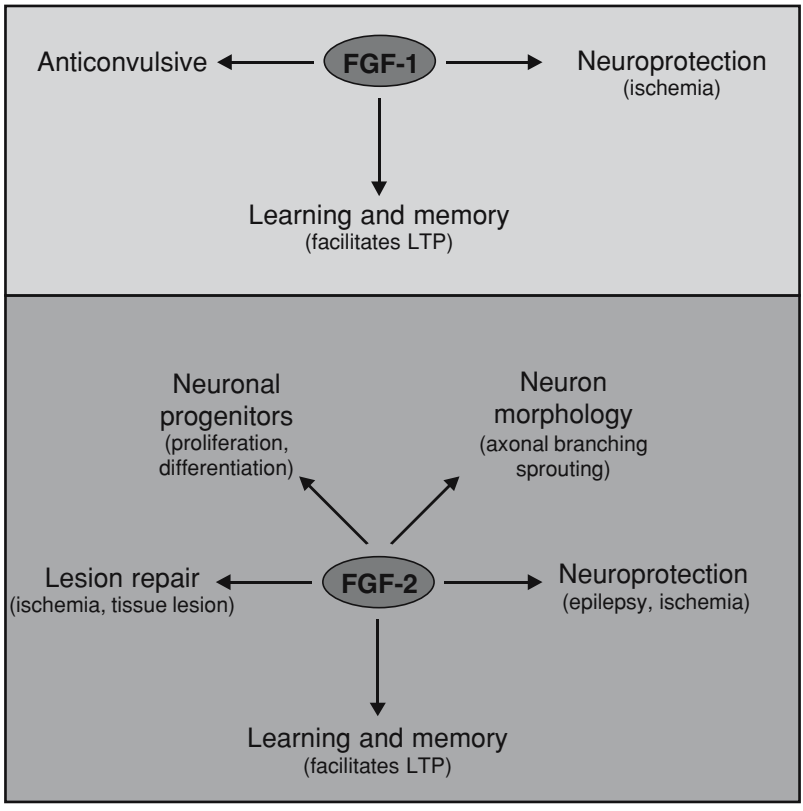
Since we wrote our last review on FGFs in the CNS (Bieger and Unsicker, 1996), significant progress has been made in the field. Most of the work has been done on neural functions of FGF-1 and -2 (cf. › *Figure 5-6*), implying that understanding the roles of the other members of the FGF family expressed in the brain is still relatively fragmentary.

**Table 5-3**
**Viability and phenotypes of available FGF-gene-deficient mice**

| Gene | Null mutant | Phenotype | References |
|---|---|---|---|
| FGF-1 | Viable | No detectable changes | Miller et al. (2000) |
| FGF-2 | Viable | Mild cardiovascular and skeletal disturbances, disturbed layering of the cerebral cortex, disturbed healing of skin wounds | Dono et al. (1998), Zhou et al. (1998) |
| FGF-3 | Viable | Disturbed inner ear and tail development | Mansour et al. (1993) |
| FGF-4 | Lethal (E4–5) | Disturbed inner cell mass proliferation, defects in limb development | Feldman et al. (1995), Sun et al. (2002) |
| FGF-5 | Viable | Increased hair growth | Hebert et al. (1994) |
| FGF-6 | Viable | Mild disturbances in muscle regeneration | Fiore et al. (1997, 2000), Floss et al. (1997) |
| FGF-7 | Viable | Disturbed growth of hair follicles, disturbed growth of ureteric bud | Guo et al. (1996), Qiao et al. (1999) |

For those FGF knockouts that are not available (N.A.), their predominant expression loci are listed

**Figure 5-6**
**In the central nervous system (CNS) various positive effects of fibroblast growth factors (FGFs) on neuronal structures have been demonstrated. In this figure, a schematic overview for effects of FGF-1 and FGF-2 on hippocampal neurons is given (adapted from Reuss and von Bohlen und Halbach, 2003)**



## 3.1 FGF Expression Patterns

Numerous studies support the notion that FGFs affect a large variety of developmental processes in the nervous system (Molteni et al., 2001; Vaccarino et al., 2001). These include, among others, cell fate determination (Anderson, 1993; Grothe et al., 2004), migration, differentiation (Kalcheim, 1996), cell survival (Grothe and Wewetzer, 1996; Mufson et al., 1999; Perrone-Capano and Di Porzio, 2000), and regeneration (Timmer et al., 2003, 2004; Jungnickel et al., 2004). FGF-1 and -2 are expressed from the earliest stages of nervous system development through into adulthood in distinct expression patterns (Eckenstein, 1994; Vaccarino et al., 1999; Gremo and Presta, 2000). The most prominent difference between FGF-1 and -2 is related to their cellular localization. FGF-2 is expressed by neurons and nonneuronal cells, while FGF-1 is localized predominantly in neurons.

In the adult CNS, FGF-1 is found throughout the brainstem in neurons of the oculomotor nucleus, the pons, the lateral geniculate nucleus, the reticular formation, the ventral tegmental area, and the substantia nigra. In the diencephalon both thalamus and hypothalamus (e.g., the magnocellular preoptic area) harbor FGF-1-positive neuron populations. In the telencephalon, neurons in the medial septum, diagonal bands of Broca, nucleus basalis of Meynert, striatum, cerebral cortex, and hippocampus express FGF-1 (Bean et al., 1991; Stock et al., 1992; Bizon et al., 1996). Spinal cord motoneurons and sensory ganglia also stain for FGF-1 (Elde et al., 1991). In situ hybridization studies have revealed FGF-1 messenger RNA (mRNA) in the cerebellum, locus coeruleus, hippocampus, and neocortex (Wilcox and Unnerstall, 1991).

Like FGF-1, FGF-2 is widely distributed throughout the CNS and the PNS (Eckenstein et al., 1991). FGF-2 is prominently expressed in the brainstem, midbrain including substantia nigra, thalamus, olfactory bulb, striatum, hippocampus, and cerebral cortex (Ernfors et al., 1990; Bean et al., 1991; Gomez-Pinilla et al., 1994; Grothe and Janet, 1995). FGF-2 also occurs in motor and sensory nuclei (Grothe et al., 1991) as well as in the neural and anterior lobes of the pituitary (Gonzalez et al., 1994).

FGF-3 mRNA is expressed in distinct locations of the embryonic mouse brain, including forebrain, midbrain–hindbrain junction, rhombomere boundaries, and in the otic placode. The initially high levels of FGF-3 transcripts in the otic placode are downregulated as the placode invaginates to form the otic pit (Mahmood et al., 1996). Expression of FGF-4 in CNS neurons and glia is controversial and needs to be investigated in more detail (Ozawa et al., 1996). However, its expression in tissues relevant to inner ear development (periplacodal and placodal ectoderm) has been reported repeatedly (Wright et al., 2003). FGF-5 mRNA has been reported to be more abundant in the postnatal than in the embryonic mouse brain (Ozawa et al., 1998). Although FGF-5 mRNA expression levels are low, compared with FGF-1 and -2 mRNAs, the FGF-5 mRNA is widely distributed, including in portions of the cerebral cortex, hippocampus, and thalamus. In general, FGF-5 mRNA can be detected in many limbic areas (olfactory bulb, hippocampal formation, entorhinal cortex), where expression levels are higher than in the neocortex (Gomez-Pinilla and Cotman, 1993), suggesting roles in limbic functions or dysfunctions. FGF-6 has been reported to be CNS- and skeletal-muscle-specific in mice at the time of birth (Ozawa et al., 1996). FGF-7 mRNA is localized in the ventricular layer of the embryonic mouse forebrain (Mason et al., 1994); it seems to be absent from the adult rat brain (Hattori et al., 1997), but can be detected in rat dorsal root ganglia (DRG) (Li et al., 2002). Although FGF-8 is widely expressed in adult tissues (e.g., brain, heart, lung, kidney, testis, ovary, prostate; Schmitt et al., 1996), the CNS location, in which it has been most intensely studied, seems to be the isthmic organizer of the midbrain–hindbrain junction (cf. Mason et al., 2000; Holzschuh et al., 2003). FGF-9 is moderately or weakly expressed, mostly in neurons, in widespread regions of the CNS including the olfactory bulb, caudate putamen, cerebral cortex, hippocampus, thalamus hypothalamus, and nuclei of the midbrain, brainstem, cerebellum, and ventral spinal cord (Tagashira et al., 1995). In the PNS, DRG neurons can be immunostained for FGF-9 (Nakamura et al., 1999). High levels of FGF-10 mRNA have been reported to be expressed by brainstem motor nuclei, while low levels are found in the hippocampus and thalamus (Hattori et al., 1997). FGF-12 and -13 mRNAs both occur in the developing mouse CNS in cells outside the proliferating ependymal layer (Hartung et al., 1997). FGF-13 is additionally expressed throughout the PNS (Hartung et al., 1997; Li et al., 2002). FGF-14 has been shown to be widely expressed in brain and spinal cord. A detailed study on its cerebellar expression has revealed that it is first observed in

postmitotic granule cells and that its developmental expression pattern is complementary to Math1 (Wang et al., 2000). A study that addressed FGF-15 reported a regionally restricted pattern of expression in the developing CNS (McWhirter et al., 1997). Like FGF-4, FGF-16 is expressed in tissues related to inner ear development (posterior otic cup and vesicle; Wright et al., 2003). FGF-17 mRNA is preferentially expressed in the neuroepithelium of the isthmus and septum in the embryonic rat brain (Hoshikawa et al., 1998). FGF-18, a predominantly neuronal FGF, has been reported to be transiently expressed at early postnatal stages in various CNS regions, including cortex and hippocampus (Hoshikawa et al., 2002). FGF-20 is preferentially expressed in the substantia nigra pars compacta (Ohmachi et al., 2000), while FGF-23 mRNA is predominant in the thalamic ventrolateral nucleus (Yamashita et al., 2000). Together, the available data support the notion of both discrete and broad expression patterns of FGFs in the nervous system, with distinct temporal and spatial regulations during development.

## 3.2 FGFR Expression Patterns

The different subtypes of FGFRs are widely expressed in the CNS. FGFR-1, -2, and -3 mRNAs are highly expressed in the diencephalon and telencephalon. Lower levels of expression have been reported for the mesencephalon, metencephalon, and myelencephalon (Belluardo et al., 1997). FGFR-1 is expressed in specific neuronal populations in the adult CNS (Asai et al., 1993; Yazaki et al., 1994), but has also been detected in astrocytes of white matter tracts (Takami et al., 1998). In contrast to FGFR-1, which is predominantly expressed on neurons, FGFR-2 and -3 are primarily on glial cells (Asai et al., 1993; Yazaki et al., 1994; Miyake et al., 1996). The fourth member of the FGFRs, FGFR-4, seems to be predominant during early development and is not detectable in the adult CNS with the exception of the lateral habenular nucleus (Fuhrmann et al., 1999).

## 3.3 Neurogenesis and Differentiation

While many neuronal differentiation genes have been identified, little is known about what determines where and when neurons are formed. Onset of neuronal differentiation first occurs in the spinal cord in a rostral to caudal sequence. A key regulatory event in this paradigm is the ability of somatic mesoderm to repress FGF-8 transcription in the prospective spinal cord (Diez et al., 2000). At a cellular level, differentiation of neuroepithelial precursor cells into neurons has been shown to be potently inhibited by FGF-1 and -2 (Faux et al., 2001). The underlying mechanism involves upregulation of *notch* and downregulation of *Delta1*.

Generally, the actual developmental status of a cell crucially determines the type of effect elicited by a given FGF family member. Thus, it has been shown that at an early developmental time point FGF-2 is able to expand the period of dopamine precursor division in vitro well beyond the period of cell division occurring in vivo. Temporal expansion of cell division was accompanied by a delay in differentiation (Bouvier and Mytilineou, 1995). The role of FGF-8 for the specification of early developing CNS dopaminergic neurons has recently been elaborated in zebrafish mutants by Holzschuh et al. (2003). Interestingly and contrary to expectations based on previous studies, FGF-8, neither alone nor in combination with hedgehog signaling, is required for specification of early developing dopaminergic neurons. On neural stem cells overexpressing the nuclear orphan receptor Nurr1, FGF-8 has been shown to induce midbrain dopaminergic phenotypes in cooperation with sonic hedgehog (Kim et al., 2003). In addition, FGF-20 has been shown to promote the differentiation of Nurr1-overexpressing neural stem cells into tyrosine-hydroxylase-positive neurons (Grothe et al., 2004).

Restrictions in neuronal fate occur during the transition from a multipotential to a postmitotic cell and are determined by cell-intrinsic and -extrinsic signals. In this context, FGF-2 has been shown to be mitogenic for those embryonic spinal cord cells that are already committed to a neuronal pathway (Ray and Gage, 1994). FGF-2 also regulates the proliferative fate of neural progenitor cells in the striatum. This is done in cooperation with insulin-like growth factor (IGF), which is a key factor in the regulation of neuronal stem cell activation, and epidermal growth factor (EGF; Arsenijevic et al., 2001). EGF induces the

proliferation of putative stem cells, which give rise to spheres of undifferentiated cells that can generate neurons and astrocytes. These spheres of undifferentiated cells contain FGFR-1 mRNA and protein (Vescovi et al., 1993). Exogenously applied FGF-2 regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells, indicating that sequential actions of growth factors play a role in regulating the generation of neurons and astrocytes in the developing CNS (Vescovi et al., 1993). In the hippocampus, FGF-2 is mitogenic for stem cells (Vicario-Abejon et al., 1995) and regulates the size of the hippocampal granule neuron population that is generated from progenitor cells (Cheng et al., 2002). Many other neuron populations, e.g., GABAergic neurons, are generated by FGF-2 (Deloulme et al., 1991).

In postmitotic hippocampal neurons, FGF-2 promotes survival (e.g., Walicke et al., 1986) and differentiation, e.g., of calbindin-expressing cells (Vicario-Abejon et al., 1995). Substantial evidence suggests that FGF-2 affects neurogenesis in the hippocampus (Nakagami et al., 1997; Palmer et al., 1999; Yoshimura et al., 2001). In primary cultures of hippocampal cells, FGF-2 not only triggers phenotypic differentiation, but also drives the formation of neurons of various developmental stages. These different phenotypes include, on the one hand, neurons with precursor and juvenile neuron morphologies that are unable to fire action potentials, and on the other, age-matched polarized neurons firing multiple action potentials (Eubanks et al., 1996).

An increasing number of studies tried to exploit neural stem cells for the repair of CNS lesions, e.g., in the spinal cord (cf. Enomoto et al., 2003). In this context, FGF-2 has been shown to crucially control both proliferation and differentiation phenotypes of neural stem cells from hippocampus and spinal cord after grafting into the neonatal spinal cord (Enomoto et al., 2003). A specific novel role of nuclear FGF and nuclear FGFR-1 has been reported for human neuronal progenitor cells (Stachowiak et al., 2003). In these cells, FGFR-1 mediates cAMP-induced neuronal differentiation by regulating CREB and CREB-binding protein. FGF-2 signaling onto FGFR-1-positive neural stem cells from the embryonic rat telencephalon, in conjunction with EGF, has also been shown to permit both self-renewal and differentiation into neuronal, astroglial, and oligodendroglial phenotypes. FGF-2 triggering self-renewal, but not EGF, induced cytosolic calcium responses, whereas in FGF-2 and EGF-containing medium both FGF-2 and EGF evoked calcium signals only in the differentiating progeny of these cells. These results suggest that FGF-2, but not EGF, sustains calcium-dependent self-renewal of neural stem cells, whereas together the two growth factors permit the initial commitment of neural stem cells into neuronal and glial phenotypes (Maric et al., 2003).

## 3.4 Effects on Adult Neuronal Precursor Cells

Proliferation of neural stem cells and differentiation into mature neurons do not occur only during development, but can also be detected in the adult brain (Altman and Das, 1965). Proliferation of adult neuron precursor cells has been demonstrated for the forebrain in vitro (Alvarez-Buylla and Lois, 1995), as well as for the hippocampus and the subventricular zone of the forebrain in vivo (Goldman et al., 1997). FGF-2 is an important regulator of pre-, peri- and postnatal neurogenesis. FGF-2 induces proliferation of neural progenitor cells in the hippocampus and in the subventricular zone (Wagner et al., 1999). Subcutaneous injection of FGF-2 at P1 increases [$^3$H]thymidine incorporation by 70% in hippocampal and subventricular zone homogenates and elicits a twofold increase in mitotic nuclei in the dentate gyrus and the dorsolateral subventricular zone, suggesting that FGF-2 penetrates the blood–brain barrier to regulate adult neurogenesis (Wagner et al., 1999).

**Neurogenesis in the Hippocampus.** Hippocampal cells from adult rats are capable of proliferating and generating neurons in culture containing FGF-2. These cells express a variety of neuronal and glial markers, including O4, NSE, MAP2, NF150, GAD, and calretinin. Two months after labeling and grafting these cells to the adult rat hippocampus, their descendents can be found in the dentate gyrus, where they differentiate into neurons exclusively in the granule cell layer (Gage et al., 1995). Electrophysiological properties of hippocampal progenitor cells passaged in the presence of FGF-2 are distinctly different from cells grown in the absence of FGF-2. Thus, FGF-2 elicited low levels of sodium, calcium, *N*-methyl-D-aspartate (NMDA),

and kainate currents as compared with other growth conditions. After multiple passages in the continued presence of FGF-2, sodium, calcium, and NMDA, responses declined further, whereas kainate and gamma amino butyric acid (GABA) responses remained substantial (Sah et al., 1997).

**Neurogenesis in the Striatum.** The striatum is another prominent brain structure containing substantial numbers of neuronal progenitor cells (Reynolds et al., 1992; Vescovi et al., 1993). Multipotential progenitors isolated from the adult mouse striatum proliferate and differentiate into astrocytes, oligodendrocytes, and neuron-like cells when exposed to FGF-2 (Vescovi et al., 1993; Gritti et al., 1996). The neuron-like cells exhibit neuronal electrophysiological properties, and are immunoreactive for GABA, substance P, choline acetyltransferase, and glutamate (Gritti et al., 1996).

**Neurogenesis in the Cortex of the Adult Human Brain.** Multipotent neural precursors residing in the adult human cortex have recently been isolated. Precursors have also been found in the hippocampus and amygdala and can reside far from ventricles (Arsenijevic et al., 2001).

**Neurogenesis in the Cerebellum.** The cerebellum is a long-known brain region with postnatal neurogenesis. A single injection of FGF-2 into the neonatal cerebellum stimulates cell division of neuronal precursors in the external granular layer (EGL; Cheng et al., 2001). As a result of increased proliferation in the EGL, numbers of neuronal cells in the internal granular layer (IGL), the final destination of the EGL precursors, is significantly increased (Cheng et al., 2001).

**Neurogenesis in the Peripheral Nervous System.** With regard to peripheral neurons, FGF-2 can induce proliferation of cells cultured from postnatal mouse DRG (Namaka et al., 2001). Dissociated neurons die under the culture conditions employed. Following FGF-2 withdrawal and addition of trophic factors, new DRG neurons develop and differentiate (Namaka et al., 2001). DRG neurons require FGF-2 during distinct developmental time windows. For a subpopulation of DRG neurons (termed P-neurons for their "pear-like" shape), for example, it was shown that they sequentially required first NGF and subsequently FGF-2 for their survival (Acosta et al., 2001).

**Neurogenesis in FGF-Deficient Mouse Mutants.** On the basis of evidence that exogenous FGF-2 prominently interferes with the generation and differentiation of neuronal precursor cells, respective phenotypes were expected in FGF-2-deficient mice. FGF-2-deficient mice are viable, and display distinct neuronal defects, e.g., a numerical deficit in cortical neurons (Dono et al., 1998). Despite a 40% decrease in frontal and parietal cortical gluatmatergic pyramidal neurons and reductions in neuronal soma size, no change in pyramidal or granule cell number has been detected in the hippocampus and occipital cortex of *Fgf2*(−/−) mice (Korada et al., 2002). This suggests that FGF-2 is necessary to regulate cell number and size in the anterior cerebral cortex. In contrast to pyramidal neurons, cortical GABA interneurons are unaffected by the lack of FGF-2. Raballo et al. (2000) have reported that the volume of the dorsal cerebral cortical anlage of *Fgf2*(−/−) mice is substantially smaller, whereas the volume of the basal cerebral cortical anlage is unchanged. Furthermore, the dorsal cerebral cortical anlage of FGF-2 knockout mice seems to have less founder cells and a reduced expansion of the progenitor pool over the first portion of neurogenesis.

## 3.5 Axon Growth and Branching

FGFs can have profound effects on neuronal morphologies, including axon and dendritic length and arborization. In this capacity, FGFs resembles other growth factors, e.g., the neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NT-4), and ciliary neurotrophic factor (CNTF; Patel and McNamara, 1995; Kalil et al., 2000). FGF-2 significantly increases axon branching of dissociated cortical neurons (Kalil et al., 2000). Within the hippocampus, multiple factors have been identified that enhance axonal branching of pyramidal axons (but not dendrites). FGF-2 appears to be the most effective factor stimulating axonal branching (Patel and McNamara, 1995). FGF-2 seems to specifically affect bifurcation and growth of axonal branches, without affecting the elongation rate of primary axons. The effect of FGF-2 is reversible, indicating that the continuous presence of the factor is required (Aoyagi et al., 1994). Several lines of evidence suggest that FGFR signaling can collaborate with signaling through extracellular matrix components, e.g., fibronectin (Choung et al., 2002).

Studies on cultured cortical neurons have revealed that growth cones pause and enlarge in regions, from which at later time points axon branches develop (Szebenyi et al., 1998). Application of FGF-2 to cultured cortical neurons increases the size of growth cones and inhibits advancement of growth cones, thereby causing a threefold increase in axon branching. FGF-2 also affects growth cone morphology and promotes rapid extension of filopodia within minutes (Szebenyi et al., 2001). Furthermore, FGF-2 administered to the growth cone increases the probability for axonal branching, suggesting that distal regions of the axon are more responsive to FGF-2 than other regions of the axon shaft (Szebenyi et al., 2001). The observation that FGF-2 is a potent trigger of axonal branching of cultured neurons has recently been carried to the in vivo situation. In an injury model of the entorhinal cortex–hippocampal connection, denervation of the hippocampal formation induces axonal sprouting. This results in an increase in the expression of several growth factors, including FGF-2 (Ramirez et al., 1999). Infusion of FGF-2 into rats with unilateral entorhinal lesions increased sprouting of axon terminals of the cholinergic septodentate pathway (Ramirez et al., 1999), suggesting a role of FGF-2 in the regulation of injury-related axonal remodeling of this cholinergic pathway.

## 3.6 Neuroprotection and Lesion Repair

**Effects and Underlying Mechanisms.** Among the earliest discoveries concerning putative roles of FGFs in the nervous system was the neurotrophic survival-promoting effect of FGF-2 on many different neuron populations in vitro (Morrison et al., 1986; Walicke et al., 1986; Unsicker et al., 1987) and following brain lesions (cf. Bieger and Unsicker, 1995). Many subsequent studies have addressed putative mechanisms underlying these effects of FGF-2. Thus, Mattson et al. (1989, 1993) showed that FGF-2 decreases glutamate-induced neuronal cell death by regulating glutamate receptor subunits, leading to a suppression of the 71-kDa NMDA receptor protein (NMDARP-71) but not of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor GluR1. Moreover, FGF-2 potentiates quisqualate-induced inositol phosphate formation in hippocampal neurons. This effect is blocked by addition of the AMPA/kainate receptor antagonist 6,7-dinitro-quinoline-2,3-dione (DNQX), suggesting an involvement of an AMPA/kainate receptor subtype distinct from GluR1 (Blanc et al., 1999). Recent evidence suggests that the neuroprotective effect of FGF-2 may require the presence of additional growth factors, e.g., glial-cell-line-derived neurotrophic factor (GDNF; Lenhard et al., 2002). Signaling of FGF-1-mediated protection against glutamate toxicity has been shown to imply inactivation of glycogen synthase kinase-3β by phosphorylation at serine 9, which requires PI3K-Akt (Hashimoto et al., 2002).

Similarly, cerebellar granule neurons are also protected by Fgf-1 against excitotoxictiy in a PI3-kinase/Akt-dependent and MAP-kinase/CREB-independent manner (Hossain et al., 2002).

It has been speculated that the neuroprotective effects of FGF-2 results, in part, from a prevention or attenuation of oxidative damage (Zhang et al., 1993). It has also been found that FGF-2, as well as some other factors, is effective in suppressing oxidative impairment of synaptic transporter functions, and that FGF-2 suppresses oxidative stress and mitochondrial dysfunction induced by amyloid beta peptide and $Fe^{2+}$ in synaptosomes (Guo and Mattson, 2000).

**Forebrain Septal and Midbrain Dopaminergic Neurons.** In addition to hippocampal neurons, forebrain septal and midbrain dopaminergic neurons are supported by FGF. FGF-2 promotes survival of both cholinergic (Otto et al., 1989) and noncholinergic septal neurons (Cummings et al., 1992) following fimbria–fornix transection. The effect on the cholinergic population, which projects to the hippocampus, may be indirect and mediated by glial cells, as indicated by an in vitro study (Perkins and Cain, 1995). Mesencephalic dopaminergic neurons are immunoreactive for FGF-2 in vivo and in vitro (Tooyama et al., 1992; Casper et al., 1994) and respond to exogenous FGF-2 with increased survival both in vitro and in vivo. FGF-2 also significantly augments their survival (Otto and Unsicker, 1993a) following exposure to the dopaminotoxic substance 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or to its active form, the methyl pyridinium ion ($MPP^+$). Importantly, FGF-2 also partially protects in vivo against the deleterious chemical and morphological consequences of an MPTP lesion (Otto and Unsicker, 1990, 1993b). Similarly, resistance of dopaminergic neurons to L-glutamate-mediated toxicity was greatly enhanced in the presence of FGF-2 (Casper and Blum, 1995). Together, these data identify FGF-2 as a potent neuroprotective agent

for dopaminergic nigral neurons. Interestingly, both the low- (18 kDa) and the high-molecular weight FGF-2 isoforms (21 and 23 kDa) seem to carry survival and neurite-promoting activities for dopaminergic neurons (Claus et al., 2000, 2004a, b). This is consistent with the expression of all three isoforms as well as FGFR-1, -2, and -3 in the intact and toxin-lesioned rat dopaminergic nigrostriatal system (Claus et al., 2004b). As far as underlying mechanisms of the protective effects of FGF-2 for midbrain dopaminergic neurons are concerned, two possibilities have been discussed and experimentally tested. One important mechanism is the FGF-2-mediated induction of other growth factors, such as TGF-β and others (Krieglstein et al., 1998). Interestingly, regulation of gap junctions may be implicated in the mediation of the neuroprotective effects of FGF-2 (Leung et al., 2001): In mixed cultures of midbrain neurons and nonneuronal cells, blockade of gap junction communication by oleamide causes a significant reduction of survival rates of dopaminergic midbrain neurons in the presence of FGF-2.

FGF-8, which plays a well-documented role in early pattern formation of the midbrain/hindbrain, can also promote survival of postmitotic neurons as efficiently as FGF-2. For example, FGF-8 protects rat hippocampal neurons from oxidative stress (Mark et al., 1999). In a time course study, it has been reported that FGF-8 is neuroprotective when added as a pretreatment, cotreatment, and even at 2-h postinsult (Mark et al., 1999). This may indicate that FGF-8 might be useful in treatment of oxidative insults, such as stroke.

**FGF-Mediated Neuroprotection In Vivo.** As summarized above, neuroprotective effects are a prominent feature of FGF functions. The in vivo capacity of exogenous FGFs for protecting lesioned neurons and orchestrating responses to lesions has been substantially documented. Protection of retinal ganglion cells following transection of the optic nerve has been the first paradigm, in which the neurotrophic potential of FGF-1 and -2 has been demonstrated in vivo (Sievers et al., 1987). Axotomized cholinergic neurons of the septo-hippocampal pathway and toxin-lesioned nigrostriatal dopaminergic neurons were the next populations, for which a protective effect of FGF-2 was reported (Anderson et al., 1988; Otto et al., 1989; Otto and Unsicker, 1990). A putative physiological relevance of FGF-2 has been suggested by the finding that endogenous FGF is significantly upregulated after lesioning. Cortical lesions induce a rise in FGF-2 mRNA and protein for up to 2 weeks, with microglia and reactive astrocytes being the principal sources of FGF-2 (Frautschy et al., 1991). Similarly, entorhinal cortex lesions have been shown to elicit an increase of FGF-2 in the outer molecular layer of the dentate gyrus ipsilateral to the lesion (Gomez-Pinilla et al., 1992). Importantly, FGF-2-mediated repair processes can improve behavioral scores of mice after lesioning (Ishihara et al., 1992), suggesting an important capacity of FGF-2 for restoring brain functions.

## 3.7 Ischemia

Stroke resulting from brain ischemia is a major cause of death and long-term disability, annually affecting more than 700,000 people in the USA. Depending on the site of vascular occlusion, distinct brain regions may be affected. Several members of the FGF family, in particular FGF-2, are intimately involved in neuronal protection and repair after ischemic, excitotoxic, and metabolic injury (cf. Alzheimer and Werner, 2002 for review).

Hippocampal ischemia elicits rapid neuronal cell death (Kirino, 2000; Martone et al., 2000), which can be overcome by neuroprotective growth factors, including FGF-1, -2, and -7 (Nakata et al., 1993; Cuevas et al., 1998; Sadohara et al., 2001). As with other CNS lesion paradigms, FGF-2 has been shown to be upregulated following brain ischemia (Masumara et al., 1996; Martinez et al., 2001). In animal models of brain ischemia, FGF-1 and -2 have been widely documented to prevent cell death resulting from ischemic damage. Thus, application of FGF-1 into the lateral cerebral ventricles before or even shortly after an ischemic insult prevents the death of hippocampal pyramidal cells (Sasaki et al., 1992). Similarly, application of FGF-2 also prevented CA1 neuronal damage in a dose-dependent manner (Nakata et al., 1993). Unexpectedly, even systemic administration of FGF-2 can ameliorate acute focal ischemic injury in the cerebral cortex without increasing blood flow following middle cerebral artery occlusion (MCAO; Bethel et al., 1997). Two hours after MCAO and a 24-h reperfusion interval, FGF-2-like immunoreactivity was upregulated in the striatum and the frontoparietal cortex. In the core of the infarct and in the surrounding region, the so-called penumbra, astroglial cells were the predominant source of FGF-2; only few neurons in

the penumbra were FGF-2-immunoreactive (Wei et al., 2000). Together, these data suggest that FGF-2 is upregulated and may possibly act as a protective factor in cerebral ischemia. Consistent with this assumption, postischemic administration of FGF-2 improves sensorimotor function and reduces infarct size (Li and Stephenson, 2002). Beneficial effects have also been reported following adenovirus-mediated gene transfer (Matsuoka et al., 2003) and conjugation to a blood–brain barrier delivery vector (Song et al., 2002). Synergistic protective effects of caspase inhibitors and FGF-2 against brain injury induced by transient focal ischemia argue in favor of putative combinatorial therapeutic strategies (Ma et al., 2001). Despite the well-documented benefits of FGF-2 in animal models of stroke, there is currently no clinical development in stroke, after a phase II/III trial with FGF-2 in acute stroke patients was discontinued because of an unfavorable risk-to-benefit ratio (Alzheimer and Werner, 2002).

With regard to other FGFs, application of FGF-18 shortly after MCAO produces a dose-dependent reduction in infarct volume and amelorites behavioral deficits (Ellsworth et al., 2003, 2004).

**FGF-2 Knockout Mice.** Mice deficient for FGF-2 have greatly added to our understanding of FGF-2 functions in the normal and lesioned brain. An impact of FGF-2 for the generation and differentiation of neural stem cells has been consolidated by the observation that endogenously synthesized FGF-2 is necessary and sufficient to stimulate proliferation and differentiation of neural progenitor cells in the adult hippocampus following a brain insult (Yoshimura et al., 2001). FGF-2-deficient mice exhibit a significant decline in BrdU-labeling kainic acid treatment or MCAO in the hippocampal formation as compared with wild-type littermates. This phenotype could be rescued by an intraventricular injection of a herpes simplex virus-1 amplicon vector carrying the FGF-2 gene into FGF-2(−/−) mice. Mice lacking FGF-2 display a significant increase in infarct volume after MCAO and lack the ischemia-mediated induction of BDNF and its cognate receptor trkB in the hippocampal formation (Kiprianova et al., 2004), suggesting BDNF and trkB as potential targets of FGF-2 actions.

## 3.8 Seizures and Seizure-Induced Brain Damage

Glutamate is a major factor in ischemia-mediated neuronal degeneration in the CNS (Rothman and Olney, 1986; Kiessling and Gass, 1994; Ikonomidou and Turski, 1995). Its excessive release and extracellular accumulation causes persistent activation of glutamate receptors, followed by acute neurotoxic degeneration of the hyperstimulated neuron (Ikonomidou and Turski, 1996). Glutamate-mediated neurotoxicity is also a major cause of neuron death in epilepsy (Olney et al., 1986). A model system for eliciting brain seizures is the application of kainic acid (kainate) or bicuculline (Olney et al., 1986). Bicuculline is a GABA receptor antagonist, whereas kainic acid acts as an agonist of a specific set of glutamate receptor (kainate receptors; von Bohlen und Halbach and Dermietzel, 2002).

Depending on the experimental paradigm and the FGF isoform, FGFs may have anticonvulsant or proconvulsant properties. FGF-2 does not induce major anticonvulsive effects when administered before or after kainic-acid-induced seizures (Liu et al., 1993; Liu and Holmes, 1997a), but can induce seizures on its own (Liu and Holmes, 1997b). In contrast, FGF-1 may decrease convulsions in the kainate model (Cuevas and Gimenez-Gallego, 1996). In addition, both FGF-1 (Cuevas et al., 1994) and FGF-2 (Liu et al., 1993) prevent hippocampal cell losses in the kainate model. Kainate induces a robust increase in FGF-2 gene expression in the hippocampus, with a peak at 24 h (Riva et al., 1994). Similarly, bicuculline increases FGF-2 mRNA (Riva et al., 1992). This increase in FGF-2 expression is supposed to be due to an upregulation in astroglial rather than neuronal cells. Upregulation of astroglial FGF-2 may result from released excessive glutamate acting on astroglial glutamate receptors (Humpel et al., 1993).

Kindling is a widely studied animal model for temporal lobe epilepsy in which daily electrical stimulation of certain brain regions results in the gradual progression and intensification of limbic motor seizures (Kalynchuk, 2000). After a single kindling stimulation, FGF-2 mRNA levels, but not FGF-1 mRNA levels, have been found to be increased in the hippocampus. Fully kindled animals left unstimulated for 1 week, however, did not exhibit any alteration in the mRNA levels for FGF-1 or -2 (Simonato et al., 1998; Bregola et al., 2000). These data, in combination with the data obtained by using the other epilepsy models, indicate that the observed changes in FGF-2 mRNA may represent short-term effects.

Studies addressing time-dependent regulation of FGF-2 protein in limbic regions following electroshock seizures have revealed that chronic, but not acute, treatments cause a significant rise in frontal and rhinal cortices (Gwinn et al., 2002).

## 3.9 Glial Cells as an Important Source of FGFs

Glial cells are an important source for the synthesis of FGFs in the CNS. In particular, astrocytes synthesize and release FGF-2 (cf. Bieger and Unsicker, 1996; Reuss and von Bohlen und Halbach, 2003, for reviews). Regulation of astroglial FGF-2 expression is regulated by numerous stimuli and cytokines. For example, oxygen deprivation causes a massive increase in FGF-2 protein expression in cultured astrocytes, which peaks at 24 h (Liu and Zhu, 1999). Similarly, serum and glucose deprivations induce astroglial FGF-2 immunoreactivity and moderately increase FGFR-1 expression (Liu and Zhu, 1999). In the PNS, Schwann cells are an important source of FGF-2. Reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ hybridization have revealed expression of the mRNAs for FGF-2 and the FGFR-1, -2, and -3 in Schwann cells and macrophages. FGF-2 and all four FGFRs were expressed in neurons of the spinal ganglia. A crush lesion resulted in no upregulation of FGFR transcripts in the nerve (Grothe et al., 2001). Schwann cells overexpressing high-molecular-weight isoforms have been reported to enhance axonal regeneration through peripheral nerve gaps (Timmer et al., 2003) as well as reinnervation and functional recovery of intrastriatal dopamine grafts following their transplantation to the respective elsion sites (Timmer et al., 2004).

**Paracrine Actions of FGF on Neurons.** FGF-2 released from astroglial cells acts in both paracrine and autocrine fashions. Signaling toward neurons can significantly increase the length of axons and dendrites of loate embryonic cortical neurons, as shown by treatment of cultures with astroglial-conditioned medium (Le and Esquenazi, 2002). Antibody-mediated blocking of FGF-2 signaling significantly reduces astroglial-dependent neurite growth (Le and Esquenazi, 2002). Astroglial FGF-2 also plays an important role in neuron survival. In mixed cultures of embryonic midbrain dopaminergic neurons and astroglial cells, dopamine stimulates release of FGF-2, which then promotes survival and neurite growth. Antibodies to FGF-2 specifically block this effect (Reuss and Unsicker, 2000).

**FGF-9 Is Expressed by Both Astroglial Cells and Oligodendrocytes.** Using double immunofluorescence and in situ hybridization, Nakamura et al. (1999) have detected FGF-9-specific signals in glia fibrillary acidic protein (GFAP)-positive white matter astrocytes of adult rat spinal cord and brainstem. FGF-9 is also expressed by CNPase-positive cerebellar and callosal oligodendrocytes (Nakamura et al., 1999).

In contrast to the FGFs mentioned above, FGF-18 is preferentially expressed in neurons but not in glial cells in the brain. FGF-18 was found to have mitogenic activity for both astrocytes and microglia, but seems to lack neurotrophic activity. These findings suggest that FGF-18 is a unique FGF having a role as a neuron-derived glial cell growth factor (Hoshikawa et al., 2002).

At early stages during development FgfR1–3 expression overlaps with that of Olig2 (an oligodendrocyte progenitor marker) in the embryonic ventricular zone of the lateral and medial ganglionic eminences (Bansal et al., 2003), suggesting a role of respective ligands in oligodendroglial differentiation. With regard to myelination, transient exposure of cultured brain cells to FGF-2 has been reported to increase myelination (Magy et al., 2003). In vivo, FGF-2 has been shown to inhibit oligodendrocyte lineage differentiation and myelin production by oligodendrocytes (Goddard et al., 2001; Armstrong et al., 2002).

**Autocrine/Paracrine Effects of FGF on Astroglia and Oligodendrocytes.** Glial cells are also a prominent target for different FGF family members. Autocrine or paracrine actions of FGF-2 on astrocytes include regulation of gap junction (connexin43) expression and function, neurotransmitter sensitivity, and intermediate filament density (Reuss et al., 1998, 2000a, b; Gomes et al., 1999).

In an in vitro study about the effects of FGF-2 and TGF-β1 on astrocytes, these factors have been claimed to act antagonistically on astrocyte differentiation as monitored by GFAP expression (Reilly et al., 1998). In this study, treatment with TGF-β1 leads to a significant increase in GFAP mRNA and protein, whereas FGF-2 changed astrocytes from a polygonal to a stellate morphology and suppressed GFAP expression. In addition, both factors seemed to interfere with each other since FGF-2 inhibited the TGF-β1-mediated increase in GFAP mRNA and protein. However, suppression of GFAP expression

reported by Reilly et al. (1998) was contradictory to earlier reports about FGF-2-dependent induction of GFAP in astrocytes (Eclancher et al., 1990; Perraud et al., 1990).

A special feature of FGF-2 in astrocytes is its subcellular localization during regulation of astrocyte proliferation. As proposed by Stachowiak et al. (1997), astroglial activation leads to nuclear translocation and accumulation of FGF-2 and its receptor. This finding has been confirmed by Joy et al. (1997).

Besides their effects on astrocyte differentiation, FGFs have also been shown to regulate other astroglial cell functions, including gap junction coupling and neurotransmitter sensitivity (Reuss et al., 1998, 2000a, b). Cultured cortical and striatal, but not mesencephalic, astrocytes respond to FGF-2 with a reduction of connexin43 protein, connexin43 mRNA, and intercellular communication as revealed by dye spreading (Reuss et al., 1998). Interestingly, FGF-5 and -9 can perform identical functions, but with different brain region specificity, in that FGF-5 exclusively addresses midbrain astroglial cells, and FGF-9, cortical, striatal, and midbrain astroglia (Reuss et al., 2000a). Identical region specificity is seen with regard to the astroglial phenotype of FGF-2, -5, and FGF-2/FGF-5 double-deficient mice (Reuss et al., 2003). FGF-2(−/−) mice displayed a dramatic reduction in GFAP expression of gray matter, but not white matter astrocytes in cortex and striatum, and not in midbrain. FGF-5(−/−) mice show an identical phenotype for midbrain, but not cortical and striatal astroglia, while FGF2/FGF-5 double deficiency affects astroglial GFAP in all three regions. Brain region specificity is not matched by a respective distributional pattern of the ligands, or FGFR-2 and -3; both receptors can be mapped to astroglial cells in a brain-region-independent fashion (Reuss et al., 1998). Possibly, differential splice forms of the receptors or regional specificity of coligands/coreceptors may account for the observed region specificity. Another prominent effect of FGF-2 on astroglial functions is its influence on astroglial sensitivity to dopamine. Treatment of astrocytes with FGF-2 leads to an increase in numbers of dopamine-sensitive astrocytes and to an induction of the D1 dopamine receptor (Reuss et al., 2000b).

In addition to astrocytes, oligodendrocytes are important target cells for FGF actions. Responsiveness of astrocytes and oligodendroglial cells to FGFs is further supported by the fact that both cell types express the IgIIIc splice variant of FGFR-2 and -3 (Miyake et al., 1996; Reuss et al., 2000). Another glial cell type that is affected by FGFs is microglia (Goddard et al., 2002). Activation of all three glial subpopulations, astrocytes, oligodendrocytes, and microglia, can be observed after injection of FGF-2 in the cerebrospinal fluid (Goddard et al., 2002), resulting in increased GFAP expression, swelling of the cell bodies, and enhanced formation of processes in astrocytes. The same study demonstrates a significant increase in the number of ED1-labeled microglia and a change in microglial morphology toward a multipolar and granular appearance after FGF-2 injections. Finally, in oligodendrocytes a loss of myelin sheaths was observed after injections of FGF-2 (Goddard et al., 2002). As demonstrated by Cohen and Chandross (2000), FGF-2 is not the only FGF family member to influence oligodendrocyte performances, as FGF-9 is able to modulate the expression of myelin related proteins and multiple FGFRs in developing oligodendrocytes.

## 3.10 Learning and Memory

Increasing evidence suggests that FGFs may be important modulators of processes attributed to learning and memory. Continuous superfusion of in vitro brain slices with FGF-1 has been shown to decrease the basal amplitude of spikes and significantly increase paired-pulse facilitation in the hippocampus (Sasaki et al., 1994). Tetanic stimulation can induce long-term potentiation (LTP). When FGF-1 is continuously applied, tetanic stimulation leads to an enhancement of the magnitude of short-term potentiation after the tetanus and facilitates the generation of LTP. Furthermore, FGF-1 also enhances dose-dependent posttetanic potentiation directly after the tetanus (Sasaki et al., 1994). This suggests that FGF-1 may be involved in mechanisms related to the generation of LTP. Since LTP is thought to be linked to mechanisms involved in memory formation and learning (Bliss and Collingridge, 1993), FGF-1 might have a role in such mechanisms. Evidence supporting this view came from a study, using i.c.v. injections of FGF-1. LTP can be induced using a subthreshold stimulation in combination with administration of FGF-1; however, LTP cannot be induced using the subthreshold stimulation alone (Hisajima et al., 1992). Further evidence came from a study using an FGF-1 fragment analog (amino acid residues 1 through 29 of FGF-1 with an alanine

substituted at position 16) on accelerated senescence-prone mice. Subcutaneous injection of this FGF-1 fragment analog was found to prolong the mean retention latency and to shorten the latency in a passive avoidance test. In addition, improved performance was also obtained in several other behavioral tests, indicating a beneficial effect of the FGF-1 fragment analog on learning and memory in accelerated senescence-prone mice (Sasaki et al., 1999).

In addition to FGF-1, FGF-2 seems to be involved in neuronal signaling. In the dentate gyrus, subthreshold stimulation (20 pulses at 60 Hz) normally fails to induce LTP; however, after administration of FGF-2, LTP can be induced using this protocol (Ishiyama et al., 1991). A similar model system demonstrating growth factor effects on LTP is provided by the following example: Transection of the fimbria–fornix pathway decreases the frequency of LTP generation. Intracerebroventricular injection of EGF or FGF-2 facilitated LTP generation in the fimbria–fornix lesioned rats, suggesting that EGF and FGF-2 can improve hippocampal LTP impairment after loss of subcortical afferents (Abe et al., 1992).

## 4 Conclusions and Perspectives

The great diversity of effects generated by FGFs and reviewed in this chapter results from a great diversity of ligands, receptors, receptor splice variants, and intracellular messenger cascades that eventually lead to alterations in gene expression. In the CNS at least ten FGF family members are present. Surprisingly, the past decade (cf. review by Bieger and Unsicker, 1996) has added relatively little, with the exception of FGF-1 and -2, to the neural functions of the other FGFs in the nervous system. It seems that the analysis of established FGF mouse mutants and the generation of conditional FGF and FGFR mutants is gaining speed now, triggering expectations that in a decade from now we shall have a more detailed understanding of FGF signaling to neurons and glial cells. Hopefully, this also applies to a more thorough understanding of the roles FGFs play in concert with other cytokines, e.g., TGF-βs. Finally, there has been relatively modest progress in the past decade regarding therapeutic applications of FGFs, acting agonistically or antagonistically as small peptides and drugs, raising hopes that problems such as targeting of factors to specific cells, transport through the blood–brain barrier, and long-term deliverance, for example, through growth-factor-secreting cells, may eventually be overcome.

## References


Abe K, Ishiyama J, Saito H. 1992. Effects of epidermal growth factor and basic fibroblast growth factor on generation of long-term potentiation in the dentate gyrus of fimbria–fornix-lesioned rats. Brain Res 593: 335-338.

Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, et al. 1986a. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233: 545-548.

Abraham JA, Whang JL, Tumolo A, Mergia A, Fiddes JC. 1986b. Human basic fibroblast growth factor: nucleotide sequence, genomic organization, and expression in mammalian cells. Cold Spring Harb Symp Quant Biol 51(Pt. 1): 657-668.

Acosta CG, Fabrega AR, Masco DH, Lopez HS. 2001. A sensory neuron subpopulation with unique sequential survival dependence on nerve growth factor and basic fibroblast growth factor during development. J Neurosci 21: 8873-8885.

Altman J, Das GD. 1965. Autoradiographic and histological evidence for postnatal hippocampal neurogenesis in rats. J Comp Neurol 124: 319-325.

Alvarez-Buylla A, Lois C. 1995. Neuronal stem cells in the brain of adult vertebrates. Stem Cells 13: 263-272.

Alzheimer C, Werner S. 2002. Fibroblast growth factors and neuroprotection. Adv Exp Med Biol 513: 335-351.

Anderson DJ. 1993. Cell fate determination in the peripheral nervous system: the sympathoadrenal progenitor. J Neurobiol 24: 185-198.

Anderson KJ, Dam D, Lee S, Cotman CW. 1988. Basic fibroblast growth factor prevents death of lesioned cholinergic neurons in vivo. Nature 332: 360-361.

Antoine M, Reimers K, Dickson C, Kiefer P. 1997. Fibroblast growth factor 3, a protein with dual subcellular localization, is targeted to the nucleus and nucleolus by the concerted action of two nuclear localization signals and a nucleolar retention signal. J Biol Chem 272: 29475-29481.

Aoyagi A, Nishikawa K, Saito H, Abe K. 1994. Characterization of basic fibroblast growth factor-mediated acceleration of axonal branching in cultured rat hippocampal neurons. Brain Res 661: 117-126.

Armstrong RC, Le TQ, Frost EE, Borke RC, Vana AC. 2002. Absence of fibroblast growth factor 2 promotes oligodendroglial repopulation of demyelinated white matter. J Neurosci 22: 8574-8585.

Arnaud E, Touriol C, Boutonnet C, Gensac MC, Vagner S, et al. 1999. A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. Mol Cell Biol 19: 505-514.

Arsenijevic Y, Weiss S, Schneider B, Aebischer P. 2001. Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. J Neurosci 21: 7194-7202.

Asai T, Wanaka A, Kato H, Masana Y, Seo M, et al. 1993. Differential expression of two members of FGF receptor gene family, FGFR-1 and FGFR-2 mRNA, in the adult rat central nervous system. Brain Res Mol Brain Res 17: 174-178.

Bansal R, Lakhina V, Remedios R, Tole S. 2003. Expression of FGF receptors 1, 2, 3 in the embryonic and postnatal mouse brain compared with Pdgfralpha, Olig2 and Plp/dm20: implications for oligodendrocyte development. Dev Neurosci 25: 83-95.

Bean AJ, Elde R, Cao YH, Oellig C, Tamminga C, et al. 1991. Expression of acidic and basic fibroblast growth factors in the substantia nigra of rat, monkey, and human. Proc Natl Acad Sci USA 88: 10237-10241.

Belluardo N, Wu G, Mudo G, Hansson AC, Pettersson R, et al. 1997. Comparative localization of fibroblast growth factor receptor-1, -2, and -3 mRNAs in the rat brain: in situ hybridization analysis. J Comp Neurol 379: 226-246.

Bethel A, Kirsch JR, Koehler RC, Finklestein SP, Traystman RJ. 1997. Intravenous basic fibroblast growth factor decreases brain injury resulting from focal ischemia in cats. Stroke 28: 609-615.

Bieger S, Unsicker K. 1996. Functions of fibroblast growth factors (FGFs) in the nervous system. In: *Chemical Factors in Neural Growth, Degeneration, and Repair* (C. Bell, ed.), pp 339-375. Elsevier Science B. V.

Bizon JL, Lauterborn JC, Isackson PJ, Gall CM. 1996. Acidic fibroblast growth factor mRNA is expressed by basal forebrain and striatal cholinergic neurons. J Comp Neurol 366: 379-389.

Blanc EM, Jallageas M, Recasens M, Guiramand J. 1999. Potentiation of glutamatergic agonist-induced inositol phosphate formation by basic fibroblast growth factor is related to developmental features in hippocampal cultures: neuronal survival and glial cell proliferation. Eur J Neurosci 11: 3377-3386.

Blanquet PR, Patte C, Fayein N, Courtois Y. 1989. Identification and isolation from bovine epithelial lens cells of two basic fibroblast growth factor receptors that possess bFGF-enhanced phosphorylation activities. Biochem Biophys Res Commun 160: 1124-1131.

Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361: 31-39.

Bouvier MM, Mytilineou C. 1995. Basic fibroblast growth factor increases division and delays differentiation of dopamine precursors in vitro. J Neurosci 15: 7141-7149.

Bregola G, Frigati L, Zucchini S, Simonato M. 2000. Different patterns of induction of fibroblast growth factor-2 and brain-derived neurotrophic factor messenger RNAs during kindling epileptogenesis, and development of a herpes simplex vector for fibroblast growth factor-2 gene transfer in vivo. Epilepsia 41(Suppl. 6): S122-S126.

Burdine RD, Chen EB, Kwok SF, Stern MJ. 1997. Egl-17 encodes an invertebrate fibroblast growth factor family member required specifically for sex myoblast migration in *Caenorhabditis elegans*. Proc Natl Acad Sci USA 94: 2433-2437.

Burgess WH, Dionne CA, Kaplow J, Mudd R, Friesel R, et al. 1990. Characterization and cDNA cloning of phospholipase C-gamma, a major substrate for heparin-binding growth factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. Mol Cell Biol 10: 4770-4777.

Calamandrei G, Alleva E. 1995. Neuronal growth factors, neurotrophins and memory deficiency. Behav Brain Res 66: 129-132.

Casper D, Blum M. 1995. Epidermal growth factor and basic fibroblast growth factor protect dopaminergic neurons from glutamate toxicity in culture. J Neurochem 65: 1016-1026.

Casper D, Roboz GJ, Blum M. 1994. Epidermal growth factor and basic fibroblast growth factor have independent actions on mesencephalic dopamine neurons in culture. J Neurochem 62: 2166-2177.

Chellaiah AT, McEwen DG, Werner S, Xu J, Ornitz DM. 1994. Fibroblast growth factor receptor (FGFR) 3. Alternative splicing in immunoglobulin-like domain III creates a receptor highly specific for acidic FGF/FGF-1. J Biol Chem 269: 11620-11627.

Cheng Y, Black IB, DiCicco-Bloom E. 2002. Hippocampal granule neuron production and population size are regulated by levels of bFGF. Eur J Neurosci 15: 3-12.

Cheng Y, Tao Y, Black IB, DiCicco-Bloom E. 2001. A single peripheral injection of basic fibroblast growth factor (bFGF) stimulates granule cell production and increases cerebellar growth in newborn rats. J Neurobiol 46: 220-229.

Choung PH, Seo BM, Chung CP, Yamada KM, Jang JH. 2002. Synergistic activity of fibronectin and fibroblast growth factor receptors on neuronal adhesion and neurite extension through extracellular signal-regulated kinase pathway. Biochem Biophys Res Commun 295: 898-902.

Claus P, Bruns AF, Grothe C. 2004a. Fibroblast growth factor-2(23) binds directly to the survival of motoneuron protein and is associated with small nuclear RNAs. Biochem J 384: 559-565.

Claus P, Doring F, Gringel S, Muller-Ostermeyer F, Fuhlrott J, et al. 2003. Differential intranuclear localization of fibroblast growth factor-2 isoforms and specific interaction with the survival of motoneuron protein. J Biol Chem 278: 479-485.

Claus P, Werner S, Timmer M, Grothe C. 2004. Expression of the fibroblast growth factor-2 isoforms and the FGF receptor 1-4 transcripts in the rat model system of Parkinson's disease. Neurosci Lett 360: 117-120.

Cohen RI, Chandross KJ. 2000. Fibroblast growth factor-9 modulates the expression of myelin related proteins and multiple fibroblast growth factor receptors in developing oligodendrocytes. J Neurosci Res 61: 273-287.

Coughlin SR, Barr PJ, Cousens LS, Fretto LJ, Williams LT. 1988. Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. J Biol Chem 263: 988-993.

Coulier F, Pontarotti P, Roubin R, Hartung H, Goldfarb M, et al. 1997. Of worms and men: an evolutionary perspective on the fibroblast growth factor (FGF) and FGF receptor families. J Mol Evol 44: 43-56.

Cuevas P, Carceller F, Munoz-Willery I, Gimenez-Gallego G. 1998. Intravenous fibroblast growth factor penetrates the blood-brain barrier and protects hippocampal neurons against ischemia-reperfusion injury. Surg Neurol 49: 77-83.

Cuevas P, Gimenez-Gallego G. 1996. Antiepileptic effects of acidic fibroblast growth factor examined in kainic acid-mediated seizures in the rat. Neurosci Lett 203: 66-68.

Cuevas P, Revilla C, Herreras O, Largo C, Gimenez-Gallego G. 1994. Neuroprotective effect of acidic fibroblast growth factor on seizure-associated brain damage. Neurol Res 16: 365-369.

Cummings BJ, Yee GJ, Cotman CW. 1992. bFGF promotes the survival of entorhinal layer II neurons after perforant path axotomy. Brain Res 591: 271-276.

Deloulme JC, Gensburger C, Sarhan S, Seiler N, Sensenbrenner M. 1991. Effects of basic fibroblast growth factor on the development of GABAergic neurons in culture. Neuroscience 42: 561-568.

Diez DC, Breitkreuz DN, Storey KG. 2000. Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. Development 129: 1681-1691.

Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, et al. 1990. Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J 9: 2685-2692.

Dono R, Texido G, Dussel R, Ehmke H, Zeller R. 1998. Impaired cerebral cortex development and blood pressure regulation in FGF-2-deficient mice. EMBO J 17: 4213-4225.

Eckenstein FP. 1994. Fibroblast growth factors in the nervous system. J Neurobiol 25: 1467-1480.

Eckenstein F, Woodward WR, Nishi R. 1991. Differential localization and possible functions of aFGF and bFGF in the central and peripheral nervous systems. Ann N Y Acad Sci 638: 348-360.

Eclancher F, Perraud F, Faltin J, Labourdette J, Sensenbrenner M. 1990. Reactive astrogliosis after basic fibroblast growth factor (bFGF) injection in the injured rat brain. Glia 3: 502-509.

Elde R, Cao YH, Cintra A, Brelje TC, Pelto-Huikko M, et al. 1991. Prominent expression of acidic fibroblast growth factor in motor and sensory neurons. Neuron 7: 349-364.

Ellsworth JL, Garcia R, Yu J, Kindy MS. 2003. Fibroblast growth factor-18 reduced infarct volumes and behavioral deficits after transient occlusion of the middle cerebral artery in rats. Stroke 34: 1507-1512.

Ellsworth JL, Garcia R, Yu J, Kindy MS. 2004. Time window of fibroblast growth factor-18-mediated neuroprotection after occlusion of the middle cerebral artery in rats. J Cereb Blood Flow Metab 24: 114-123.

Enomoto M, Shinomiya K, Okabe S. 2003. Migration and differentiation of neural progenitor cells from two different regions of embryonic central nervous system after transplantation into the intact spinal cord. Eur J Neurosci 17: 1223-1232.

Eriksson AE, Cousens LS, Weaver LH, Matthews BW. 1991. Three-dimensional structure of human basic fibroblast growth factor. Proc Natl Acad Sci USA 88: 3441-3445.

Ernfors P, Lonnerberg P, Ayer-LeLievre C, Persson H. 1990. Developmental and regional expression of basic fibroblast growth factor mRNA in the rat central nervous system. J Neurosci Res 27: 10-15.

Eubanks JH, Perez-Velazquez JL, Kerr RG, Carlen PL, Mills LR, et al. 1996. Three distinct neuronal phenotypes exist in embryonic rat hippocampal neurons cultured in basic fibroblast growth factor. Neurosci Lett 204: 5-8.

Faham S, Linhardt RJ, Rees DC. 1998. Diversity does make a difference: fibroblast growth factor-heparin interactions. Curr Opin Struct Biol 8: 578-586.

Faux CH, Turnley AM, Epa R, Cappai R, Bartlett PF. 2001. Interactions between fibroblast growth factors and Notch regulate neuronal differentiation. J Neurosci 21: 5587-5596.

Feldman B, Poueymirou W, Papaioannou VE, DeChiara TM, Goldfarb M. 1995. Requirement of FGF-4 for postimplantation mouse development. Science 267: 246-249.

Fiore F, Planche J, Gibier P, Sebille A, deLapeyriere O, et al. 1997. Apparent normal phenotype of Fgf6−/− mice. Int J Dev Biol 41: 639-642.

Fiore F, Sebille A, Birnbaum D. 2000. Skeletal muscle regeneration is not impaired in Fgf6−/− mutant mice. Biochem Biophys Res Commun 272: 138-143.

Floss T, Arnold HH, Braun T. 1997. A role for FGF-6 in skeletal muscle regeneration. Genes Dev 11: 2040-2051.

Frautschy SA, Walicke PA, Baird A. 1991. Localization of basic fibroblast growth factor and its mRNA after CNS injury. Brain Res 553: 291-299.

Friesel R, Burgess WH, Mehlman T, Maciag T. 1986. The characterization of the receptor for endothelial cell growth factor by covalent ligand attachment. J Biol Chem 261: 7581-7584.

Friesel R, Maciag T. 1999. Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling. Thromb Haemost 82: 748-754.

Friesel RE, Maciag T. 1995. Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction. FASEB J 9: 919-925.

Fuhrmann V, Kinkl N, Leveillard T, Sahel J, Hicks D. 1999. Fibroblast growth factor receptor 4 (FGFR4) is expressed in adult rat and human retinal photoreceptors and neurons. J Mol Neurosci 13: 187-197.

Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, et al. 1995. Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci USA 92: 11879-11883.

Goddard DR, Berry M, Kirvell SL, Butt AM. 2001. Fibroblast growth factor-2 inhibits myelin production by oligodendrocytes in vivo. Mol Cell Neurosci 18: 557-569.

Goddard DR, Berry M, Kirvell SL, Butt AM. 2002. Fibroblast growth factor-2 induces astroglial and microglial reactivity in vivo. J Anat 200: 57-67.

Goldman SA, Nedergaard M, Crystal RG, Fraser RA, Goodman R, et al. 1997. Neural precursors and neuronal production in the adult mammalian forebrain. Ann N Y Acad Sci 835: 30-55.

Gomes FC, Paulin D, Moura Neto V. 1999. Glial fibrillary acidic protein (GFAP): modulation by growth factors and its implication in astrocyte differentiation. Braz J Med Biol Res 32: 619-631.

Gomez-Pinilla F, Cotman CW. 1993. Distribution of fibroblast growth factor 5 mRNA in the rat brain: an in situ hybridization study. Brain Res 606: 79-86.

Gomez-Pinilla F, Lee JW, Cotman CW. 1992. Basic FGF in adult rat brain: cellular distribution and response to entorhinal lesion and fimbria–fornix transection. J Neurosci 12: 345-355.

Gomez-Pinilla F, Lee JW, Cotman CW. 1994. Distribution of basic fibroblast growth factor in the developing rat brain. Neuroscience 61: 911-923.

Gonzalez AM, Logan A, Ying W, Lappi DA, Berry M, et al. 1994. Fibroblast growth factor in the hypothalamic–pituitary axis: differential expression of fibroblast growth factor-2 and a high affinity receptor. Endocrinology 134: 2289-2297.

Gremo F, Presta M. 2000. Role of fibroblast growth factor-2 in human brain: a focus on development. Int J Dev Neurosci 18: 271-279.

Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, et al. 1996. Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. J Neurosci 16: 1091-1100.

Grothe C, Janet T. 1995. Expression of FGF-2 and FGF receptor type 1 in the adult rat brainstem: effect of colchicine. J Comp Neurol 353: 18-24.

Grothe C, Meisinger C, Holzschuh J, Wewetzer K, Cattini P. 1998. Over-expression of the 18 kD and 21/23 kD fibroblast growth factor-2 isoforms in PC12 cells and Schwann cells results in altered cell morphology and growth. Mol Brain Res 57: 97-105.

Grothe C, Nikkhah G. 2001. The role of basic fibroblast growth factor in peripheral nerve regeneration. Anat Embryol (Berl) 204: 17-171.

Grothe C, Timmer M, Scholz T, Winkler C, Nikkah G, et al. 2004. Fibroblast growth factor-20 promotes the differentiation of Nurr1-overexpressing neural stem cells into tyrosine hydroxylase-positive neurons. Neurobiol Dis 17: 163-170.

Grothe C, Wewetzer K. 1996. Fibroblast growth factor and its implications for developing and regenerating neurons. Int J Dev Biol 40: 403-410.

Grothe C, Zachmann K, Unsicker K. 1991. Basic FGF-like immunoreactivity in the developing and adult rat brainstem. J Comp Neurol 305: 328-336.

Guo L, Degenstein L, Fuchs E. 1996. Keratinocyte growth factor is required for hair development but not for wound healing. Genes Dev 10: 165-175.

Guo ZH, Mattson MP. 2000. Neurotrophic factors protect cortical synaptic terminals against amyloid and oxidative stress-induced impairment of glucose transport, glutamate transport and mitochondrial function. Cereb Cortex 10: 50-57.

Gwinn RP, Kondratyev A, Gale K. 2002. Time-dependent increase in basic fibroblast growth factor protein in limbic regions following electroshock seizures. Neuroscience 114: 403-409.

Hartung H, Feldman B, Lovec H, Coulier F, Birnbaum D, et al. 1997. Murine FGF-12 and FGF-13: expression in embryonic nervous system, connective tissue and heart. Mech Dev 64: 31-39.

Hattori Y, Yamasaki M, Konishi M, Itoh N. 1997. Spatially restricted expression of fibroblast growth factor-10 mRNA in the rat brain. Brain Res Mol Brain Res 47: 139-146.

Haynes LW. 1988. Fibroblast (heparin-binding) growing factors in neuronal development and repair. Mol Neurobiol 2: 263-289.

Hebert JM, Rosenquist T, Gotz J, Martin GR. 1994. FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. Cell 78: 1017-1025.

Hisajima H, Saito H, Abe K, Nishiyama N. 1992. Effects of acidic fibroblast growth factor on hippocampal long-term potentiation in fasted rats. J Neurosci Res 31: 549-553.

Holzschuh J, Hauptmann G, Driever W. 2003. Genetic analysis of the roles of Hh, FGF8, and nodal signaling during catecholaminergic system development in the zebrafish brain. J Neurosci 23: 5507-5519.

Hashimoto M, Sagara Y, Langford D, Everall IP, Mallory M, Everson A, Digicaylioglu M, Masliah E. 2002. Fibroblast growth factor 1 regulates signalling via the glycogen synthase kinase-3beta pathway, Implications for neuroprotection. J Biol Chem 277: 32985-32991.

Hoshikawa M, Ohbayashi N, Yonamine A, Konishi M, Ozaki K, Fukui S, Itoh N. 1998. Structure and expression of a novel fibroblast growth factor, FGF-17, preferentially expressed in the embryonic brain. Biochem Biophys Res Commun 244: 187-191.

Hoshikawa M, Yonamine A, Konishi M, Itoh N. 2002. FGF-18 is a neuron-derived glial cell growth factor expressed in the rat brain during early postnatal development. Brain Res Mol Brain Res 105: 60-66.

Hossain MA, Russell JC, Gomez R, Laterra J, Gomes R. 2002. Neuroprotection by scatter factor/hepatocyte growth factor and FGF-1 in cerebellar granule neurons is phosphatidylinositol 3-kinase/akt-dependent and MAPK/CREB-independent. J Neurochem 81: 365-378.

Humpel C, Lippoldt A, Chadi G, Ganten D, Olson L, et al. 1993. Fast and widespread increase of basic fibroblast growth factor messenger RNA and protein in the forebrain after kainate-induced seizures. Neuroscience 57: 913-922.

Ikonomidou C, Turski L. 1995. Excitotoxicity and neurodegenerative diseases. Curr Opin Neurol 8: 487-497.

Ikonomidou C, Turski L. 1996. Neurodegenerative disorders: clues from glutamate and energy metabolism. Crit Rev Neurobiol 10: 239-263.

Ishihara A, Saito H, Nishiyama N. 1992. Basic fibroblast growth factor ameliorates learning deficits in basal forebrain-lesioned mice. Jpn J Pharmacol 59: 7-13.

Ishiyama J, Saito H, Abe K. 1991. Epidermal growth factor and basic fibroblast growth factor promote the generation of long-term potentiation in the dentate gyrus of anaesthetized rats. Neurosci Res 12: 403-411.

Itoh N, Yazaki N, Tagashira S, Miyake A, Ozaki K, et al. 1994. Rat FGF receptor-4 mRNA in the brain is expressed preferentially in the medial habenular nucleus. Brain Res Mol Brain Res 21: 344-348.

Johnson DE, Lee PL, Lu J, Williams LT. 1990. Diverse forms of a receptor for acidic and basic fibroblast growth factors. Mol Cell Biol 10: 4728-4736.

Johnson DE, Lu J, Chen H, Werner S, Williams LT. 1991. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. Mol Cell Biol 11: 4627-4634.

Johnson DE, Williams LT. 1993. Structural and functional diversity in the FGF receptor multigene family. Adv Cancer Res 60: 1-41.

Joy A, Moffett J, Neary K, Mordechai E, Stachowiak EK, et al. 1997. Nuclear accumulation of FGF-2 is associated with proliferation of human astrocytes and glioma cells. Oncogene 14: 171-183.

Jungnickel J, Claus P, Gransalke K, Timmer M, Grothe C. 2004. Targeted disruption of the FGF-2 gene affects the response to peripheral nerve injury. Mol Cell Neurosci 25: 444-452.

Kalcheim C. 1996. The role of neurotrophins in development of neural-crest cells that become sensory ganglia. Philos Trans R Soc Lond B Biol Sci 351: 375-381.

Kalil K, Szebenyi G, Dent EW. 2000. Common mechanisms underlying growth cone guidance and axon branching. J Neurobiol 44: 145-158.

Kalynchuk LE. 2000. Long-term amygdala kindling in rats as a model for the study of interictal emotionality in temporal lobe epilepsy. Neurosci Biobehav Rev 24: 691-704.

Kelley MJ, Pech M, Seuanez HN, Rubin JS, O'Brien SJ, et al. 1992. Emergence of the keratinocyte growth factor multigene family during the great ape radiation. Proc Natl Acad Sci USA 89: 9287-9291.

Kiefer P, Acland P, Pappin D, Peters G, Dickson C. 1994. Competition between nuclear localization and secretory signals determines the subcellular fate of a single CUG-initiated form of FGF3. EMBO J 13: 4126-4136.

Kiessling M, Gass P. 1994. Stimulus–transcription coupling in focal cerebral ischemia. Brain Pathol 4: 77-83.

Kim TE, Lee HS, Lee YB, Hong SH, Lee YS, et al. 2003. Sonic hedgehog and FGF8 collaborate to induce dopaminergic phenotypes in the Nurr1-overexpressing neural stem cell. Biochem Biophys Res Commun 305: 1040-1048.

Kiprianova I, Schindowski K, von Bohlen und Halbach O, Krause S, Dono R, et al. 2004. Enlarged infract volume and loss of BDNF mRNA induction following brain ischemia in mice lacking FGF-2. Exp Neurol 189: 252-260.

Kirino T. 2000. Delayed neuronal death. Neuropathology 20: 95-97.

Korada S, Zheng W, Basilico C, Schwartz ML, Vaccarino FM. 2002. Fibroblast growth factor 2 is necessary for the growth of glutamate projection neurons in the anterior neocortex. J Neuroscience 22: 863-875.

Kornbluth S, Paulson KE, Hanafusa H. 1988. Novel tyrosine kinase identified by phosphotyrosine antibody screening of cDNA libraries. Mol Cell Biol 8: 5541-5544.

Kouhara H, Hadari YR, Spivak-Kroizman T, Schilling J, Bar-Sagi D, et al. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. Cell 89: 693-702.

Krieglstein K, Reuss B, Maysinger D, Unsicker K. 1998. Transforming growth factor-beta mediates the neurotrophic effect of fibroblast growth factor-2 on midbrain dopaminergic neurons. Eur J Neurosci 10: 2746-2750.

Langren E, Klint P, Landgren E und Claesson-Welsh L. 1998. Fibroblast growth factor receptor-1 mediates chemotaxis independently of direct SH2-domain protein binding. Oncogene 17: 283-291.

Larsson H, Klint P, Landgren E, Claesson-Welsh L. 1999. Fibroblast growth factor receptor-1-mediated endothelial cell proliferation is dependent on the Src homology (SH) 2/SH3 domain-containing adaptor protein Crk. J Biol Chem 274: 25726-25734.

Le R, Esquenazi S. 2002. Astrocytes mediate cerebral cortical neuronal axon and dendrite growth, in part, by release of fibroblast growth factor. Neurol Res 24: 81-92.

Lee PL, Johnson DE, Cousens LS, Fried VA, Williams LT. 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science 245: 57-60.

Lenhard T, Schober A, Suter-Crazzolara C, Unsicker K. 2002. Fibroblast growth factor-2 requires glial-cell-line-derived neurotrophic factor for exerting its neuroprotective actions on glutamate-lesioned hippocampal neurons. Mol Cell Neurosci 20: 181-197.

Leung DSY, Unsicker K, Reuss B. 2001. Gap junctions modulate survival-promoting effects of fibroblast growth factor-2 on cultured midbrain dopaminergic neurons. Mol Cell Neurosci 18: 44-55.

Li L, Donly C, Li Q, Willis LG, Keddie BA, Erlandson MA, Theilmann DA. 2002. Identification and genomic analysis of a second species of nucleopolyhedrovirus isolated from *Mamestra configurata*. Virology 297: 226-244.

Li Q, Stephenson D. 2002. Postischemic administration of basic fibroblast growth factor improves sensorimotor function and reduces infarct size following permanent focal cerebral ischemia in the rat. Exp Neurol 177: 531-537.

Liu Z, D'Amore PA, Mikati M, Gatt A, Holmes GL. 1993. Neuroprotective effect of chronic infusion of basic fibroblast growth factor on seizure-associated hippocampal damage. Brain Res 626: 335-338.

Liu Z, Holmes GL. 1997a. Basic fibroblast growth factor is highly neuroprotective against seizure-induced long-term behavioural deficits. Neuroscience 76: 1129-1138.

Liu Z, Holmes GL. 1997b. Basic fibroblast growth factor-induced seizures in rats. Neurosci Lett 233: 85-88.

Liu X, Zhu XZ. 1999. Increased expression and nuclear accumulation of basic fibroblast growth factor in primary cultured astrocytes following ischemic-like insults. Brain Res Mol Brain Res 71: 171-177.

Ma J, Qiu J, Hirt L, Dalkara T, Moskowitz MA. 2001. Synergistic protective effect of caspase inhibitors and bFGF against brain injury induced by transient focal ischaemia. Br J Pharmacol 133: 345-350.

Magy L, Keita M, Richard L, Piaser M, Vallat JM. 2003. Transient exposure to FGF2 enhances myelination in embryonic brain cell cocultures. Exp Neurol 181: 17-24.

Mahmood R, Mason IJ, Morriss-Kay GM. 1996. Expression of Fgf-3 in relation to hindbrain segmentation, otic pit position and pharyngeal arch morphology in normal and retinoic acid-exposed mouse embryos. Anat Embryol (Berl) 194: 13-22.

Mansour SL, Goddard JM, Capecchi MR. 1993. Mice homozygous for a targeted disruption of the proto-oncogene int-2 have developmental defects in the tail and inner ear. Development 117: 13-28.

Maric D, Maric I, Chang YH, Barker JL. 2003. Prospective cell sorting of embryonic rat neural stem cells and neuronal and glial progenitors reveals selective effects of basic fibroblast growth factor and epidermal growth factor on self-renewal and differentiation. J Neurosci 23: 240-251.

Mark RJ, Fuson KS, Keane-Lazar K, May PC. 1999. Fibroblast growth factor-8 protects cultured rat hippocampal neurons from oxidative insult. Brain Res 830: 88-93.

Martinez G, Di Giacomo C, Sorrenti V, Camazza ML, Ragusa N, et al. 2001. Fibroblast growth factor-2 and transforming growth factor beta 1 immunostaining in rat brain after cerebral postischemic reperfusion. J Neurosci Res 63: 136-142.

Martone ME, Hu BR, Ellisman MH. 2000. Alterations of hippocampal postsynaptic densities following transient ischemia. Hippocampus 10: 610-616.

Mason I, Chambers D, Shamim H, Walshe J, Irving C. 2000. Regulation and function of FGF8 in patterning of midbrain and anterior hindbrain. Biochem Cell Biol 78: 577-584.

Mason IJ, Fuller-Pace F, Smith R, Dickson C. 1994. FGF-7 (keratinocyte growth factor) expression during mouse

development suggests roles in myogenesis, forebrain regionalisation and epithelial-mesenchymal interactions. Mech Dev 45: 15-30.

Masumara M, Murayama N, Inoue T, Ohno T. 1996. Selective induction of fibroblast growth factor receptor-1 mRNA after transient focal ischemia in the cerebral cortex of rats. Neurosci Lett 213: 119-122.

Matsuoka N, Nozaki K, Takagi Y, Nishimura M, Hayashi J, et al. 2003. Adenovirus-mediated gene transfer of fibroblast growth factor-2 increases BrdU-positive cells after forebrain ischemia in gerbils. Stroke 34: 1519-1525.

Mattson MP, Kumar KN, Wang H, Cheng B, Michaelis EK. 1993. Basic FGF regulates the expression of a functional 71 kDa NMDA receptor protein that mediates calcium influx and neurotoxicity in hippocampal neurons. J Neurosci 13: 4575-4588.

Mattson MP, Murrain M, Guthrie PB, Kater SB. 1989. Fibroblast growth factor and glutamate: opposing roles in the generation and degeneration of hippocampal neuroarchitecture. J Neurosci 9: 3728-3740.

McWhirter JR, Goulding M, Weiner JA, Chun J, Murre C. 1997. A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. Development 124: 3221-3232.

Mignatti P, Morimoto T, Rifkin DB. 1992. Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum–Golgi complex. J Cell Physiol 151: 81-93.

Miller DL, Ortega S, Bashayan O, Basch R, Basilico C. 2000. Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. Mol Cell Biol 20: 2260-2268.

Miyagawa T, Saito H, Nishiyama N. 1993. Branching enhancement by basic fibroblast growth factor in cut neurite of hippocampal neurons. Neurosci Lett 153: 29-31.

Miyakawa K, Hatsuzawa K, Kurokawa T, Asada M, Kuroiwa T, et al. 1999. A hydrophobic region locating at the center of fibroblast growth factor-9 is crucial for its secretion. J Biol Chem 274: 29352-29357.

Miyake A, Hattori Y, Ohta M, Itoh N. 1996. Rat oligodendrocytes and astrocytes preferentially express fibroblast growth factor receptor-2 and -3 mRNAs. J Neurosci Res 45: 534-541.

Miyake A, Konishi M, Martin FH, Hernday NA, Ozaki K, et al. 1998. Structure and expression of a novel member, FGF-16, on the fibroblast growth factor family. Biochem Biophys Res Commun 243: 148-152.

Miyamoto M, Naruo K, Seko C, Matsumoto S, Kondo T, et al. 1993. Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. Mol Cell Biol 13: 4251-4259.

Mocchetti I, Wrathall JR. 1995. Neurotrophic factors in central nervous system trauma. J Neurotrauma 12: 853-870.

Moenner M, Chevallier B, Badet J, Barritault D. 1986. Evidence and characterization of the receptor to eye-derived growth factor I, the retinal form of basic fibroblast growth factor, on bovine epithelial lens cells. Proc Natl Acad Sci USA 83: 5024-5028.

Mohammadi M, Dionne CA, Li W, Li N, Spivak T, et al. 1992. Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature 358: 681-684.

Mohammadi M, Honegger AM, Rotin D, Fischer R, Bellot F, et al. 1991. A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. Mol Cell Biol 11: 5068-5078.

Mohammadi M, Schlessinger J, Hubbard SR. 1996. Structure of the FGF receptor tyrosine kinase domain reveals a novel autoinhibitory mechanism. Cell 86: 577-587.

Molteni R, Fumagalli F, Magnaghi V, Roceri M, Gennarelli M, et al. 2001. Modulation of fibroblast growth factor-2 by stress and corticosteroids: from developmental events to adult brain plasticity. Brain Res Brain Res Rev 37: 249-258.

Morrison RS, Sharma A, DeVellis J, Bradshaw RA. 1986. Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. Proc Natl Acad Scie USA 83: 7537-7541.

Moscatelli D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. J Cell Physiol 131: 123-130.

Moy FJ, Seddon AP, Bohlen P, Powers R. 1996. High-resolution solution structure of basic fibroblast growth factor determined by multidimensional heteronuclear magnetic resonance spectroscopy. Biochemistry 35: 13552-13561.

Mufson EJ, Kroin JS, Sendera TJ, Sobreviela T. 1999. Distribution and retrograde transport of trophic factors in the central nervous system: functional implications for the treatment of neurodegenerative diseases. Prog Neurobiol 57: 451-484.

Muslin AJ, Peters KG, Williams LT. 1994. Direct activation of phospholipase C-gamma by fibroblast growth factor receptor is not required for mesoderm induction in Xenopus animal caps. Mol Cell Biol 14: 3006-3012.

Nakagami Y, Saito H, Matsuki N. 1997. Basic fibroblast growth factor and brain-derived neurotrophic factor promote survival and neuronal circuit formation in organotypic hippocampal culture. Jpn J Pharmacol 75: 319-326.

Nakamura S, Todo T, Motoi Y, Haga S, Aizawa T, et al. 1999. Glial expression of fibroblast growth factor-9 in rat central nervous system. Glia 28: 53-65.

Nakata N, Kato H, Kogure K. 1993. Protective effects of basic fibroblast growth factor against hippocampal neuronal damage following cerebral ischemia in the gerbil. Brain Res 605: 354-356.

Namaka MP, Sawchuk M, MacDonald SC, Jordan LM, Hochman S. 2001. Neurogenesis in postnatal mouse dorsal root ganglia. Exp Neurol 172: 60-69.

Neufeld G, Gospodarowicz D. 1985. The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells. J Biol Chem 260: 13860-13868.

Neufeld G, Gospodarowicz D. 1986. Basic and acidic fibroblast growth factors interact with the same cell surface receptors. J Biol Chem 261: 5631-5637.

Ohmachi S, Watanabe Y, Mikami T, Kusu N, Ibi T, et al. 2000. FGF-20, a novel neurotrophic factor, preferentially expressed in the substantia nigra pars compacta of rat brain. Biochem Biophys Res Commun 277: 355-360.

Olney JW, Collins RC, Sloviter RS. 1986. Excitotoxic mechanisms of epileptic brain damage. Adv Neurol 44: 857-877.

Ong SH, Guy GR, Hadari YR, Laks S, Gotoh N, et al. 2000. FRS2 proteins recruit intracellular signaling pathways by binding to diverse targets on fibroblast growth factor and nerve growth factor receptors. Mol Cell Biol 20: 979-989.

Ornitz DM. 2000. FGFs, heparan sulfate and FGFRs: complex interactions essential for development. Bioessays 22: 108-112.

Ornitz DM, Itoh N. 2001. Fibroblast growth factors. Genome Biol 2: 3005.1-3005.12.

Ornitz DM, Xu J, Colvin JS, McEwen DG, MacArthur CA, et al. 1996. Receptor specificity of the fibroblast growth factor family. J Biol Chem 271: 15292-15297.

Otto D, Frotscher M, Unsicker K. 1989. Basic fibroblast growth factor and nerve growth factor administered in gel foam rescue medial septal neurons after fimbria fornix transection. J Neurosci Res 22: 83-91.

Otto D, Unsicker K. 1990. Basic FGF reverses chemical and morphological deficits in the nigrostriatal system of MPTP-treated mice. J Neurosci 10: 1912-1921.

Otto D, Unsicker K. 1993a. FGF-2-mediated protection of cultured mesencephalic dopaminergic neurons against MPTP and MPP+: specificity and impact of culture conditions, non-dopaminergic neurons, and astroglial cells. J Neurosci Res 34: 382-393.

Otto D, Unsicker K. 1993b. FGF-2 modulates dopamine and dopamine-related striatal transmitter systems in the intact and MPTP-lesioned mouse. Eur J Neurosci 5: 927-932.

Ozawa K, Uruno T, Miyakawa K, Seo M, Imamura T. 1996. Expression of the fibroblast growth factor family and their receptor family genes during mouse brain development. Brain Res Mol Brain Res 41: 279-288.

Ozawa K, Suzuki S, Asada M, Tomooka Y, Li AJ, et al. 1998. An alternatively spliced fibroblast growth factor (FGF)-5 mRNA is abundant in brain and translates into a partial agonist/antagonist for FGF-5 neurotrophic activity. J Biol Chem 273: 29262-29271.

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. 1999. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci 19: 8487-8497.

Patel MN, McNamara JO. 1995. Selective enhancement of axonal branching of cultured dentate gyrus neurons by neurotrophic factors. Neuroscience 69: 763-770.

Pellegrini L, Burke DF, von Delft F, Mulloy B, Blundell TL. 2000. Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. Nature 407: 1029-1034.

Perkins LA, Cain LD. 1995. Basic fibroblast growth factor (bFGF) increases the survival of embryonic and postnatal basal forebrain cholinergic neurons in primary culture. Int J Dev Neurosci 13: 51-61.

Perraud F, Labourdette G, Eclancher F, Sensenbrenner M. 1990. Primary cultures of astrocytes from different brain areas of newborn rats and effects of basic fibroblast growth factor. Dev Neurosci 12: 11-21.

Perrone-Capano C, Di Porzio U. 2000. Genetic and epigenetic control of midbrain dopaminergic neuron development. Int J Dev Biol 44: 679-687.

Peters KG, Marie J, Wilson E, Ives HE, Escobedo J, et al. 1992. Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca2+ flux but not mitogenesis. Nature 358: 678-681.

Plotnikov AN, Hubbard SR, Schlessinger J, Mohammadi M. 2000. Crystal structures of two FGF–FGFR complexes reveal the determinants of ligand-receptor specificity. Cell 101: 413-424.

Powers CJ, McLeskey SW, Wellstein A. 2000. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer 7: 165-197.

Qiao J, Uzzo R, Obara-Ishihara T, Degenstein L, Fuchs E, et al. 1999. FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. Development 126: 547-554.

Raballo R, Rhee J, Lyn-Cook R, Leckman JF, Schwartz ML, et al. 2000. Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. J Neurosci 20: 5012-5023.

Raffioni S, Thomas D, Foehr ED, Thompson LM, Bradshaw RA. 1999. Comparison of the intracellular signaling

responses by three chimeric fibroblast growth factor receptors in PC12 cells. Proc Natl Acad Sci USA 96: 7178-7183.

Ramirez JJ, Finklestein SP, Keller J, Abrams W, George MN, et al. 1999. Basic fibroblast growth factor enhances axonal sprouting after cortical injury in rats. Neuroreport 10: 1201-1204.

Ray J, Gage FH. 1994. Spinal cord neuroblasts proliferate in response to basic fibroblast growth factor. J Neurosci 14: 3548-3564.

Reilly JF, Maher PA, Kumari VG. 1998. Regulation of astrocyte GFAP expression by TGF-beta1 and FGF-2. Glia 22: 202-210.

Reuss B, Dermietzel R, Unsicker K. 1998. Fibroblast growth factor 2 (FGF-2) differentially regulates connexin (cx) 43 expression and function in astroglial cells from distinct brain regions. Glia 22: 19-30.

Reuss B, Hertel M, Werner S, Unsicker K. 2000a. Fibroblast growth factors-5 and -9 distinctly regulate expression and function of the gap junction protein connexin43 in cultured astroglial cells from different brain regions. Glia 30: 231-241.

Reuss B, Leung DS, Ohlemeyer C, Kettenmann H, Unsicker K. 2000b. Regionally distinct regulation of astroglial neurotransmitter receptors by fibroblast growth factor-2. Mol Cell Neurosci 16: 42-58.

Reuss B, Unsicker K. 2000. Survival and differentiation of dopaminergic mesencephalic neurons are promoted by dopamine-mediated induction of FGF-2 in striatal astroglial cells. Mol Cell Neurosci 16: 781-792.

Reuss B, von Bohlen und Halbach O. 2003. Fibroblast growth factors and their receptors in the central nervous system. Cell Tissue Res 313: 139-157.

Revest JM, DeMoerlooze L, Dickson C. 2000. Fibroblast growth factor 9 secretion is mediated by a non-cleaved amino-terminal signal sequence. J Biol Chem 275: 8083-8090.

Reynolds BA, Tetzlaff W, Weiss S. 1992. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12: 4565-4574.

Riva MA, Donati E, Tascedda F, Zolli M, Racagni G. 1994. Short- and long-term induction of basic fibroblast growth factor gene expression in rat central nervous system following kainate injection. Neuroscience 59: 55-65.

Riva MA, Gale K, Mocchetti I. 1992. Basic fibroblast growth factor mRNA increases in specific brain regions following convulsive seizures. Brain Res Mol Brain Res 15: 311-318.

Rothman SM, Olney JW. 1986. Glutamate and the pathophysiology of hypoxic–ischemic brain damage. Ann Neurol 19: 105-111.

Ruta M, Burgess W, Givol D, Epstein J, Neiger N, et al. 1989. Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the fms-like gene (FLG). Proc Natl Acad Sci USA 86: 8722-8726.

Sadohara T, Sugahara K, Urashima Y, Terasaki H, Lyama K. 2001. Keratinocyte growth factor prevents ischemia-induced delayed neuronal death in the hippocampal CA1 field of the gerbil brain. Neuroreport 12: 71-76.

Sah DW, Ray J, Gage FH. 1997. Regulation of voltage- and ligand-gated currents in rat hippocampal progenitor cells in vitro. J Neurobiol 32: 95-110.

Sasaki K, Oomura Y, Figurov A, Yagi H. 1994. Acidic fibroblast growth factor facilitates generation of long-term potentiation in rat hippocampal slices. Brain Res Bull 33: 505-511.

Sasaki K, Oomura Y, Suzuki K, Hanai K, Yagi H. 1992. Acidic fibroblast growth factor prevents death of hippocampal CA1 pyramidal cells following ischemia. Neurochem Int 21: 397-402.

Sasaki K, Tooyama I, Li AJ, Oomura Y, Kimura H. 1999. Effects of an acidic fibroblast growth factor fragment analog on learning and memory and on medial septum cholinergic neurons in senescence-accelerated mice. Neuroscience 92: 1287-1294.

Schmitt JF, Hearn MT, Risbridger GP. 1996. Expression of fibroblast growth factor-8 in adult rat tissues and human prostate carcinoma cells. J Steroid Biochem Mol Biol 57: 173-178.

Seddon AP, Yayon A. 1994. Diminished heparin binding of a basic fibroblast growth factor mutant is associated with reduced receptor binding, mitogenesis, plasminogen activator induction, and in vitro angiogenesis. Biochemistry 33: 10999-11007.

Sievers J, Hausmann B, Unsicker K, Berry M. 1987. Fibroblast growth factors promote the survival of adult rat retinal ganglion cells after transection of the optic nerve. Neurosci Lett. 76: 157-162.

Simonato M, Molteni R, Bregola G, Muzzolini A, Piffanelli M, et al. 1998. Different patterns of induction of FGF-2, FGF-1 and BDNF mRNAs during kindling epileptogenesis in the rat. Eur J Neurosci 10: 955-963.

Song BW, Vinters HV, Wu D, Pardridge WM. 2002. Enhanced neuroprotective effects of basic fibroblast growth factor in regional brain ischemia after conjugation to a blood–brain barrier delivery vector. J Pharmacol Exp Ther 301: 605-610.

Spivak-Kroizman T, Mohammadi M, Hu P, Jaye M, Schlessinger J, et al. 1994b. Point mutation in the fibroblast growth factor receptor eliminates phosphatidylinositol hydrolysis without affecting neuronal differentiation of PC12 cells. J Biol Chem 269: 14419-14423.

Stauber DJ, DiGabriele AD, Hendrickson WA. 2000. Structural interactions of fibroblast growth factor receptor with its ligands. Proc Natl Acad Sci USA 97: 49-54.

Stachowiak MK, Moffett J, Maher P, Tucholski J, Stachowiak EK. 1997. Growth factor regulation of cell growth and proliferation in the nervous system. A new intracrine nuclear mechanism. Mol Neurobiol 15: 257-283.

Stachowiak EK, Fang X, Myers J, Dunham S, Stachowiak MK. 2003. cAMP-induced differentiation of human neuronal progenitor cells is mediated by nuclear fibroblast growth factor receptor-1 (FGFR1). J Neurochem 84: 1296-1312.

Stock A, Kuzis K, Woodward WR, Nishi R, Eckenstein FP. 1992. Localization of acidic fibroblast growth factor in specific subcortical neuronal populations. J Neurosci 12: 4688-4700.

Sun X, Mariani FV, Martin GR. 2002. Functions of FGF signalling from the apical ectodermal ridge in limb development. Nature 418: 501-508.

Sun X, Meyers EN, Lewandoski M, Martin GR. 1999. Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. Genes Dev 13: 1834-1846.

Sutherland D, Samakovlis C, Krasnow MA. 1996. Branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. Cell 87: 1091-1101.

Szebenyi G, Callaway JL, Dent EW, Kalil K. 1998. Interstitial branches develop from active regions of the axon demarcated by the primary growth cone during pausing behaviors. J Neurosci 18: 7930-7940.

Szebenyi G, Dent EW, Callaway JL, Seys C, Lueth H, et al. 2001. Fibroblast growth factor-2 promotes axon branching of cortical neurons by influencing morphology and behavior of the primary growth cone. J Neurosci 21: 3932-3941.

Tagashira S, Ozaki K, Ohta M, Itoh N. 1995. Localization of fibroblast growth factor-9 mRNA in the rat brain. Brain Res Mol Brain Res 30: 233-241.

Takami K, Matsuo A, Terai K, Walker DG, McGeer EG, et al. 1998. Fibroblast growth factor receptor-1 expression in the cortex and hippocampus in Alzheimer's disease. Brain Res 802: 89-97.

Timmer M, Muller-Ostermeyer F, Kloth V, Winkler C, Grothe C, et al. 2004. Enhanced survival, reinnervation, and functional recovery of intrastriatal dopamine grafts cotransplanted with Schwann cells overexpressing high molecular weight FGF-2 isoforms. Exp Neurol 187: 118-136.

Timmer M, Robben S, Muller-Ostermeyer F, Nikkah G, Grothe C. 2003. Axonal regeneration across long gaps in silicone chambers filled with Schwann cells overexpressing high molecular weight FGF-2. Cell Transplant 12: 265-277.

Tooyama I, Walker D, Yamada T, Hanai K, Kimura H, McGeer EG, McGeer PL. 1992. High molecular weight basic fibroblast growth factor-like protein is localized to a subpopulation of mesencephalic dopaminergic neurons in the rat brain. Brain Res 593: 274-280.

Unsicker K, Reichert-Preibsch H, Schmidt R, Pettmann B, Labourdette G, Sensenbrenner M. 1987. Astroglial and fibroblast growth factors have neurotrophic functions for cultured peripheral and central nervous system neurons. Proc Natl Acad Sci USA 84: 5459-5463.

Vaccarino FM, Ganat Y, Zhang Y, Zheng W. 2001. Stem cells in neurodevelopment and plasticity. Neuropsychopharmacology 25: 805-815.

Vaccarino FM, Schwartz ML, Raballo R, Rhee J, Lyn-Cook R. 1999. Fibroblast growth factor signaling regulates growth and morphogenesis at multiple steps during brain development. Curr Top Dev Biol 46: 179-200.

Vainikka S, Partanen J, Bellosta P, Coulier F, Birnbaum D, et al. 1992. Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. EMBO J 11: 4273-4280.

Venkataraman G, Raman R, Sasikharan V, Sasikharan R. 1999. Fibroblast growth factors 1 and 2 are distinct in oligomerization in the presence of heparin-like glycosaminoglycans. Proc Natl Acad Sci USA 96: 1892-1897.

Vescovi AL, Reynolds BA, Fraser DD, Weiss S. 1993. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron 11: 951-966.

Vicario-Abejon C, Johe KK, Hazel TG, Collazo D, McKay RD. 1995. Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. Neuron 15: 105-114.

von Bohlen und Halbach O, Dermietzel R. 2002. Neurotransmitters and neuromodulators. Weinheim: Wiley-VCH.

Wagner JP, Black IB, DiCicco-Bloom E. 1999. Stimulation of neonatal and adult brain neurogenesis by subcutaneous injection of basic fibroblast growth factor. J Neurosci 19: 6006-6016.

Walicke P, Cowan WM, Ueno N, Baird A, Guillemin R. 1986. Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc Natl Acad Sci USA 83: 3012-3016.

Wang JK, Xu H, Li HC, Goldfarb M. 1996. Broadly expressed SNT-like proteins link FGF receptor stimulation to activators of Ras. Oncogene 13: 721-729.

Wang Q, McEwen DG, Ornitz DM. 2000. Subcellular and developmental expression of alternatively spliced forms of fibroblast growth factor 14. Mech Dev 90: 283-287.

Wei OY, Huang YL, Da CD, Cheng JS. 2000. Alteration of basic fibroblast growth factor expression in rat during cerebral ischemia. Acta Pharmacol Sin 21: 296-300.

Wilcox BJ, Unnerstall JR. 1991. Expression of acidic fibroblast growth factor mRNA in the developing and adult rat brain. Neuron 6: 397-409.

Wright TJ, Hatch EP, Karabagli H, Karabagli P, Schoenwolf GC, et al. 2003. Expression of mouse fibroblast growth factor and fibroblast growth factor receptor genes during early inner ear development. Dev Dyn 228: 267-272.

Wu H, Reynolds AB, Kanner SB, Vines RR, Parsons JT. 1991. Identification and characterization of a novel cytoskeleton-associated pp60src substrate. Mol Cell Biol 11: 5113-5124.

Xu H, Lee KW, Goldfarb M. 1998. Novel recognition motif on fibroblast growth factor receptor mediates direct association and activation of SNT adapter proteins. J Biol Chem 273: 17987-17990.

Xu J, Liu Z, Ornitz DM. 2000. Temporal and spatial gradients of Fgf8 and Fgf17 regulate proliferation and differentiation of midline cerebellar structures. Development 127: 1833-1843.

Yamashita T, Yoshioka M, Itoh N. 2000. Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. Biochem Biophys Res Commun 277: 494-498.

Yan KS, Kuti M, Yan S, Mujtaba S, Farooq A, Goldfarb MP, Zhou MM. 2002. FRS2 PTB domain conformation regulates interactions with divergent neurotrophic receptors. J Biol Chem 277: 17088-17094.

Yazaki N, Hosoi Y, Kawabata K, Miyake A, Minami M, et al. 1994. Differential expression patterns of mRNAs for members of the fibroblast growth factor receptor family, FGFR-1-FGFR-4, in rat brain. J Neurosci Res 37: 445-452.

Yenush L, White MF. 1997. The IRS-signalling system during insulin and cytokine action. Bioessays 19: 491-500.

Yoshimura S, Takagi Y, Harada J, Teramoto T, Thomas SS, et al. 2001. FGF-2 regulation of neurogenesis in adult hippocampus after brain injury. Proc Natl Acad Sci USA 98: 5874-5879.

Zhan X, Plourde C, Hu X, Friesel R, Maciag T. 1994. Association of fibroblast growth factor receptor-1 with c-Src correlates with association between c-Src and cortactin. J Biol Chem 269: 20221-20224.

Zhang Y, Tatsuno T, Carney JM, Mattson MP. 1993. Basic FGF, NGF, and IGFs protect hippocampal and cortical neurons against iron-induced degeneration. J Cereb Blood Flow Metab 13: 378-388.

Zhou M, Sutliff RL, Paul RJ, Lorenz JN, Hoying JB, et al. 1998. Fibroblast growth factor 2 control of vascular tone. Nat Med 4: 201-207.

Zhu X, Komiya H, Chirino A, Faham S, Fox GM, et al. 1991. Three-dimensional structures of acidic and basic fibroblast growth factors. Science 251: 90-93.

# 6 Transforming Growth Factor-βs in the Brain

*K. Krieglstein*

**Abstract:** Transforming growth factors-β (TGF-βs) regulate numerous cell functions in the developmental and adult brain. TGF-βs are secreted dimeric proteins that signal via heteromeric transmembrane serine–threonine kinase receptors. Phosphorylation of R-Smads leads to the formation of complexes with the common Smad4, which translocates to the nucleus to regulate, as a larger transcriptional complex, immediate early gene and target gene expression. In the nervous system, TGF-βs have roles in neurons and glia and are involved in the regulation of proliferation, differentiation, and neuron survival and death, as well as in orchestrating its response to lesion.

**List of Abbreviations:** Aβ, amyloid β; AD, Alzheimer's disease; Alk, activin-receptor-like kinase; BMP, bone morphogenetic protein; CNS, central nervous system; DRG, dorsal root ganglion; E, embryonic day; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; GDF, growth/differentiation factor; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; IEG, immediate early gene; IL, interleukin; JNK, c-Jun NH(2)-terminal kinase; ir, immunoreactivity/immunoreactive; LAP, latency-associated protein; LLC, large latent protein complex; LTBP, latent-TGF-β-binding protein; MAPK, mitogen-activated protein kinase; MEKK, MAPK/ERK kinase kinase; MIS, müllerian inhibiting substance; MMP, matrix metalloprotease; NGF, nerve growth factor; PAI, plasminogen activator inhibitor; PD, Parkinson's disease; PDGF, platelet-derived growth factor; PNS, peripheral nervous system; Shh, sonic hedgehog; TAK, TGF-β-activated kinase; TGF-β, transforming growth factor-β; TβR, TGF-β receptor; t-PA, tissue plasminogen activator

## 1 Introduction

Transforming growth factors-β (TGF-βs) are multifunctional cytokines with widespread distribution and contextual activity. There are three unique TGF-β isoforms expressed in mammals encoded by three different genes regulated by unique promoters. TGF-βs are synthesized as preproproteins containing a signal peptide and a C-terminally located mature part. TGF-βs form disulfide-bridged homodimers and are folded in a cystine-knot-like motif. During processing the mature protein stays noncovalently bound to its proprotein, building a latent biologically inactive form. TGF-βs signal via a heteromeric transmembrane serine–threonine kinase receptor, whereby the signal may be intracellularly mediated via Smad proteins that translocate into the nucleus to form, in combination with other components, the transcriptional complex. The biological effects of TGF-βs in the nervous system cover regulation of proliferation, migration, differentiation, survival, and death.

## 2 TGF-β and Receptors

TGF-β was initially isolated from human platelets and resulted in the purification of a 25-kDa homodimeric protein, now called TGF-β1 (Assoian et al., 1983). In due course, a second protein, TGF-β2, was purified (Seyedin et al., 1985, 1987; Cheifetz et al., 1987; and others). The cloning of these proteins revealed that the precursors are encoded as preproproteins consisting of 390 aa for TGF-β1 and 412 aa for TGF-β2, each carrying a 20–30 aa signal peptide on its N terminus and resulting in a 112 aa processed mature form (Derynck et al., 1985; De Martin et al., 1987; Madison et al., 1988). Screening of cDNA libraries revealed a third form, TGF-β3 (Derynck et al., 1988; Jakowlew et al., 1988a; ten Dijke et al., 1988a), as well as TGF-β4 from chicken (Jakowlew et al., 1988b; now considered as the TGF-β1 homolog from chicken) and TGF-β5 from Xenopus (Kondaiah et al., 1990). Each TGF-β isoform is encoded by a distinct gene located on different chromosomes: TGF-β1 on human chromosome arm 19q13, TGF-β2 on 1q41, and TGF-β3 on 14q24 and on mouse chromosomes 7, 1, and 12, respectively (Fujii et al., 1986; Barton et al., 1988; ten Dijke et al., 1988b).

### 2.1 TGF-β Superfamily

The TGF-β superfamily of proteins includes about 30 members in mammals. They are divided into two major subgroups, the first one comprising, inter alia, TGF-βs, activins, nodal and myostatin/GDF8,

müllerian inhibiting substance (MIS), and a second group including the bone morphogenetic proteins (BMP) and growth/differentiation factors (GDF) (Chang et al., 2002; Miyazono et al., 2002). TGF- β/BMP-like proteins are found in vertebrates and invertebrates, including *Caenorhabditis elegans* and *Drosophila melanogaster* (Newfeld et al., 1999; Patterson and Padgett, 2000). Many of these signaling proteins harbor important functions during early embryogenesis, organogenesis, after birth, and in the adult, as well as for tissue repair and homeostasis (Kingsley, 1994; Hogan, 1996; Chang et al., 2002; Reddi, 2005). Dysregulated functions of TGF-β superfamily members have been attributed to the pathogenesis of diseases such as cancer (Mummery and van den Eijnden-van Raaij, 1999; Blobe et al., 2000; Miyazono et al., 2003; Siegel and Massague, 2003), immune tolerance and inflammation (Wahl and Chen, 2003; Schmidt-Weber and Blaser, 2004), skeletal dysplasias (Thomas et al., 1997; Stelzer et al., 2003), cachexia (Lee and McPherron, 2001; Zimmers et al., 2002), osteoporosis (Turgeman et al., 2002, Wu et al., 2003), infertility (Dong et al., 1996; Galloway et al., 2000), abnormal vascularization (Paques et al., 1997; Blobe et al., 2000; He et al., 2003), atherosclerosis (Grainger, 2004), psoriasis (Blessing et al., 1996), fibrotic disease (Bartram and Speer, 2004; Bataller and Brenner, 2005), and renal disease (Böttinger and Bitzer, 2002; Schena and Gesualdo, 2005).

## 2.2 TGF-β Structure

TGF-β2 was the first protein of the TGF-β superfamily for which the protein structure was solved upon crystallographic determination (Daopin et al., 1992; Schlunegger and Grutter, 1992). The monomer represents an elongated structure consisting of two pairs of double-stranded β-sheets, tied together by four disulfide bonds in the core region of the protein homomer. This cystine cluster may replace the hydrophobic core of globular proteins. TGF-β dimers are formed via two identical hydrophobic interfaces and stabilized by the ninth cystine on each side building an interchain disulfide bond. Although the structure seemed unusual at first, the structural motif, coined the cystine knot motif (McDonald and Hendrickson, 1993), could be extended to describe a structural superfamily of growth factors, including nerve growth factor (NGF) and platelet-derived growth factor (PDGF) (although NGF and PDGF differ from TGF-βs in their dimer structure; Daopin et al., 1992; Swindells, 1992; McDonald and Hendrickson, 1993; Sebald et al., 2004). By following the resolution of the crystal structures of TGF- β3 (Mittl et al., 1996), BMP7 (Griffith et al., 1996), and BMP2 (Scheufler et al., 1999), it can be concluded that despite the sequence similarity of as little as 30%, the TGF-β superfamily is characterized by a typical tertiary structure of two fingertip loops and a third helical structure including a specific mode of dimerization resulting in a similar three-dimensional structure (for a review see Sebald et al., 2004).

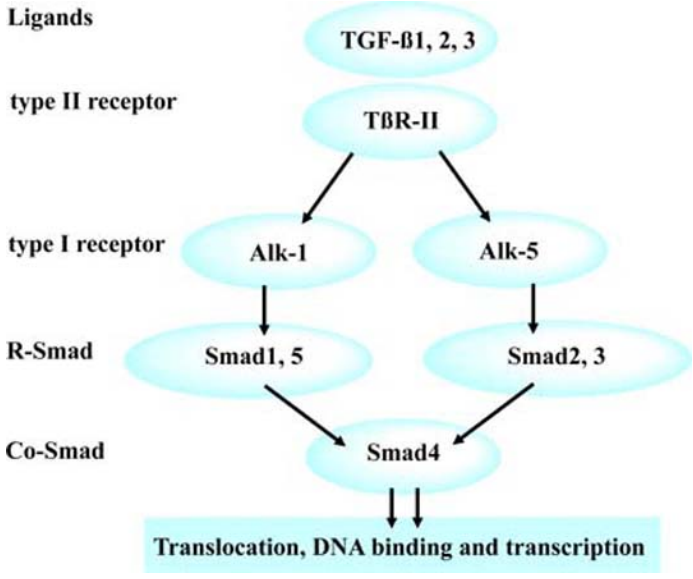
## 2.3 TGF-β Activation

TGF-βs are secreted as large latent protein complexes (LLC; for a review see Annes et al., 2003) and targeted to the extracellular matrix (ECM). TGF-β1, -β2, and -β3 are synthesized as homodimeric proproteins (pro-TGF-β; 75 kDa). In the trans-Golgi network, the precursors are cleaved by furin into the N-terminal propeptides, also referred to as latency-associated proteins (LAPs), which still strongly bind the C-terminal mature TGF-β dimer in a noncovalent manner. This small latency complex is released; however, within this complex TGF-β cannot bind its extracellular receptor. LAP, on the other hand, forms a specific disulfide bridge with another gene product, the latent-TGF-β-binding protein (LTBP/fibrillin protein family; for reviews see Ramirez and Pereira, 1999; Hyytiäinen et al., 2004). Thus LLC includes mature TGF-β noncovalently bound to an LAP–LTBP complex. TGF-β activity may therefore not be regulated via synthesis or release from the cell but rather by release from the LLC. Mechanisms known to activate TGF-β include proteoloytic activation of LAP, for example by plasmin, matrix metalloproteinase-2 (MPP-2) or MPP-9, by activation by thrombospondin, integrins, reactive oxygen species, and by pH (reviewed in and references therein: Annes et al., 2003). Therefore, orchestration of TGF-β availability is the crucial step in TGF-β biology (Rifkin, 2005).

## 3 TGF-β Receptors and Signaling

Signaling by TGF-β family members occurs through a heteromeric transmembrane serine–threonine kinase receptor complex, consisting of type II and type I receptors. There are five type II receptors and seven type I receptors (activin-receptor-like kinases, Alk) known in vertebrates (Miyazono et al., 2000; Shi and Massague, 2003); different combinations of type II and type I receptors may be responsible for ligand-specific signaling. TGF-βs signal via TGF-β receptors type II (TβR-II) and ALK5, and in endothelial cells via TβR-II/ALK1 (◉ *Figures 6-1* and ◉ *6-2*; DaCosta Byfield and Roberts, 2004; Lebrin et al., 2005). Ligand

**◘ Figure 6-1**
**TGF-β signal transduction. TGF-β ligands bind to TβR-II, which then recruits a type I receptor, which may be either Alk-5 or Alk-1. Recruitment of Alk-1 could be shown for TGF-β signaling in endothelial cells as well as in the lesion impaired neurons. TβR-II/Alk-5 signaling is further mediated via R-Smads Smad2 and 3, whereas TβR-II/Alk-1 signaling is mediated via Smad1,5. Modified from Waite and Eng, 2003**

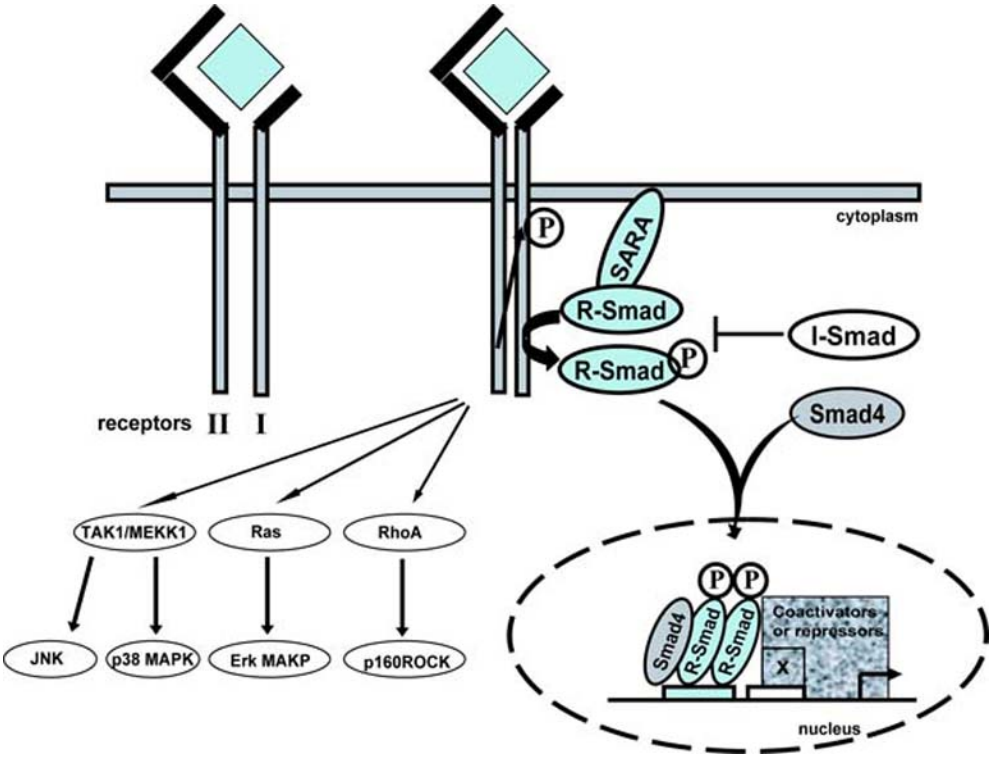



binding to the TβR-II induces recruitment of TβR-I into the complex. Next, the type II receptor phosphorylates the type I receptor at its GS domain, thereby further propagating the signal via phosphorylation of receptor-regulated Smads (R-Smads: Smad2, Smad3; ten Dijke and Hill, 2004). R-Smad phosphorylation is facilitated by presentation of SARA, i.e., Smad anchor for receptor activation (Tsukazaki et al., 1998). Upon activation, R-Smads form heteromeric complexes with Smad4 in the cytoplasm and translocate to the nucleus, where they control gene expression. Inhibitory Smads (I-Smads, e.g., Smad7) act in an opposing manner to R-Smads by binding to the activated type I receptor, thereby inhibiting phosphorylation of R-Smads. Signaling is terminated either by I-Smad or by recruitment of E3-ubiquitin ligase (Smurf1) to the activated type I receptor, resulting in receptor ubiquitination and degradation. Alternatively, signaling may be terminated through protein phosphatase-1-dependent dephosphorylation (Shi and Massague, 2003). For further reading regarding numerous molecular details in TGF-β signaling, please refer to current reviews and references therein (Waite and Eng, 2003; de Caestecker, 2004; Feng and Derynck, 2005; Le Roy and Wrana, 2005).

Other proteins have been characterized that can associate with the TβR complex and regulate TGF-β-dependent signaling independent from Smad activation. These include TGF-β-activated kinase 1 (TAK1)/

**Figure 6-2**

**Smad and non-Smad signaling. The active heterotetrameric receptor complex phosphorylate SARA-presented R-Smads. Phosphorylated R-Smads associate with Smad4 in the cytoplasm and translocate into the nucleus. The R-Smad/Smad4 complex binds to a Smad-binding DNA element (SBE) and cooperates with other transcription factors (X), coactivators like CBF/p300, or repressors like c-ski/SnoN, Tgif of Tieg, to regulate gene transcription. Independent of Smad, other signaling pathways like RhoA, Ras, and TAK1/MEKK1 have been shown to be activated via the TGF-β receptor complex. Modified from Derynck and Zhang, 2003**




MEKK1-dependent JNK or mitogen-activated protein kinase (MAPK) activation, rapid ras activation, and RhoA activation (*Figure 6-2*; for further review see Mulder, 2000; Derynck and Zhang, 2003; Moustakas and Heldin, 2005).

## 4 TGF-β Target Genes

TGF-β-dependent transcriptional regulation is mediated via interaction of DNA-binding Smads with sequence-specific transcription factors in combination with the coactivators CBP and p300 (Jonk et al., 1998; Massague, 2000; Derynck and Zhang, 2003). Sequence-specific transcription factors that cooperate with the R-Smad–Smad4 complex include AP-17bZIP, and members of the transcription factor families AML/Runx, bHLH, homeodomain, SP1, and winged helix. Besides the essential coactivator CBP or p300, other coactivators (e.g., SMIF, MDG1, ARC105) and corepressors (e.g., s-Ski/SnoN, cMyc, TGIF, SNIP1, SIP1) that interact with Smads define the level of transcriptional activation (Itoh et al., 2000; Massague, 2000; Moustakas et al., 2001; Derynck and Zhang, 2003). The multitude of possible transcriptional complexes strongly supports the earlier assumption of a context-dependent TGF-β activity, now envisionable at the level of transcriptional regulation.

With the advent of genomic expression profiling using microarray techniques, TGF-β target gene expression has been widely studied using epithelial, mesenchymal, and endothelial cells (Verrecchia et al., 2001; Zavadil et al., 2001; Ota et al., 2002; Kang et al., 2003; Xie et al., 2003; Yang et al., 2003; Zhao et al., 2004; please see also the corresponding supporting material). In several screens, gene expression has been analyzed in the context of immediate early gene (IEG) expression, i.e., within 30 min, and further distal target gene expression possibly mediated via transcriptional targets of TGF-β. It also turned out that all IEGs seem to be under the control of Smad3, whereas Smad2 may be involved in the modulation of target gene expression. Furthermore, all IEGs seem to be Smad3 induced rather than suppressed (Xie et al., 2003; Yang et al., 2003). Comparing the gene expression results derived from several cellular systems, it is obvious that TGF-β-dependent transcription is strongly cell type dependent (Zavadil et al., 2001; Yang et al., 2003). In any case, the screens support the identification of already well established direct target genes of TGF-β, including Smad7, Myc, JunB, Pai-1, p21/Cip1, p15/INK4B, collagen type 4, fibronectin, Smac1, Smurf1, Snail, Tieg, and Tgif.

The array of TGF-β target genes (❯ *Table 6-1*) gives a good overview on potential signaling crosstalks of TGF-β function in development, maintenance, and repair of the nervous system, including Wnt, notch, and hedgehog pathways.

## 5 Biological Activity and Physiological Relevance of TGF-β

### 5.1 Distribution of TGF-β in Developing and Adult Brain

Localization of TGF-β isoforms in mice and rats has been performed using immunohistochemistry and in situ hybridization studies, demonstrating a widespread distribution of TGF-β2 and TGF-β3 during development (Flanders et al., 1991; Pelton et al., 1991a, b; Unsicker et al., 1991). TGF-β1 is confined to meninges and choroid plexuses. During mouse development TGF-β2 and -β3 immunoreactivities (ir) first become detectable along peripheral nerves, in radial glial cells, and along central nervous system (CNS) axon tracts at E12. Neuronal cell bodies become ir from E15 onward. Most notably, TGF-β ir is not detectable in the ventricular zone throughout the neural tube, suggesting that TGF-β may not be involved in the regulation of cell division of neural stem cells during development (Flanders et al., 1991). In contrast, cells in the subventricular zone, subplate, and lamina I of the E16 cortex stain positive for TGF-β. As they develop, astrocytes are ir for TGF-β2 and -β3. In the adult nervous system, both neurons and astroglia are ir for TGF-β and -β3. Ir neuron populations include cortical layers 2, 3, and 5, hippocampus, piriform cortex, retinal ganglionic cells, hindbrain aminergic neurons, as well as spinal and hindbrain motoneurons (Unsicker et al., 1991). TGF-β1 is most prominent within the choroid plexus and meninges; it may, however, be expressed in other cells below levels of detectability. Upon lesioning, TGF-β1 may by upregulated in astrocytes as well as in neurons in vivo. TGF-β1 also becomes detectable in tissue culture, possibly mimicking a lesion-like situation. In primary neural tissue culture, treatment with all three TGF-β isoforms usually results in identical responses, suggesting that the recombinant proteins used have similar affinities for their shared receptor complex (Krieglstein and Unsicker, 1994).

In addition to the distribution of TGF-β within the peripheral nervous system (PNS) and the CNS, its subcellular localization and mode of secretion is of importance in order to elaborate on its possible functions. Taking PC12 cells as a model to study sorting in the trans-Golgi network, Specht et al. (2003) could show that TGF-β2 may be sorted and released to a large proportion via the regulated path of secretion. Secretory vesicles provide a milieu of pH 5, which is suitable for TGF-β activation within the vesicle, enabling release of active TGF-β (Specht et al., 2003). This activity-dependent release may suggest that TGF-β2 functions as a modulator of synaptic plasticity.

### 5.2 TGF-β May Act as a Morphogen

Early in development, when the neural tube is still devoid of any TGF-β expression, TGF-β3 expression can be detected in the notochord, the wall of the dorsal aorta, and in the dermomyotome (chick stage 18/E3;

**Table 6-1**
**Genes upregulated or downregulated (d) by TGF-β in epithelial cells with potential interest in nervous system development, adulthood, and repair**

| Cell–matrix interaction | Cell–cell interaction | Signaling molecules and effectors | |
|---|---|---|---|
| Decorin | α-Catenin | Activin A | |
| HSPG/perlecan | β-Catenin | BMP4 | d |
| IGFBP5 | Connexion 37 | BMPRII | |
| IGFBP3 | Deltex1 (DTX1) | c-myc | d |
| Integrin a3 | Disheveled | CTGF | |
| Integrin a5 | Disheveled homolog | FGFR1 | |
| Integrin a4 | Ephrin type A receptor 2 | Follistatin-like 3 | |
| Integrin b3 | Ephrin type B receptor 2 | FZD1 | |
| Integrin b5 | Ephrin type B receptor 4 | FZD7 | |
| Laminin 37 kDa receptor | EphB2 | GATA2 | |
| Laminin B1 | EPH3 | Hes1 | |
| MMP-2 | Ephrin-B1 | HGF | d |
| MMP-3 | Notch 2 | Id1 | d |
| MMP-11 | Notch group protein | Id2 | d |
| MMP-14 | Smoothened | Id3 | d |
| MMP-16 | TLE3 | IGF II receptor | |
| Neogenin | Wnt-8B | IGF-2R | |
| Semaphorin I | Wnt-13 | Jagged 1 | |
| Semaphorin V | | Nedd9 | |
| Tenascin-C | | Neuregulin | |
| TIMP-1 | | p15Ink4b | |
| TIMP-3 | | p21Cip1 | |
| | | PDGF | |
| | | rhoB | |
| | | Slug | |
| | | Snail | |
| | | Sortilin | d |
| | | Sox4 | |
| | | Sox9 | |
| | | Sprouty homolog 2 | d |
| | | Tgif | |
| | | Tieg | |
| | | TNF receptor 1 | |
| | | TNFR | |

Taken from Verrecchia et al. (2001), Ota et al. (2002), Kang et al. (2003), Zavadil et al. (2001), and Zhao et al. (2004)

Unsicker et al., 1996). Furthermore, TGF-β2 and -β3 are expressed in floor plate cells (Unsicker et al., 1996). TGF-β3 may therefore have the capacity to act as a ventralizing factor on patterning and phenotype determination along the neural tube. TGF-β has been shown to specify development of mesencephalic dopaminergic neurons in vivo (Farkas et al., 2003) and promote dopaminergic differentiation from mesencephalic neural precursor cells in vitro (Roussa et al., 2005). Data also suggest that TGF-β may cooperate with sonic hedgehog (Shh) in dopaminergic neuron development (Farkas et al., 2003). Migrating neural crest cells express TGF-β1 and may be influenced by TGF-β along their migratory route. A promoting role of TGF-β for the development of a catecholaminergic phenotype is suggested by in vitro experiments (Howard and Gershon, 1993).

## 5.3 TGF-β Controls Proliferation

TGF-βs are well known for their capacity to regulate cell proliferation in a context-dependent manner. There are at least four scenarios in which regulation of cell proliferation is an important issue in nervous system development and maintenance: (1) neurogenesis, (2) proliferation of neuroblasts (neural crest cells), (3) proliferation of glial cells during development or upon lesioning, and (4) upon transformation in tumors.

Neurogenesis in the neural tube requires definite exit of progenitor cells of the cell cycle to generate postmitotic neurons. Although TGF-β is known for inhibiting cell proliferation by inducing G1 arrest, there is so far no evidence for a role of TGF-β in developmental neurogenesis. However, there is some indirect evidence that neural stem cells in the neuroepithelium need to be protected from TGF-β action, in order to prevent premature growth retardation (Hanashima et al., 2002; Seoane et al., 2004). Seoane and coworkers have demonstrated on the basis of protein interaction analysis in HaCaT cells that p21Cip1 expression is regulated by TGF-β-dependent Smad in combination with the forkhead box (Fox) family member FoxO. This FoxO–Smad complex is inhibited by FoxG1, which has been shown to be essential for proliferation of telencephalic progenitor cells (Xuan et al., 1995). Indeed, FoxG1 mutants, which display reduced proliferation of telencephalic progenitor cells, premature differentiation, and early depletion of progenitor population (Xuan et al., 1995), show high levels of p21Cip1 in TGF-β-sensitive progenitor cells (Seoane et al., 2004). Exit from the cell cycle during terminal differentiation, as required for neurogenesis, has been described to be regulated by Ink4d and Kip1 inhibitors of cyclin-dependent kinases (Zindy et al., 1999; Cunningham and Roussel, 2001). p27Kip1 has been identified as a TGF-β-dependent target gene; however, there is no evidence for a TGF-β-dependent regulation of p19Ink4d. This suggests that TGF-β may serve as an extracellular regulator to induce cell cycle G1 arrest, but may probably not regulate cell cycle exit required for terminal differentiation.

By affecting the cell cycle before terminal differentiation, TGF-β may, of course, regulate proliferation of neuroepithelial cells, including neuroblasts, neural crest cells, and glial progenitors (Anchan and Reh, 1995; Zhang et al., 1997b). Furthermore, TGF-β2 has been shown to regulate cell proliferation in neural crest-derived chromaffin cells (Rahhal et al., 2004) with the capacity of lifelong proliferation.

## 5.4 TGF-β in Neuronal Survival and Death

TGF-β has been shown to promote neuron survival of several neuron populations in vitro (Martinou et al., 1990; Poulson et al., 1994; Krieglstein et al., 1995). However, it is now well established that TGF-β may modulate the neurotrophic capacities of numerous growth factors including neurotrophins (Krieglstein and Unsicker, 1996) and, most importantly, glial cell line-derived neurotrophic factor (GDNF; Krieglstein et al., 1998b). GDNF was shown to crucially depend on TGF-β to exert its neurotrophic activities on peripheral as well as mesencephalic dopaminergic neurons in vitro. In vivo, its neuroprotective effect on target-deprived preganglionic sympathetic neurons also depends on the presence of TGF-β (Schober et al., 1999). GDNF/TGF-β cooperativity on chick ciliary ganglionic neurons has now been characterized in detail (Peterziel et al., 2002), whereby TGF-β is required for appropriate GDNF receptor recruitment to the plasma membrane (see also Peterziel and Strelau, this volume).

Depending on the cellular context, TGF-β has also been shown to regulate ontogenetic neuron death. Upon immunoneutralization of all TGF-β isoforms in ovo (E6–E10), ontogenetic cell death of chick parasympathetic ciliary ganglionic neurons, sensory dorsal root ganglionic (DRG) neurons as well as lumbar spinal motoneurons could be prevented (Krieglstein et al., 2000). Similarly, TGF-β regulates ontogenetic, morphogenetic cell death in the developing retina of chick and mouse embryos (Dünker et al., 2001; Dünker and Krieglstein, 2003). Another classical model for morphogenetic cell death during embryogenesis represents the removal of interdigital tissue to form individual fingers. Similarly, double deletion of TGF-β2 and -β3 in the mouse resulted in lack of cell death (Dünker et al., 2002). Furthermore, induced neuron death following embryonic limb bud ablation in chick embryos resulted in significant neuroprotection upon immunoneutralization of TGF-β (Krieglstein et al., 2000). Together, these data

suggest TGF-β as a key regulator of ontogenetic cell death in vivo. Although TGF- β-induced apoptosis and underlying signaling pathways have been well characterized in many cells types, little is known about TGF-β-induced apoptosis in neurons (Schuster and Krieglstein, 2002; Bender et al., 2004; Sanchez-Capelo, 2005).

## 5.5 TGF-β in Neuronal Differentiation and Synaptogenesis

TGF-βs have been implicated in the regulation of neurite outgrowth, transmitter synthesis, and synapse formation. TGF-β has been reported to cause neurite sprouting and elongation of hippocampal axons as well as to promote reelongation of injured axons of hippocampal neurons in vitro (Ishihara et al., 1994; Abe et al., 1996). On DRG explants, TGF-β was shown to increase number of neurites, as well as neurite length (Unsicker et al., 1996). Extracellular signaling factors such as Wnt and TGF-βs are recognized as target-derived signals in synaptogenesis (Packard et al., 2003; Salinas, 2005). In the past years all components of the TGF-β signaling system have been localized in the presynaptic terminal of the neuromuscular junction, whereby TGF-β ligands are synthesized and localized on the postsynaptic side (Toepfer et al., 1999; McLennan and Koishi, 2002). In chick ciliary ganglionic neurons, developmental expression of $K_{Ca}$ channels coincides with synaptogenesis. Dryer and coworkers have shown that target-derived TGF-β1 regulates the developmental expression of $Ca^{2+}$-activated $K^+$ currents in vitro and in vivo (Cameron et al., 1999). The acute effect of TGF-β1 relies on the translocation of $K_{Ca}$ channels from intracellular stores to the plasma membrane involving signaling via Ras, Erk, and PI4 kinase (for review see Dryer et al., 2003). TGF-β is also known to have a prominent role in long-term synaptic facilitation in isolated Aplysia ganglia (Zhang et al., 1997a). Within minutes, TGF-β1 stimulated MAPK-dependent phosphorylation of synapsin, which appeared to modulate synapsin distribution and resulted in a reduced magnitude of synaptic depression (Chin et al., 2002).

## 5.6 TGF-β in Glial Cell Function

Astroglial cells express TGF-β and are responsive to it. Culturing or in vivo lesioning elicits expression of TGF-β1 in addition to expression of TGF-β2 and -β3 (Flanders et al., 1993a). Astroglial expression of TGF-β may be regulated by a large number of cytokines, including FGFs and interleukins (ILs) (see Eddlestone and Mucke, 1993; Krieglstein et al., 1998b). TGF-β has been profoundly investigated for its role in orchestrating the response to brain lesions (for a review see Flanders et al., 1998). With regard to astrocytes, this includes regulation of astrocytic growth, astroglial scar formation, and antiinflammatory responses. In most contexts studied, TGF-β inhibits growth of astrocytes (Flanders et al., 1993a; Hunter et al., 1993). Most importantly, TGF-β counteracts mitogenic signals by astroglial mitogens such as fibroblast growth factor-2 (FGF-2) or PDGF. However, effects may vary depending on astrocyte culture conditions in vitro or may be brain region dependent in vivo (Labourdette et al., 1990; Johns et al., 1992). TGF-β may also affect cell adhesion, migration, and ECM production of astrocytes, being important in the cascade of shaping the reactive astrocyte phenotype. TGF-β-treated astrocytes show a slight increase in actin content, the appearance of actin stress fibers, a slight increase in the glial fibrillary acidic protein (GFAP), and an increased production of laminin and fibronectin (cf. Baghdassarian et al., 1993). Thus, treatment of cerebral wounds with anti-TGF-β2 antibodies was shown to lead to a marked reduction of glial scarring (Logan et al., 1999). Many effects of TGF-β on astroglia are antiinflammatory and immunosuppressive, as TGF-β modulates the expression of important cytokines involved in CNS immune reactions. These include upregulation of interleukin-6 (IL-6) and NGF (Aderka et al., 1989; Lindholm et al., 1992), blocking interferon-γ-mediated upregulation of major histocompatibility complex (MHC) class II (Dong et al., 2001), and the TNF-α- and IL-1β-mediated upregulation of intracellular adhesion molecule-1 (Shrikant et al., 1996).

Oligodendrocytes arise from a bipotential progenitor cell, the O2A progenitor. TGF-β restricts their PDGF-driven proliferation and induces oligodendroglial differentiation (McKinnon et al., 1993) but may also induce apoptosis (Schuster et al., 2002). In the PNS, TGF-β mediates developmental cell death of

Schwann cells (Parkinson et al., 2001) and blocks Schwann cell myelination and expression of myelin-related proteins (Awatramani et al., 2002; and references therein). However, in adult mice, TGF-β seems to stabilize compact myelin, as TGF-β1-null mice have grossly abnormal myelin (Day et al., 2003). Ski, a repressor of Smad-mediated TGF- β signaling, controls Schwann cell proliferation and myelination, whereas absence of Ski abolished the formation of peripheral myelin, and myelinating Schwann cells upregulate Ski in development as well as during remyelination upon injury (Atanasoski et al., 2004).

Microglia constitutes the resident immune cell in the CNS (Block and Hong, 2005). It has been suggested that activation of microglia leads to the production of toxic factors that propagate neuronal injury. TGF-β may act as a suppressor of functions of activated microglia, thereby fulfilling an antiinflammatory role in the CNS (Brionne et al., 2003). Specifically, TGF-β1 blocks microglial proliferation (Jones et al., 1998) and free radical induction (Herrera-Molina and von Bernhardi, 2005), and induces microglial apoptosis (Xiao et al., 1997; Jung et al., 2003).

## 6 TGF- β-Dependent Pathologies and Clinical Relevance

### 6.1 Tumors of the CNS

The role of TGF-β in cancer biology is complex and involves aspects of both tumor suppression and tumor promotion (Roberts and Wakefield, 2003). Tumors of the CNS include primitive neuroectodermal tumors, such as medulloblastomas and gliomas (Nieder et al., 2003; Fogarty et al., 2005). CNS tumors are characterized by rapid and infiltrative growth, angiogenesis, and immune suppression. TGF-βs are well characterized for their antiproliferative effects on many cell types, including astrocytes. However, there are many possibilities to circumvent this effect. First, as TGF-β actions are context dependent, the presence of certain mitogens, such as TGF-α/EGF or PDGF, may turn TGF-β into a growth stimulating factor (Roberts et al., 1981; Leof et al., 1986). Second, transformed cells may become insensitive to TGF-β due to overproduction of TGF-β or due to mutations of TGF-β receptors, their signaling components, or even their target genes responsible for G1 arrest (see Lyons et al., 1990; Markowitz et al., 1995; Hahn et al., 1996; Rich et al., 1999; Rich, 2003; Seoane et al., 2004 for review).

TGF-β's ability to regulate ECM composition implicated TGF-β in the regulation of tumor invasion and metastasis. In this context, TGF-β has been shown to regulate integrin expression, e.g., integrin $\alpha_V\beta_3$ expression, which has been shown to play a role in glioma propagation (Uhm et al., 1999). TGF-β has also been shown to upregulate MMP-2 and MMP-9 at the cell surface (Rooprai et al., 2000) that may interact with $\alpha_V\beta_3$ integrin (for review see Platten et al., 2001).

TGF-β is a potent immunosuppressive cytokine. Secretion of TGF-β by tumor cells may generate an environment encapsulating the tumor and protecting it against antitumor immune responses resulting in tumor promotion (Gorelik and Flavell, 2001). This immunosuppressive role has been attributed to TGF-β2, which is also the preferentially expressed isoform by many glioblastomas (Bodmer et al., 1989). On this basis, TGF-β2-specific antisense gene therapy strategies have been established to make tumor cells accessible to an effective antitumor immune response and counteract TGF-β dependent-tumor metastasis (Jachimczak et al., 1993; Lou, 2004; Schlingensiepen et al., 2005). Along this line, there is extensive research going on to identify TGF-β signaling inhibitors for cancer therapy (DaCosta Byfield et al., 2004; Yingling et al., 2004; Lahn et al., 2005).

### 6.2 Ischemia

TGF-β1 expressed at low levels in adult brain is rapidly upregulated following insults such as ischemia, excitatory injury, or traumatic brain injury (Klempt et al., 1992; Knuckey et al., 1996; Morganti-Kossmann et al., 1999; Yamashita et al., 1999; Zhu et al., 2000; Boche et al., 2003). As TGF-β is a good candidate in organizing the response to degeneration of neurons as well as in mediating antiinflammatory reactions, its neuroprotective potential has been widely analyzed (for review see Pratt and McPherson, 1997; Flanders

et al., 1998). TGF-β1 has been shown to reduce infarct size after focal cerebral ischemia and to prevent hippocampal neuronal damage after transient global ischemia (Gross et al., 1993; Prehn et al., 1993; Heinrich-Noack et al., 1996; for review see Buisson et al., 2003). Furthermore, TGF-β may also mediate tolerance of ischemic preconditioning toward subsequent ischemic insult (Boche et al., 2003). The molecular mechanism(s) by which TGF-β protects neurons from ischemic cell death relies on a signaling crosstalk between neurons and astrocytes (Prehn et al., 1994; Docagne et al., 1999), and involves the maintenance of $Ca^{2+}$ homeostasis, modulation of the t-plasminogen activator (t-PA)/plasminogen activator inhibitor (PAI-1) axis, as well as inhibition of proapoptotic pathways, such as Bad and caspase-3 (Zhu et al., 2001, 2002) and upregulation of antiapoptotic proteins such as Bcl-2 (Prehn et al., 1994). Most recently, an additional TGF-β-dependent antiapoptotic pathway involving NF-κB activation has been described (Zhu et al., 2004). This pathway seems to be downstream of Alk1, which has been shown to be upregulated in neurons in an injury-dependent manner (König et al., 2005). Injury-dependent upregulation of Alk1, an alternative type I receptor first described on endothelial cells with signaling preference toward Smad1, may also explain numerous opposing effects of TGF-β in brain development and lesions.

### 6.3 Alzheimer's Disease

There are several lines of evidence suggesting that TGF-β1 may contribute to the pathology of Alzheimer's disease (AD), particularly in promoting amyloid β (Aβ) precursor expression and Aβ deposition (van der Wal et al., 1993; Flanders et al., 1995; Wyss-Coray et al., 1997a, b; Burton et al., 2002). Mice expressing TGF-β1 under the control of GFAP develop AD-like vascular and meningeal abnormalities with age (Gaertner et al., 2005). These chronic alterations could be correlated with reduced brain tissue perfusion, leading to an increased amount of fibrillar and soluble Aβ peptides. However, in brain parenchyma, astroglial TGF-β1 expression leads to a reduction of overall Aβ as well as decreased numbers of dystrophic neurites (Wyss-Coray et al., 2001). The reduced plaque burden in brain parenchyma is thought to depend on TGF-β-dependent microglial activation and microglial Aβ clearance. Furthermore, an associated study of three polymorphisms of the human TGF-β1 gene with AD suggests that there is no correlation of TGF-β1 with AD on the basis of TGF-β1 gene variability (Araria-Goumidi et al., 2002).

### 6.4 Parkinson's Disease

Parkinson's disease (PD) is associated with a marked reduction of striatal dopamine as a consequence of loss of nigostriatal dopaminergic neurons (for review see Braak et al., 2004). TGF-β2 and -β3 are expressed in adult nigral dopaminergic neurons (Unsicker et al., 1991) and TGF-β1 and -β2 were elevated in biopsies of PD patients (Nagatsu et al., 2000). TGF-βs have been shown to promote midbrain dopaminergic neuron survival in vitro and in vivo (Krieglstein and Unsicker, 1994; Poulsen et al., 1994; Roussa et al., 2004), as well as protection against MPP+ intoxication (Krieglstein et al., 1995). Most importantly, TGF-β cooperates with GDNF to promote dopaminergic neuron survival (Krieglstein et al., 1998b). GDNF is well known as a potential therapeutic agent for PD (for review see Björklung and Lindvall, 2000). However, in vivo GDNF-dependent neuroprotective effects are based on the cooperativity with TGF-β, as shown in the animal model of PD (Schober et al., 2005). This neuroprotective strategy has also already been used by grafting chromaffin cells obtained from Zuckerkandl's organ, releasing both GDNF and TGF-β1 (Fernandez-Espejo et al., 2005).

## 7 Conclusions

TGF-βs are multifunctional cytokines acting in a contextual manner. Individual isoforms may become specifically important in distinct situations during nervous system development, maturation, and adult maintenance as well as in acute and chronic brain lesions. TGF-βs signal via a distinct heteromeric receptor system, which again, may chance upon lesioning. Many aspects of TGF-β function are beginning to be

understood. Others, however, such as regulation of neural stem cell and progenitor regulation, would have to be addressed in the future. Lateral signaling and crosstalk with other signaling pathways as well as modulation of other signaling pathways are the open issues in the future. The results to be obtained will shed light on many of the scenarios with presumable opposing effects of TGF-β. TGF-β has been and continues to be a fascinating regulator of development, adulthood, and aging.

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## References

Abe K, Chu PJ, Ishihara A, Saito H. 1996. Transforming growth factor-beta 1 promotes re-elongation of injured axons of cultured rat hippocampal neurons. Brain Res 723: 206-209.

Aderka D, Le JM, Vilcek J. 1989. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. J Immunol 143: 3517-3523.

Anchan RM, Reh TA. 1995. Transforming growth factor-beta-3 is mitogenic for rat retinal progenitor cells in vitro. J Neurobiol 28: 133-145.

Annes JP, Munger JS, Rifkin DB. 2003. Making sense of latent TGFbeta activation. J Cell Sci 116: 217-224.

Araria-Goumidi L, Lambert JC, Mann DM, Lendon C, Frigard B, et al. 2002. Association study of three polymorphisms of TGF-beta1 gene with Alzheimer's disease. J Neurol Neurosurg Psychiatry 73: 62-64.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. 1983. Transforming growth factor-beta in human platelets. J Biol Chem 258: 7155-7160.

Atanasoski S, Notterpek L, Lee HY, Castagner F, Young P, et al. 2004. The protooncogene Ski controls Schwann cell proliferation and myelination. Neuron 43: 499-511.

Awatramani R, Shumas S, Kamholz J, Scherer SS. 2002. TGFbeta1 modulates the phenotype of Schwann cells at the transcriptional level. Mol Cell Neurosci 19: 307-319.

Baghdassarian D, Toru-Delbauffe D, Gavaret JM, Pierre M. 1993. Effects of transforming growth factor-β1 on the extracellular matrix and cytoskeleton of cultured astrocytes. Glia 7: 193-202.

Barton DE, Foellmer BE, Du J, Tamm J, Derynck R, et al. 1988. Chromosomal localisation of TGF-βs 2 and 3 in man and mouse: dispersion of the TGF-β family. Oncogene Res 3: 323-331.

Bartram U, Speer CP. 2004. The role of transforming growth factor beta in lung development and disease. Chest 125: 754-765.

Bataller R, Brenner DA. 2005. Liver fibrosis. J Clin Invest 115: 209-218.

Bender H, Wang Z, Schuster N, Krieglstein K. 2004. TIEG1 facilitates transforming growth factor-beta-mediated apoptosis in the oligodendroglial cell line OLI-neu. J Neurosci Res 75: 344-352.

Björklund A, Lindvall O. 2000. Parkinson's disease gene therapy moves towards the clinic. Nat Med 6: 1207-1208.

Blessing M, Schirmacher P, Kaiser S. 1996. Overexpression of bone morphogenetic protein-6 (BMP-6) in the epidermis of transgenic mice: inhibition or stimulation of proliferation depending on the pattern of transgene expression and formation of psoriatic lesions. J Cell Biol 135: 227-239.

Blobe GC, Schiemann WP, Lodish HF. 2000. Role of transforming growth factor beta in human disease. N Engl J Med 342: 1350-1358.

Block ML, Hong JS. 2005. Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. Prog Neurobiol 76: 77-98.

Boche D, Cunningham C, Gauldie J, Perry VH. 2003. Transforming growth factor-beta 1-mediated neuroprotection against excitotoxic injury in vivo. J Cereb Blood Flow Metab 23: 1174-1182.

Bodmer S, Strommer K, Frei K, Siepl C, de Tribolet N, et al. 1989. Immunosuppression and transforming growth factor-beta in glioblastoma. Preferential production of transforming growth factor-beta 2. J Immunol 143: 3222-3229.

Böttinger EP, Bitzer M. 2002. TGF-beta signaling in renal disease. J Am Soc Nephrol 13: 2600-2610.

Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K. 2004. Stages in the development of Parkinson's disease-related pathology. Cell Tissue Res 318: 121-134.

Brionne TC, Tesseur I, Masliah E, Wyss-Coray T. 2003. Loss of TGF-beta 1 leads to increased neuronal cell death and microgliosis in mouse brain. Neuron 40: 1133-1145.

Buisson A, Lesne S, Docagne F, Ali C, Nicole O, et al. 2003. Transforming growth factor-beta and ischemic brain injury. Cell Mol Neurobiol 23: 539-550.

Burton T, Liang B, Dibrov A, Amara F. 2002. Transcriptional activation and increase in expression of Alzheimer's beta-amyloid precursor protein gene is mediated by TGF-beta in normal human astrocytes. Biochem Biophys Res Commun 295: 702-712.

Cameron JS, Dryer L, Dryer SE. 1999. Regulation of neuronal K(+) currents by target-derived factors: opposing actions of two different isoforms of TGFbeta. Development 126: 4157-4164.

Chang H, Brown CW, Matzuk MM. 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev 23: 787-823.

Cheifetz S, Weatherbee JA, Tsang MLS, Anderson JK, Mole JE, et al. 1987. The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. Cell 48: 409-415.

Chin J, Angers A, Cleary LJ, Eskin A, Byrne JH. 2002. Transforming growth factor beta1 alters synapsin distribution and modulates synaptic depression in Aplysia. J Neurosci 22: RC220.

Cunningham JJ, Roussel MF. 2001. Cyclin-dependent kinase inhibitors in the development of the central nervous system. Cell Growth Differ 12: 387-396.

DaCosta Byfield S, Major C, Laping NJ, Roberts AB. 2004. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. Mol Pharmacol 65: 744-752.

DaCosta Byfield S, Roberts AB. 2004. Lateral signalling enhances TGF-β response complexity. Trends Cell Biol 14: 107-111.

Daopin S, Piez KA, Ogawa Y, Davies DR. 1992. Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. Science 257: 369-373.

Day WA, Koishi K, McLennan IS. 2003. Transforming growth factor beta 1 may regulate the stability of mature myelin sheaths. Exp Neurol 184: 857-864.

de Caestecker M. 2004. The transforming growth factor-β superfamily of receptors. Cytokine Growth Factor Rev 15: 1-11.

De Martin R, Haendler B, Hofer-Warbinek R, Gaugitsch H, Wrann M, et al. 1987. Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the member of the transforming growth factor-β gene family. EMBO J 6: 3673-3677.

Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell JR, et al. 1985. Human transforming growth factor-beta cDNA sequence and expression in tumor cell lines. Nature 316: 701-705.

Derynck R, Lindquist PR, Lee A, Wen D, Tamm J, et al. 1988. A new type of transforming growth factor beta, TGF-β3. EMBO J 7: 3737-3743.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. Nature 425: 577-584.

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425: 577-584.

Docagne F, Nicole O, Marti HH, Mac Kenzie ET, Buisson A, et al. 1999. Transforming growth factor-beta1 as a regulator of the serpins/t-PA axis in cerebral ischemia. FASEB J 13: 1315-1324.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, et al. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383: 531-535.

Dong Y, Tang L, Letterio JJ, Benveniste EN. 2001. The Smad3 protein is involved in TGF-beta inhibition of class II transactivator and class II MHC expression. J Immunol 167: 311-319.

Dryer SE, Lhuillier L, Cameron JS, Martin-Caraballo M. 2003. Expression of K(Ca) channels in identified populations of developing vertebrate neurons: role of neurotrophic factors and activity. J Physiol Paris 97: 49-58.

Dünker N, Krieglstein K. 2003. Reduced programmed cell death in the retina and defects in lens and cornea of Tgfbeta2(−/−) Tgfbeta3(−/−) double-deficient mice. Cell Tissue Res 313: 1-10.

Dünker N, Schmitt K, Krieglstein K. 2002. TGF-beta is required for programmed cell death in interdigital webs of the developing mouse limb. Mech Dev 113: 111-120.

Dünker N, Schuster N, Krieglstein K. 2001. TGF-beta modulates programmed cell death in the retina of the developing chick embryo. Development 128: 1933-1942.

Eddleston M, Mucke L. 1993. Molecular profile of reactive astrocytes—implications for their role in neurologic disease. Neuroscience 54: 15-36.

Farkas LM, Dünker N, Roussa E, Unsicker K, Krieglstein K. 2003. Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. J Neurosci 23: 5178-5186.

Feng XH, Derynck R. 2005. Specificity and versatility in TGF-signaling through Smads. Annu Rev Cell Dev Biol. 21: 659-693.

Fernandez-Espejo E, Armengol JA, Flores JA, Galan-Rodriguez B, Ramiro S. 2005. Cells of the sympathoadrenal lineage: biological properties as donor tissue for cell-replacement therapies for Parkinson's disease. Brain Res Rev 49: 343-354.

Flanders KC, Lippa CF, Smith TW, Pollen DA, Sporn MB. 1995. Altered expression of transforming growth factor-beta in Alzheimer's disease. Neurology 45: 1561-1569.

Flanders KC, Lüdecke G, Engels S, Cissel DS, Roberts AB, et al. 1991. Localization and actions of transforming growth factor-betas in the embryonic nervous system. Development 113: 183-191.

Flanders KC, Lüdecke G, Renzig J, Hamm C, Cissel DS, Unsicker K. 1993a. Effect of TGF-βs and bFGF on astroglial cell growth and gene expression in vitro. Mol Cell Neurosci 4: 406-417.

Flanders KC, Ren RF, Lippa CF. 1998. Transforming growth factor-betas in neurodegenerative disease. Prog Neurobiol 54: 71-85.

Flanders KC, Winokur TS, Holder MG, Sporn MB. 1993b. Hyperthermia induces expression of transforming growth factor-beta s in rat cardiac cells in vitro and in vivo. J Clin Invest 92: 404-410.

Fogarty MP, Kessler JD, Wechsler-Reya RJ. 2005. Morphing into cancer: the role of developmental signaling pathways in brain tumor formation. J Neurobiol 64: 458-475.

Fujii D, Brissenden JE, Derynck R, Franke U. 1986. Transforming growth factor-β gene maps to human chromosomes 19 long arm and to mouse chromosome 7. Somat Cell Mol Genet 12: 281-288.

Gaertner RF, Wyss-Coray T, Von Euw D, Lesne S, Vivien D, et al. 2005. Reduced brain tissue perfusion in TGF-beta 1 transgenic mice showing Alzheimer's disease-like cerebrovascular abnormalities. Neurobiol Dis 19: 38-46.

Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, et al. 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nat Genet 25: 279-283.

Gorelik L, Flavell RA. 2001. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. Nat Med 7: 1118-1122.

Grainger DJ. 2004. Transforming growth factor beta and atherosclerosis: so far, so good for the protective cytokine hypothesis. Arterioscler Thromb Vasc Biol 24: 399-404.

Griffith DL, Keck PC, Sampath TK, Rueger DC, Carlson WD. 1996. Three-dimensional structure of recombinant human osteogenic protein 1: structural paradigm for the transforming growth factor beta superfamily. Proc Natl Acad Sci USA 93: 878-883.

Gross CE, Bednar MM, Howard DB, Sporn MB. 1993. Transforming growth factor-beta 1 reduces infarct size after experimental cerebral ischemia in a rabbit model. Stroke 24: 558-562.

Hahn SA, Schutte M, Hoque AT, Moskaluk CA, da Costa LT, et al. 1996. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271: 350-353.

Hanashima C, Shen L, Li SC, Lai E. 2002. Brain factor-1 controls the proliferation and differentiation of neocortical progenitor cells through independent mechanisms. J Neurosci 22: 6526-6536.

He S, Jin ML, Worpel V, Hinton DR. 2003. A role for connective tissue growth factor in the pathogenesis of choroidal neovascularization. Arch Ophthalmol 121: 1283-1288.

Heinrich-Noack P, Prehn JH, Krieglstein J. 1996. TGF-beta 1 protects hippocampal neurons against degeneration caused by transient global ischemia. Dose-response relationship and potential neuroprotective mechanisms. Stoke 27: 1609-1614.

Herrera-Molina R, von Bernhardi R. 2005. Transforming growth factor-beta 1 produced by hippocampal cells modulates microglial reactivity in culture. Neurobiol Dis 19: 229-236.

Hogan BL. 1996. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 10: 1580-1594.

Howard MJ, Gershon MD. 1993. Role of growth factors in catecholaminergic expression by neural crest cells: in vitro effects of transforming growth factor beta 1. Dev Dyn 196: 1-10.

Hunter KE, Sporn MB, Davies AM. 1993. Transforming growth factor-betas inhibit mitogen-stimulated proliferation of astrocytes. Glia 7: 203-211.

Hyytiäinen M, Penttinen C, Keski-Oja J. 2004. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. Crit Rev Clin Lab Sci 41, 233-264.

Ishihara A, Saito H, Abe K. 1994. Transforming growth factor-beta 1 and -beta 2 promote neurite sprouting and elongation of cultured rat hippocampal neurons. Brain Res 639: 21-25.

Itoh S, Itoh F, Goumans MJ, ten Dijke P. 2000. Signaling of transforming growth factor-beta family members through Smad proteins. Eur J Biochem 267: 6954-6967.

Jachimczak P, Bogdahn U, Schneider J, Behl C, Meixensberger J, et al. 1993. The effect of transforming growth factor-beta 2-specific phosphorothioate-anti-sense oligodeoxynucleotides in reversing cellular immunosuppression in malignant glioma. J Neurosurg 78: 944-951.

Jakowlew SB, Dillard PJ, Kondaiah P, Sporn MB, Roberts AB. 1988a. Complementary DNA cloning of a novel transforming growth factor-β mRNA from chick embryo chondrocytes. Mol Endocrinol 2: 747-755.

Jakowlew SB, Dillard PJ, Sporn MB, Roberts AB. 1988b. Complementary DNA cloning of an mRNA encoding transforming growth factor-beta 4 from chick embryo chondrocytes. Mol Endocrinol 2: 1186-1195.

Johns LD, Babcock G, Green D, Freedman M, Sriram S, et al. 1992. Transforming growth factor-beta 1 differentially regulates proliferation and MHC class-II antigen expression in forebrain and brainstem astrocyte primary cultures. Brain Res 585: 229-236.

Jones LL, Kreutzberg GW, Raivich G. 1998. Transforming growth factor beta's 1, 2 and 3 inhibit proliferation of ramified microglia on an astrocyte monolayer. Brain Res 795: 301-306.

Jonk LJ, Itoh S, Heldin CH, ten Dijke P, Kruijer W. 1998. Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer. J Biol Chem 273: 21145-21152.

Jung B, Kim MO, Yun SJ, Lee EH. 2003. Down-regulation of the expression of rat inhibitor-of-apoptosis protein-1 and -3 during transforming growth factor-beta1-mediated apoptosis in rat brain microglia. Neuroreport 14: 857-860.

Kang Y, Chen C-R, Massague J. 2003. A self-enabling TGF-β response coupled to stress signalling: Smad engages stress response factor ATF3 for Id1 repression in epithelial cells. Mol Cell 11: 915-926.

Kingsley DM. 1994. The TGF-β superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev 8: 133-146.

Klempt ND, Sirimanne E, Gunn AJ, Klempt M, Singh K, et al. 1992. Hypoxia–ischemia induces transforming growth factor beta 1 mRNA in the infant rat brain. Mol Brain Res 13: 93-101.

Knuckey NW, Finch P, Palm DE, Primiano MJ, Johanson CE, et al. 1996. Differential neuronal and astrocytic expression of transforming growth factor beta isoforms in rat hippocampus following transient forebrain ischemia. Mol Brain Res 40: 1-14.

Kondaiah P, Sands MJ, Smith JM, Fields A, Roberts AB, et al. 1990. Identification of a novel transforming growth factor-β mRNA in *Xenopus laevis*. J Biol Chem 265: 1089-1093.

Konig HG, Kogel D, Rami A, Prehn JH. 2005. TGF-β1 activates two distinct type I receptors in neurons: implications for neuronal NF-κB signaling. J Cell Biol 168: 1077-1086.

Krieglstein K, Farkas L, Unsicker K. 1998a. TGF-beta regulates the survival of ciliary ganglionic neurons synergistically with ciliary neurotrophic factor and neurotrophins. J Neurobiol 37: 563-572.

Krieglstein K, Henheik P, Farkas L, Jaszai J, Galter D, et al. 1998b. Glial cell line-derived neurotrophic factor requires transforming growth factor-beta for exerting its full neurotrophic potential on peripheral and CNS neurons. J Neurosci 18: 9822-9834.

Krieglstein K, Reuss B, Maysinger D, Unsicker K. 1998c. Short communication: transforming growth factor-beta mediates the neurotrophic effect of fibroblast growth factor-2 on midbrain dopaminergic neurons. Eur J Neurosci 10: 2746-2750.

Krieglstein K, Richter S, Farkas L, Schuster N, Dünker N, et al. 2000. Reduction of endogenous transforming growth factors beta prevents ontogenetic neuron death. Nat Neurosci 3: 1085-1090.

Krieglstein K, Suter-Crazzolara C, Fischer WH, Unsicker K. 1995. TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. EMBO J 14: 736-742.

Krieglstein K, Unsicker K. 1994. Transforming growth factor-beta promotes survival of midbrain dopaminergic neurons and protects them against *N*-methyl-4-phenylpyridinium ion toxicity. Neuroscience 63: 1189-1196.

Krieglstein K, Unsicker K. 1996. Distinct modulatory actions of TGF-beta and LIF on neurotrophin-mediated survival of developing sensory neurons. Neurochem Res 21: 843-850.

Labourdette G, Janet T, Laeng P, Perraud F, Lawrence D, et al. 1990. Transforming growth factor type beta 1 modulates the effects of basic fibroblast growth factor on growth and phenotypic expression of rat astroblasts in vitro. J Cell Physiol 144: 473-484.

Lahn M, Kloeker S, Berry BS. 2005. TGF-beta inhibitors for the treatment of cancer. Expert Opin Investig Drugs 14: 629-643.

Lebrin F, Deckers M, Bertolino P, ten Dijke P. 2005. TGF-beta receptor function in the endothelium. Cardiovasc Res 65: 599-608.

Lee SJ, McPherron AC. 2001. Regulation of myostatin activity and muscle growth. Proc Natl Acad Sci USA 98: 9306-9311.

Le Roy C, Wrana JL. 2005. Signaling and endocytosis: a team effort for cell migration. Dev Cell 9: 167-168.

Leof EB, Proper JA, Goustin AS, Shipley GD, Di Corleto PE, et al. 1986. Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. Proc Natl Acad Sci USA 83: 2453-2457.

Lindholm D, Castren D, Kiefer R, Zafra F, Thoenen H. 1992. Transforming growth factor-β1 in the rat brain: increase after injury and inhibition of astrocyte proliferation. J Cell Biol 117: 395-400.

Logan A, Green J, Hunter A, Jackson R, Berry M. 1999. Inhibition of glial scarring in the injured rat brain by a recombinant human monoclonal antibody to transforming growth factor-beta2. Eur J Neurosci 11: 2367-2374.

Lou E. 2004. Oncolytic viral therapy and immunotherapy of malignant brain tumors: two potential new approaches of translational research. Ann Med 36: 2-8.

Lyons RM, Gentry LE, Purchio AF, Moses HL. 1990. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. J Cell Biol 110: 1361-1367.

Madison L, Webb NR, Rose TM, Marquardt H, Ikeda T, et al. 1988. Transforming growth factor-β2: cDNA cloning and sequence analysis. DNA 7: 1-8.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, et al. 1995. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268: 1336-1338.

Martinou JC, Le Van Thai A, Valette A, Weber MJ. 1990. Transforming growth factor beta 1 is a potent survival factor for rat embryo motoneurons in culture. Dev Brain Res 52: 175-181.

Massague J. 2000. How cells read TGF-beta signals. Nat Rev Mol Cell Biol 1: 169-178.

McDonald NQ, Hendrickson WA. 1993. A structural superfamily of growth factors containing a cystine knot motif. Cell 73: 421-424.

McKinnon RD, Piras G, Ida JA Jr, Dubois-Dalcq M. 1993. A role for TGF-beta in oligodendrocyte differentiation. J Cell Biol 121: 1397-1407.

McLennan IS, Koishi K. 2002. The transforming growth factor-betas: multifaceted regulators of the development and maintenance of skeletal muscles, motoneurons and Schwann cells. Int J Dev Biol 46: 559-567.

Mittl PR, Priestle JP, Cox DA, McMaster G, Cerletti N, et al. 1996. The crystal structure of TGF-beta 3 and comparison to TGF-beta 2: implications for receptor binding. Protein Sci 5: 1261-1271.

Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. 2002. Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells 7: 1191-1204.

Miyazono K, Suzuki H, Imamura T. 2003. Regulation of TGF-beta signaling and its roles in progression of tumors. Cancer Sci 94: 230-234.

Miyazono K, ten Dijke P, Heldin CH. 2000. TGF-beta signaling by Smad proteins. Adv Immunol 75: 115-157.

Morganti-Kossmann MC, Hans VH, Lenzlinger PM, Dubs R, Ludwig E, et al. 1999. TGF-beta is elevated in the CSF of patients with severe traumatic brain injuries and parallels blood–brain barrier function. J Neurotrauma 16: 617-628.

Moustakas A, Heldin CH. 2005. Non-Smad TGF-β signals. J Cell Sci 118: 3573-3584.

Moustakas A, Souchelnytskyi S, Heldin CH. 2001. Smad regulation in TGF-beta signal transduction. J Cell Sci 114: 4359-4369.

Mulder KM. 2000. Role of Ras and Mapks in TGFbeta signaling. Cytokine Growth Factor Rev 11: 23-35.

Mummery CL, van den Eijnden-van Raaij AJ. 1999. Developmental tumours, early differentiation and the transforming growth factor beta superfamily. Int J Dev Biol 43: 693-709.

Nagatsu T, Mogi M, Ichinose H, Togari A. 2000. Changes in cytokines and neurotrophins in Parkinson's disease. J Neural Transm Suppl 60: 277-290.

Newfeld SJ, Wisotzkey RG, Kumar S. 1999. Molecular evolution of a developmental pathway: phylogenetic analyses of transforming growth factor-beta family ligands, receptors and Smad signal transducers. Genetics 152: 783-795.

Nieder C, Schlegel J, Andratschke N, Thamm R, Grosu AL, et al. 2003. The role of growth factors in central nervous system tumours. Anticancer Res 23: 1681-1686.

Ota T, Fujii M, Sugizaki T, Ishii M, Miyazawa K, et al. 2002. Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-β in human umbilical vein endothelial cells. J Cell Physiol 193: 299-318.

Packard M, Mathew D, Budnik V. 2003. Wnts and TGF beta in synaptogenesis: old friends signalling at new places. Nat Rev Neurosci 4: 113-120.

Paques M, Massin P, Gaudric A. 1997. Growth factors and diabetic retinopathy. Diabetes Metab 23: 125-130.

Parkinson DB, Dong Z, Bunting H, Whitfield J, Meier C, et al. 2001. Transforming growth factor beta (TGFbeta) mediates Schwann cell death in vitro and in vivo: examination of c-Jun activation, interactions with survival signals, and the relationship of TGFbeta-mediated death to Schwann cell differentiation. J Neurosci 21: 8572-8585.

Patterson GI, Padgett RW. 2000. TGF beta-related pathways. Roles in *Caenorhabditis elegans* development. Trends Genet 16: 27-33.

Pelton RW, Johnson MD, Perkett EA, Gold LI, Moses HL. 1991. Expression of transforming growth factor-beta 1, -beta 2, and -beta 3 mRNA and protein in the murine lung. Am J Respir Cell Mol Biol 5: 522-530.

Pelton RW, Saxena B, Jones M, Moses HL, Gold LI. 1991. Immunohistochemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. J Cell Biol 115: 1091-1105.

Peterziel H, Unsicker K, Krieglstein K. 2002. TGFbeta induces GDNF responsiveness in neurons by recruitment of GFRalpha1 to the plasma membrane. J Cell Biol 159: 157-167.

Platten M, Wick W, Weller M. 2001. Malignant glioma biology: role for TGF-beta in growth, motility, angiogenesis, and immune escape. Microsc Res Tech 52: 401-410.

Poulsen KT, Armanini MP, Klein RD, Hynes MA, Phillips HS, et al. 1994. TGF beta 2 and TGF beta 3 are potent survival factors for midbrain dopaminergic neurons. Neuron 13: 1245-1252.

Prehn JH, Backhauss C, Krieglstein J. 1993. Transforming growth factor-beta 1 prevents glutamate neurotoxicity in rat neocortical cultures and protects mouse neocortex from ischemic injury in vivo. J Cereb Blood Flow Metab 13: 521-525.

Prehn JH, Bindokas VP, Marcuccilli CJ, Krajewski S, Reed JC, et al. 1994. Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type beta confers wide-ranging protection on rat hippocampal neurons. Proc Natl Acad Sci USA 91: 12599-12603.

Rahhal B, Dünker N, Combs S, Krieglstein K. 2004. Isoform-specific role of transforming growth factor-beta2 in the regulation of proliferation and differentiation of murine adrenal chromaffin cells in vivo. J Neurosci Res 78: 493-498.

Ramirez F, Pereira L. 1999. The fibrillins. Int J Biochem Cell Biol 31: 255-259.

Reddi AH. 2005. BMPs: from bone morphogenetic proteins to bone morphogenetic proteins. Cytokine Growth Factor Rev 16: 249-250.

Rich JN. 2003. The role of transforming growth factor-beta in primary brain tumors. Front Biosci 8: e245-e260.

Rich JN, Zhang M, Datto MB, Bigner DD, Wang XF. 1999. Transforming growth factor-beta-mediated p15(INK4B) induction and growth inhibition in astrocytes is SMAD3-dependent and a pathway prominently altered in human glioma cell lines. J Biol Chem 274: 35053-35058.

Rifkin DB. 2005. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. J Biol Chem 280: 7409-7412.

Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB. 1981. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. Proc Natl Acad Sci USA 78: 5339-5343.

Roberts AB, Sporn MB. 1990. The transforming growth factor-βs. Handbook of Experimental Pharmacology, Vol. 95. Sporn MB, Roberts AB, editors. Heidelberg: Springer-Verlag; pp. 419-472.

Roberts AB, Wakefield LM. 2003. The two faces of transforming growth factor beta in carcinogenesis. Proc Natl Acad Sci USA 100: 8621-8623.

Rooprai HK, Rucklidge GJ, Panou C, Pilkington GJ. 2000. The effects of exogenous growth factors on matrix metalloproteinase secretion by human brain tumour cells. Br J Cancer 82: 52-55.

Roussa E, Farkas LM, Krieglstein K. 2004. TGF-beta promotes survival on mesencephalic dopaminergic neurons in cooperation with Shh and FGF-8. Neurobiol Dis 16: 300-310.

Roussa E, Wiehle M, Dünker N, Becker-Katins S, Oehlke O, Krieglstein K. 2006. TGF-β isoforms (TGF-β2, TGF-β3) are required for differentiation of mouse mesencephalic progenitors into dopaminergic neurons in vitro and in vivo. Stem cells (in revision).

Salinas PC. 2005. Signaling at the vertebrate synapse: new roles for embryonic morphogens? J Neurobiol 64: 435-445.

Sanchez-Capelo A. 2005. Dual role for TGF-beta1 in apoptosis. Cytokine Growth Factor Rev 16: 15-34.

Schena FP, Gesualdo L. 2005. Pathogenetic mechanisms of diabetic nephropathy. J Am Soc Nephrol 16: S30-S33.

Scheufler C, Sebald W, Hulsmeyer M. 1999. Crystal structure of human bone morphogenetic protein-2 at 2.7 Å resolution. J Mol Biol 287: 103-115.

Schlingensiepen R, Goldbrunner M, Szyrach MN, Stauder G, Jachimczak P, et al. 2005. Intracerebral and intrathecal infusion of the TGF-beta2-specific antisense phosphorothioate oligonucleotide AP 12009 in rabbits and primates: toxicology and safety. Oligonucleotides 15: 94-104.

Schlunegger MP, Grutter MG. 1992. An unusual feature revealed by the crystal structure at 2.2 Å resolution of human transforming growth factor-beta 2. Nature 358: 430-434.

Schmidt-Weber CB, Blaser K. 2004. Regulation and role of transforming growth factor-beta in immune tolerance induction and inflammation. Curr Opin Immunol 16: 709-716.

Schober A, Hertel R, Arumae U, Farkas L, Jaszai J, et al. 1999. Glial cell line-derived neurotrophic factor rescues target-deprived sympathetic spinal cord neurons but requires transforming growth factor-beta as cofactor in vivo. J Neurosci 19: 2008-2015.

Schuster N, Bender H, Philippi A, Subramaniam S, Strelau J, et al. 2002. TGF-beta induces cell death in the oligodendroglial cell line OLI-neu. Glia 40: 95-108.

Schuster N, Bender H, Rossler OG, Philippi A, Dünker N, et al. 2003. Transforming growth factor-beta and tumor necrosis factor-alpha cooperate to induce apoptosis in the oligodendroglial cell line OLI-neu. J Neurosci Res 73: 324-333.

Schuster N, Krieglstein K. 2002. Mechanisms of TGF-beta-mediated apoptosis. Cell Tissue Res 307: 1-14.

Sebald W, Nickel J, Zhang JL, Mueller TD. 2004. Molecular recognition in bone morphogenetic protein (BMP)/receptor interaction. Biol Chem 385: 697-710.

Seoane J, Le HV, Shen L, Anderson SA, Massague J. 2004. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell 117: 211-223.

Seyedin PR, Segarini PR, Rosen DM, Thompson AY, Bentz H, et al. 1987. Cartilage-inducing factor-B is a unique protein structurally and functionally related to transforming growth factor-beta. J Biol Chem 262: 1946-1949.

Seyedin PR, Thomas TC, Thompson AY, Rosen DM, Piez KA. 1985. Purification and characterization of two cartilage-inducing factors from bovine demineralised bone. Proc Natl Acad Sci USA 82: 2267-2271.

Shi Y, Massague J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113: 685-700.

Shrikant P, Lee SJ, Kalvakolanu I, Ransohoff RM, Benveniste EN. 1996. Stimulus-specific inhibition of intracellular adhesion molecule-1 gene expression by TGF-beta. J Immunol 157: 892-900.

Siegel PM, Massague J. 2003. Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer 3: 807-821.

Specht H, Peterziel H, Bajohrs M, Gerdes HH, Krieglstein K, et al. 2003. Transforming growth factor beta2 is released from PC12 cells via the regulated pathway of secretion. Mol Cell Neurosci 22: 75-86.

Stelzer C, Winterpacht A, Spranger J, Zabel B. 2003. Grebe dysplasia and the spectrum of CDMP1 mutations. Pediatr Pathol Mol Med 22: 77-85.

Swindells MB. 1992. Structural similarity between transforming growth factor-beta 2 and nerve growth factor. Science 258: 1160-1161.

ten Dijke P, Geurts van Kessel AHM, Foulkes JG, Le Beau MM. 1988b. Transforming growth factor-beta type 3 maps to human chromosome 14, region q23–q24. Oncogene 3: 721-724.

ten Dijke P, Hanson P, Iwata KK, Pieler C, Foulkes JC. 1988a. Identification of a new member of the transforming growth factor-β gene family. Proc Natl Acad Sci USA 85: 4715-4719.

ten Dijke P, Hill CS. 2004. New insights into TGF-beta-Smad signalling. Trends Biochem Sci 29: 265-273.

Thomas JT, Kilpatrick MW, Lin K, Erlacher L, Lembessis P, et al. 1997. Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1. Nat Genet 17: 58-64.

Toepfer M, Fischer P, Abicht A, Lochmuller H, Pongratz D, et al. 1999. Localization of transforming growth factor beta in association with neuromuscular junctions in adult human muscle. Cell Mol Neurobiol 19: 297-300.

Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell 95: 779-791.

Turgeman G, Zilberman Y, Zhou S, Kelly P, Moutsatsos IK, et al. 2002. Systemically administered rhBMP-2 promotes MSC activity and reverses bone and cartilage loss in osteopenic mice. J Cell Biochem 86: 461-474.

Uhm JH, Gladson CL, Rao JS. 1999. The role of integrins in the malignant phenotype of gliomas. Front Biosci 4: D188-D199.

Unsicker K, Flanders KC, Cissel DS, Lafyatis R, Sporn MB. 1991. Transforming growth factor beta isoforms in the adult rat central and peripheral nervous system. Neuroscience 44: 613-625.

Unsicker K, Meier C, Krieglstein K, Sartor BM, Flanders KC. 1996. Expression, localization, and function of transforming growth factor-beta s in embryonic chick spinal cord, hindbrain, and dorsal root ganglia. J Neurobiol 29: 262-276.

van der Wal EA, Gomez-Pinilla F, Cotman CW. 1993. Transforming growth factor-beta 1 is in plaques in Alzheimer and Down pathologies. Neuroreport 4: 69-72.

Verrecchia F, Chu M-L, Mauviel A. 2001. Identification of novel TGF-β/Smad gene targets in dermal fibroblasts using a combined cDNA microarray/promoter transactivation approach. J Biol Chem 276: 17058-17062.

Wahl SM, Chen W. 2003. TGF-beta: how tolerant can it be? Immunol Res 28: 167-179.

Waite KA, Eng C. 2003. From developmental disorder to heritable cancer: it's all in the BMP/TGF-beta family. Nat Rev Genet 4: 763-773.

Wu XB, Li Y, Schneider A, Yu W, Rajendren G, Iqbal J, et al. 2003. Impaired osteoblastic differentiation, reduced bone formation, and severe osteoporosis in noggin-overexpressing mice. J Clin Invest 112: 924-934.

Wyss-Coray T, Borrow P, Brooker MJ, Mucke L. 1997a. Astroglial overproduction of TGF-beta 1 enhances inflammatory central nervous system disease in transgenic mice. J Neuroimmunol 77: 45-50.

Wyss-Coray T, Lin C, Yan F, Yu GQ, Rohde M, et al. 2001. TGF-beta1 promotes microglial amyloid-beta clearance and reduces plaque burden in transgenic mice. Nat Med 7: 612-618.

Wyss-Coray T, Masliah E, Mallory M, McConlogue L, Johnson-Wood K, et al. 1997b. Amyloidogenic role of cytokine TGF-beta1 in transgenic mice and in Alzheimer's disease. Nature 389: 603-606.

Xiao BG, Bai XF, Zhang GX, Link H. 1997. Transforming growth factor-beta1 induces apoptosis of rat microglia without relation to bcl-2 oncoprotein expression. Neurosci Lett 226: 71-74.

Xie L, Law BK, Aakre ME, Edgerton M, Shyr Y, et al. 2003. Transforming growth factor beta-regulated gene expression in a mouse mammary gland epithelial cell line. Breast Cancer Res 5: R187-R198.

Xuan S, Baptista CA, Balas G, Tao W, Soares VC, et al. 1995. Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. Neuron 14: 1141-1152.

Yamashita K, Gerken U, Vogel P, Hossmann K, Wiessner C. 1999. Biphasic expression of TGF-beta1 mRNA in the rat brain following permanent occlusion of the middle cerebral artery. Brain Res 836: 139-145.

Yang Y-C, Piek E, Zavadil J, Liang D, Xie D, et al. 2003. Hierarchical model of gene regulation by transforming growth factor-β. Proc Natl Acad Sci USA 100: 10269-10274.

Yingling JM, Blanchard KL, Sawyer JS. 2004. Development of TGF-beta signalling inhibitors for cancer therapy. Nat Rev Drug Discov 3: 1011-1022.

Zavadil J, Bitzer M, Liang D, Yang Y-C, Massimi A, et al. 2001. Genetic programs of epithelial cell plasticity directed by transforming growth factor-β. Proc Natl Acad Sci USA 98: 6686-6691.

Zhang F, Endo S, Cleary LJ, Eskin A, Byrne JH. 1997a. Role of transforming growth factor-beta in long-term synaptic facilitation in Aplysia. Science 275: 1318-1320.

Zhang JM, Hoffmann R, Sieber-Blum M. 1997b. Mitogenic and anti-proliferative signals for neural crest cells and the neurogenic action of TGF-beta1. Dev Dyn 208: 375-386.

Zhao X, Ramsey KE, Stephan DA, Russell P. 2004. Gene and protein expression changes in human trabecular meshwork cells treated with transforming growth factor-β. Invest Ophthalmol Vis Sci 45: 4023-4034.

Zhu Y, Ahlemeyer B, Bauerbach E, Krieglstein J. 2001. TGF-beta1 inhibits caspase-3 activation and neuronal apoptosis in rat hippocampal cultures. Neurochem Int 38: 227-235.

Zhu Y, Culmsee C, Klumpp S, Krieglstein J. 2004. Neuroprotection by transforming growth factor-beta1 involves activation of nuclear factor-kappaB through phosphatidylinositol-3-OH kinase/Akt and mitogen-activated protein kinase-extracellular-signal regulated kinase1,2 signaling pathways. Neuroscience 123: 897-906.

Zhu Y, Roth-Eichhorn S, Braun N, Culmsee C, Rami A, et al. 2000. The expression of transforming growth factor-beta1 (TGF-beta1) in hippocampal neurons: a temporary upregulated protein level after transient forebrain ischemia in the rat. Brain Res 866: 286-298.

Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, et al. 2002. Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. J Neurosci 22: 3898-3909.

Zimmers TA, Davies MV, Koniaris LG, Haynes P, Esquela AF, et al. 2002. Induction of cachexia in mice by systemically administered myostatin. Science 296: 1486-1488.

Zindy F, Cunningham JJ, Sherr CJ, Jogal S, Smeyne RJ, et al. 1999. Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. Proc Natl Acad Sci USA 96: 13462-13467.

# 7 IGF-1 in Brain Growth and Repair Processes

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**Abstract:** This chapter addresses the role of insulin-like growth factor 1 (IGF-1) and the IGF-1 receptor (IGF1R) in brain development, injury response, and aging. We concentrate mainly on recent information from murine model systems, with consideration of interesting and relevant data from invertebrates and humans. IGF-1 and its cognate receptor are both highly expressed in the developing brain, supporting both autocrine and paracrine activity for this anabolic peptide. IGF-1 deletion or inhibition during brain development attenuates brain growth, with reductions in both cell number and cell size. Cell numbers are notably reduced in the olfactory system, the dentate gyrus of the hippocampus, and the striatum. Brain volume is globally decreased due to a loss of neuropil, with significant reductions in neuronal soma volume, dendritic length and complexity, and synapse number. Myelination is reduced in proportion to the decreases in neuron number and nerve processes in the IGF-1-null brain. Conversely, transgenic IGF-1 overexpression results in increased brain size with increases in cell number, cell size, and dendrite growth with proportionate increases in myelination. Metabolic activity as measured by glucose utilization is significantly decreased in the IGF-1-null brain and increased in the transgenic IGF-1-overexpressing brain. IGF-1 deletion in humans is associated with mental retardation and sensorineural deafness. IGF-1 deletion is also associated with deafness in mice, but no other obvious neurological or behavioral phenotypes have been identified.

IGF-1 prevents neuronal death in response to a variety of insults in vitro, but cell death appears to be a minor effect in the IGF-1-null brain. IGF-1's physiological effects in brain depend on when and where the peptide is expressed. For example, IGF-1 is expressed in an olfactory neuron germinal zone early in development, enhancing proliferation of these neurons, which are correspondingly reduced in number in the IGF-1-null mouse. IGF-1 is expressed in long-axon projection neurons at a later, postmitotic stage, promoting somatic and dendritic growth for these neurons, which are normal in number but small with hypotrophic dendritic arbors in the IGF-1-null brain. Increased circulating or brain IGF-1 is associated with increased hippocampal neurogenesis in adult rodents, and treatment with exogenous IGF-1 may protect against neurodegeneration in response to brain injury. IGF-1's anabolic effects in brain are executed via the IRS2-PI3K-Akt signaling system. The multifunctional enzyme glycogen synthase kinase 3 (GSK3) is a major target of this pathway. Inhibitory phosphorylation of GSK3 by IGF-1 enhances glucose utilization and protein synthesis, promoting somatic growth and dendritogenesis in IGF-1-expressing projection neurons. Brain IGF-1 also inhibits the phosphorylation of tau, a microtubule-associated protein, via the PI3K-Akt-GSK3 pathway. This neurofibrillary tangle (NFT) protein is hyperphosphorylated in both IGF-1- and IRS2-null brains. IGF-1's role in brain aging is unclear at present. Data obtained from worms to primates suggest that suppression of the IGF system slows the aging process, but it is not yet known if brain aging is altered in IGF-1-null or -deficient mice.

**List of Abbreviations:** AD, Alzheimer's disease; Akt, serine/threonine protein kinase; BAD, bcl-associated death promoter; BBB, blood–brain barrier; BRDU, bromodeoxyuridine; CNPase, 2′,3′-cyclic nucleotide, 3′-phosphodiesterase; EGF, epidermal growth factor; eIF2B, eukaryotic initiation factor 2B; FOXO, forkhead transcription factors; GH, growth hormone; GLUT, glucose transporter; GSK3, glycogen synthase kinase 3; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; IGF1R, IGF-1 receptor; IRS, insulin receptor substrate; MAG, myelin-associated protein; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor kappa B; NFT, neurofibrillary tangle; NO, nitric oxide; PCR, polymerase chain reaction; PDK1, 2, 3-phosphoinositide-dependent protein kinase 1, 2; PI3K, phosphoinositide-3 kinase; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PLP, myelin proteolipid protein; PTEN, phosphatase and tensin homolog; S6K, ribosomal protein S6 kinase

## 1 Introduction

Members of the insulin-like growth factor (IGF) family, including insulin, IGF-1, and IGF-2, promote carbohydrate, lipid, and protein metabolism in support of cell growth and survival. Insulin has a specialized role in peripheral glucose homeostasis, and a neuroendocrine role at the hypothalamic level, promoting the

integration of nutrient acquisition, storage, and expenditure. IGF-1 promotes postnatal somatic growth while IGF-2 promotes similar, proportionate growth in utero. Insulin deficiency leads to the metabolic derangements of diabetes mellitus, while IGF deficiency is associated with proportionate dwarfism. The fundamental importance of this insulin/IGF system is reflected by the fact that insulin and IGF peptides and receptor homologs are found in evolutionarily distant and diverse organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*. Recent genetic studies have shown that the insulin:IGF-1 receptor/PI3K/Akt signaling pathway is largely conserved down to the metazoan level and plays an essential role in regulating life span as well as body, organ, and cell size (Finch and Ruvkun, 2001).

An interesting feature observed in Drosophila is that insulin-like peptides are expressed in neural cells in the brain. Ablation of these "neurons" causes developmental delay and growth retardation (Rulifson et al., 2002). In fact, single-gene mutations targeting the insulin/IGF receptor or downstream signaling components result in significant extension of the life span in yeast, nematodes, fruit fly, and rodents (reviewed in Richardson et al., 2004; Katic and Kahn, 2005). In many cases, these animals are healthier than normal, like animals on food-restricted diets, although fecundity may be impaired. Thus it seems that the insulin/IGF system promotes anabolic effects that increase growth rate and fertility, but also accelerates the aging process through impaired responses to oxidative and other types of stress. Suppressed insulin/IGF signaling impairs somatic growth, but minimizes damage to and increases repair of cell macromolecules. The question is how these anabolic growth-promoting and age-accelerating peptides function in the brain. IGF-1 is clearly important in brain development and function, as individuals homozygous for mutations/deletions in *IGF1* are profoundly mentally retarded (Woods et al., 1997; Bonapace et al., 2003; Walenkamp et al., 2005).

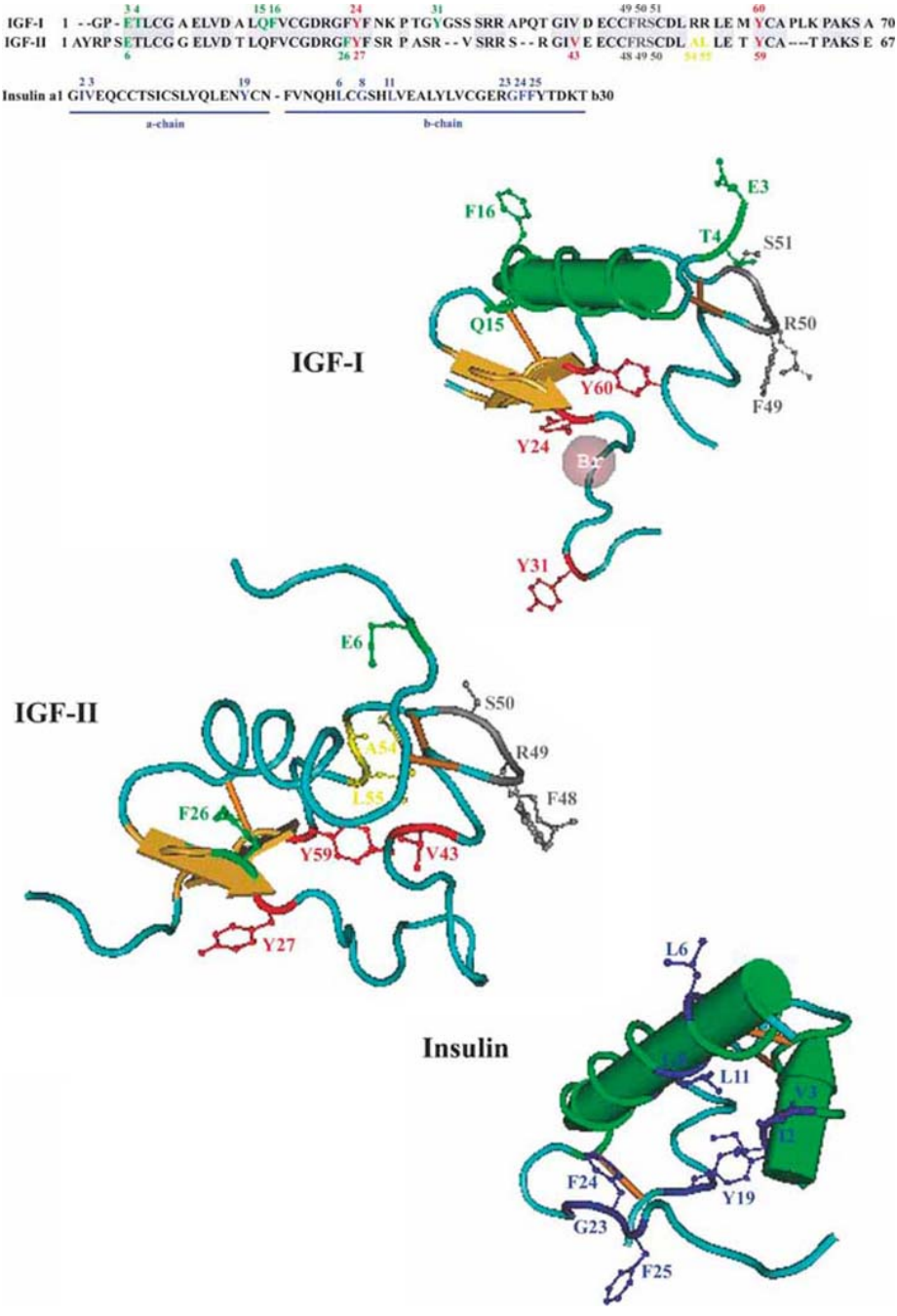
## 2 IGF/Insulin Peptides and Binding Proteins

IGF-1, IGF-2, and insulin (❯ *Figure 7-1*) belong to an ancient family of peptides sharing a common evolutionary origin (LeRoith et al., 1986; LeRoith et al., 1993; Reinecke and Collet, 1998; Navarro et al., 1999). An ancestral gene encoding an insulin-like peptide gave rise to multiple genes encoding more specialized peptides about the time gastroenteric and central nervous systems (CNS) differentiated (Reinecke and Collet, 1998). From that time insulin became progressively more specialized in terms of secondary processing (proteolytic excision of the "C" peptide and joining of the A and B peptides by disulfide bonds), packaging in acidic secretory granules, and association with the gastrointestinal tract. Insulin expression is largely restricted to pancreatic beta cells, where its synthesis and secretion are tightly coupled to ingested substrates (Tager et al., 1981). IGF-1 and IGF-2, in contrast, did not acquire such extensive posttranslational processing, and have continued to be widely expressed in many cell types demonstrating constitutive secretion (Clemmons, 1989; Sussenbach, 1989). IGF-1 and IGF-2 are single-chain polypeptides of 70 amino acids with three intramolecular disulfide bridges. The IGFs share about 50% homology with insulin in amino acid sequences in addition to very similar tertiary structures and functional binding sites (❯ *Figure 7-1*).

In mammals, insulin production is centrally localized in the beta cells of the pancreas, from which insulin is released in bolus fashion in response to nutrient stimuli. Insulin serves in classic endocrine hormone fashion to regulate glucose, lipid, and protein metabolism in many peripheral tissues, but excluding the brain. IGF-1 is produced in great abundance by the liver where its synthesis is regulated by pituitary growth hormone (GH) (Laron, 2001). IGF-1 is also synthesized locally in many tissues (Daughaday and Rotwein, 1989; Le Roith et al., 2001), including the brain (Rotwein et al., 1988; Bartlett et al., 1991; Bondy, 1991), where GH does not regulate its synthesis (Wang et al., 1999; Lupu et al., 2001; Sun et al., 2005). Circulating insulin levels peak after meals but are very low most other times, while circulating IGF-1 levels are severalfold higher than insulin, and stable around the clock. Also, in contrast to insulin, IGFs in the circulation and interstitial fluids are bound to high-affinity IGF-binding proteins (IGFBPs) that prolong IGF half-life by impeding proteolysis and renal clearance (Clemmons, 1998; Duan, 2002). Insulin levels are relatively stable throughout the life span in normal individuals, while IGF-1 levels peak during childhood and decline steadily as people age (Laron, 2001).

**Figure 7-1**

**Comparison of amino acid sequence and predicted tertiary structure of the IGF-1, IGF-2, and insulin peptides. This figure was reproduced from article "Insulin-like growth factor ligands, receptors, and binding proteins in cancer" by Foulstone et al. 2005. J Pathol 205: 148. Copyright of the Pathological Society of Great Britain and Ireland. Reproduced with permission from John Wiley and Sons**



IGFs serve as endocrine and paracrine/autocrine regulators of somatic growth both in utero (IGF-2) and during postnatal growth (IGF-1) (Sara and Carlsson-Skwirut, 1986; Daughaday and Rotwein, 1989; Baker et al., 1993). IGF-2 is important for somatic growth during embryonic development, but its role, if any, during postnatal life is unknown. Targeted gene deletion showed that IGF-2 expression is imprinted, that is, preferentially expressed from the paternal allele in most tissues, with deletion of the paternal allele producing a 30–40% reduction in somatic size (DeChiara et al., 1990), but even homozygous IGF-2 deletion produces no discernible effect on the CNS or peripheral nervous system (PNS) (C.A. Bondy and R.R. Reinhardt, unpublished data). Indeed, IGF-2 overexpression in brain appears to have no effect on brain size or structure or mouse behavior (Reijnders et al., 2004). IGF-2's lack of any apparent effect in brain development may be explained by the brain's high-level expression of the IGF-2-mannose-6-phosphate receptor, which sequesters IGF-2 into lysosomes (Hawkes and Kar, 2004).

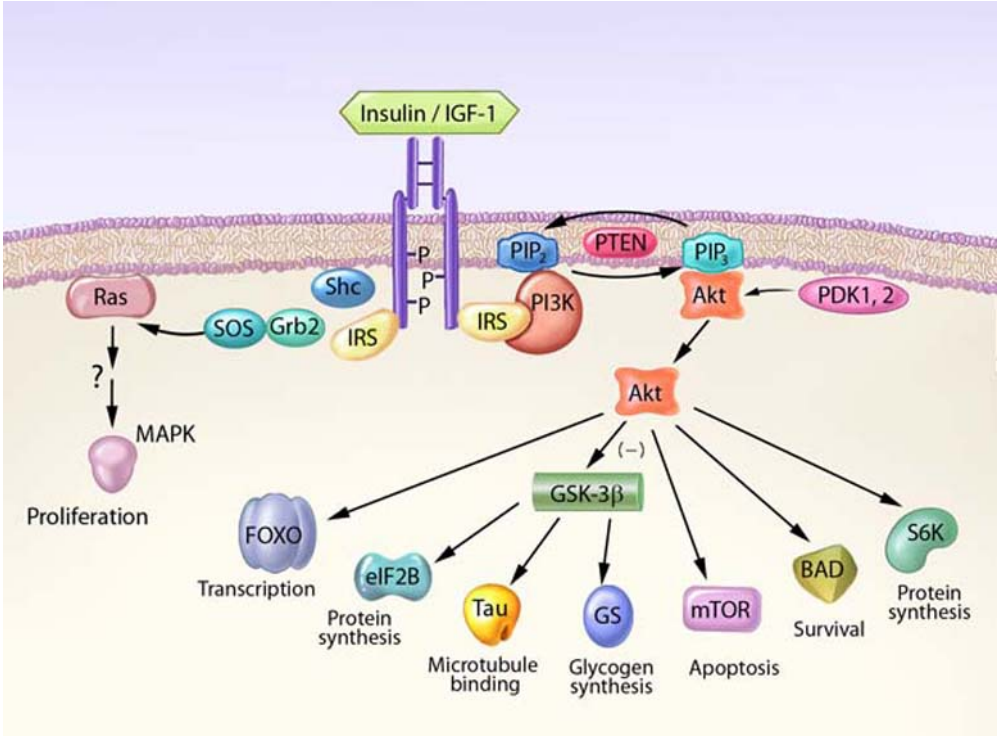
IGF-1 and IGF-2 bind with high affinity to a number of IGFBPs, which protect the IGFs from proteolysis and modulate their interaction with their receptor (Clemmons, 1998). IGFBPs are expressed in the brain in addition to being expressed in diverse peripheral tissues (Bondy and Lee, 1993a; Brar and Chernausek, 1993; Lee and Bondy, 1993; Lee et al., 1993; Logan et al., 1994; Sullivan and Feldman, 1994; Ye and D'Ercole, 1998). IGFBP2 and 5 are most abundant in the brain, and are expressed in spatiotemporal coordination with IGF-1 (Lee et al., 1992b; Lee et al., 1993). Early in development IGFBP5 messenger RNA (mRNA) is concentrated in germinal zones and is colocalized with IGF-1 in developing sensory and cerebellar relay neurons (Bondy and Lee, 1993a), and IGF-1 appears to induce IGFBP5 expression (Ye and D'Ercole, 1998). IGFBP2 mRNA is concentrated in astroglia adjacent to IGF-1-expressing neurons (Lee et al., 1993) and colocalizes with IGF-2 in the meninges and choroid plexi (Logan et al., 1994). IGFBP2 is also highly abundant in capillary endothelium, median eminence, and other circumventricular sites (Lee et al., 1993), suggesting a potential role in carrier-mediated transcytosis of circulating IGFs into the brain. Thus, each IGFBP may play a specific role in modulating IGF-1's bioactivity in brain development. These theoretical modulatory roles appear nonessential, however, since targeted deletion of IGFBPs, singly or in combination, produces no apparent neurological phenotype (J. Pintar, personal communication).

## 3 IGF-1/Insulin Receptors and Signaling Pathways

Like the cognate peptides, the insulin and IGF-1 receptors (IGF1Rs) demonstrate close structural homology and sequence identity (reviewed in Clemmons, 1989; LeRoith, 1996), having evolved from a common ancestor, in parallel with the ligands' evolution (LeRoith et al., 1993; Reinecke and Collet, 1998; Navarro et al., 1999). The type 1 IGF receptor, or the IGF1R, actually binds and transduces both IGF-1 and IGF-2 with high affinity. There is a so-called IGF-2-mannose-6-phosphate receptor unrelated to the insulin/IGF receptor family (Kiess et al., 1988) that binds and clears IGF-2 by sequestration into lysosomes (Wylie et al., 2003). Insulin and IGFs bind their cognate receptors with highest affinity, but cross-reactivity occurs at higher hormone concentrations (Clemmons, 1989). The insulin and IGF1Rs are membrane-bound tyrosine kinases that are covalent dimers in the absence of ligand. One molecule of insulin or IGF binds to the extracellular alpha-chains, triggering transautophosphorylation of the intracellular beta-chains (Luo et al., 1999). The tyrosine kinase domains of the insulin and IGF1Rs are highly conserved, with ~85% amino acid sequence identity (Hubbard, 1999) and very similar tertiary structures (Favelyukis et al., 2001). Not unexpectedly, the two receptors engage the same signaling pathways (❯ *Figure 7-2*). Receptor activation triggers phosphorylation of IRS proteins, which serve as binding sites for proteins containing src homology 2 domains, including the p85 regulatory subunit of phosphoinositide-3 kinase (PI3K). Activation of PI3K leads to the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which triggers phosphoinositide-dependent kinases to activate the ser/thr kinase Akt (also known as protein kinase B), upon recruitment to the plasma membrane (Summers and Birnbaum, 1997). The lipid phosphatase, PTEN, negatively impacts this pathway by dephosphorylating PIP3. Activated Akt, through subsequent phosphorylation of several downstream targets, is primarily responsible for the ability of this family of growth factors to stimulate glucose uptake and protein synthesis culminating in cell growth (reviewed in Saltiel and Kahn, 2001). The ras/MAPK pathway has also been associated with insulin/IGF receptor activation (❯ *Figure 7-2*) in studies on cultured

**Figure 7-2**

**Schematic diagram of signaling pathways involved in IGF-1's activity in brain. Ligand binding to insulin/IGF-1 receptors (IGF1Rs) triggers receptor autophsophorylation and association with IRS-docking proteins. Activation of phosphoinositide-3 kinase (PI3K) generates phospholipids that activate Akt. Akt may then interact with multiple downstream substrates, including GSK-3β, FOXO, eIF2B, mTOR, bcl-associated death promoter (BAD), and S6K. For example, Akt serine phosphorylates GSK3-β, causing its inhibition. Since GSK-3β normally inhibits glycogen synthase and eIF2B, inactivation of GSK-3β promotes both glycogen and protein synthesis. The microtubule-associated protein tau is also a target for GSK-3β and is hyperphosphorylated in the IGF-1-null brain, providing further evidence that IGF-1 normally inhibits brain GSK-3β activity. The MAPK pathway has been implicated in insulin/IGF action by in vitro studies and some in vivo observations on peripheral tissues, but its relevance to IGF action in brain is unknown**




cells. The in vivo significance of this association remains unclear, since most of the known physiological effects of insulin/IGF-1 involve the PI3K-Akt pathway (Katic and Kahn, 2005). This review focuses on the latter pathway, which has been specifically implicated in IGF signaling in the brain.

## 3.1 IGF-1 Signaling in Brain

Activation of PI3K and Akt kinase is central to insulin/IGF-1-induced anabolic effects. For example, Akt activation results in translocation of glucose transporters (GLUTs), from intracellular pools to the plasma membrane, promoting glucose entry into cells (Kohn et al., 1996; Summers and Birnbaum, 1997). In the periphery, glucose transport is promoted by insulin at the insulin receptor, activating the IRS/PI3K/Akt system, but in the brain, IGF-1 is responsible for local glucose transport and utilization by the same pathway (Cheng et al., 2000). IGF-1-induced Akt phosphorylation appears linked to translocation of neuronal

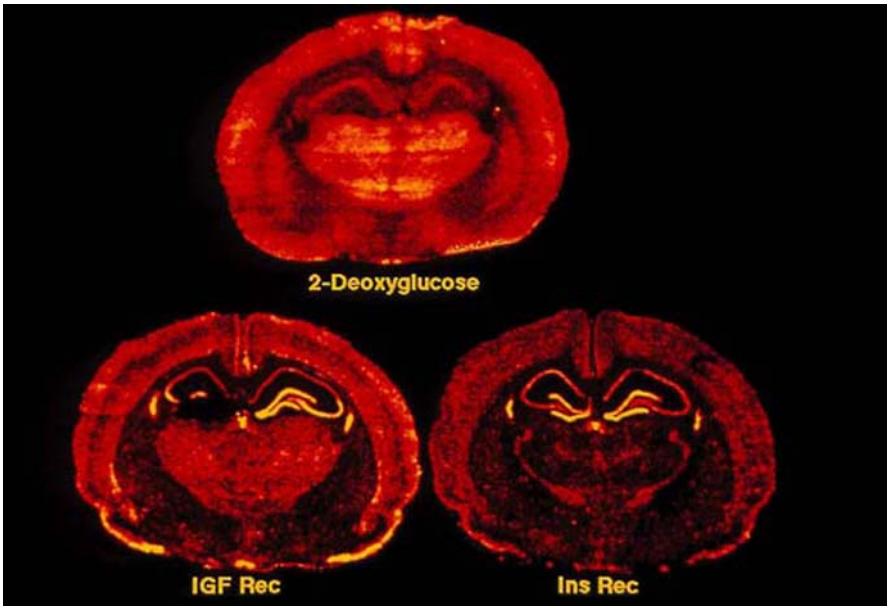
GLUT4 from intracellular pools to membranes of nerve processes in the normal developing brain. Another target of insulin/IGF signaling via Akt kinase is glycogen synthase kinase 3 (GSK3). Insulin and IGF-1 both stimulate the inhibitory serine phosphorylation of this multifunctional enzyme in neurons (Hong and Lee, 1997). Insulin/IGF-induced inhibitory phosphorylation of GSK3 (Summers et al., 1999) relieves GSK3's inhibition of glycogen synthase and of the eukaryotic translation initiation factor 2B (eIF2B), thus promoting glycogen and protein synthesis. The convergence of IGF1R, phospho-Akt, membranous GLUT4, phospho-GSK3, and abundant glycogen stores specifically in large IGF-1-expressing neurons (Cheng et al., 2000) suggests that IGF-1 acts in a cell-autonomous or in an autocrine manner, via the PI3K-Akt-GSK3 pathway, to promote nutrient acquisition, protein, and lipid synthesis supporting the growth of maturing projection neurons.

## 3.2 IGF-1 and Brain Glucose Utilization

Reflecting IGF-1's role in brain glucose utilization, IGF-1 and IGF1R expression closely parallel regional glucose utilization (see *Figure 7-3*, Cheng et al., 2000). High-level IGF-1 expression is seen in concert with

**Figure 7-3**

**Autoradiography comparing gene expression patterns for the IGF-1 receptor (IGF1R), the insulin receptor, and 2-deoxyglucose uptake in the early postnatal rat brain**




intense glucose uptake in maturing cerebellar, somatosensory, auditory–vestibular, olfactory, and visual system neurons. Glucose utilization is reduced by 30–60% in the IGF-1-null brain, with the greatest decrease in structures where IGF-1 expression is normally highest (Cheng et al., 2000). The defect in glucose utilization is demonstrable at the nerve terminal level in synaptosomes prepared from IGF-1-null brains, and is completely reversed by IGF-1, showing that the defect in glucose utilization is not due to reduced neural activity or reduced brain blood flow, neither of which affects the synaptosome preparation. Furthermore, the finding of reduced glucose uptake in isolated nerve terminals shows that IGF-1 normally

promotes glucose uptake by nerve terminals independent of glial effects, since glial cells are not present in the synaptosome preparation.

Conversely, brain glucose utilization is globally increased in IGF-1-overexpressing adult mice (Gutierrez-Ospina et al., 1997). It is not certain which cell types are responsible for the ectopic IGF-1 expression in these mice, but apparently IGF-1 is in excess through most of the brain for much of development. Thus, the generalized increase in glucose use likely reflects local field potentials originating from more highly ramified dendritic arbors with greater synaptic density in IGF-1-overexpressing brains, along with direct IGF-1-enhanced glucose transport and utilization. The fact that pentobarbital anesthesia suppressed glucose uptake in both transgenic and wild-type (WT) mice in that study was thought to suggest that IGF-1 does not promote brain glucose utilization (Gutierrez-Ospina et al., 1997). However, pentobarbital interferes with GLUT function per se (Haspel et al., 1999) and so suppresses any stimulus of glucose transport.

## 4 IGF-1 Versus Insulin in the Brain

A theory that neurodegeneration in the aging human brain may be linked to loss of insulin trophic effects has recently been put forward (Hoyer, 2004). Despite the fact that there is very little insulin within the brain, both the insulin and IGF1Rs are widely expressed in the developing and mature brain (*Figure 7-3*) (Hill et al., 1986; Bohannon et al., 1988; Bondy et al., 1992a, b). IGF-1 and insulin receptors are coexpressed in many brain regions, such as the granule cell layers of the olfactory bulb, dentate gyrus, and cerebellar cortex (Bondy et al., 1992a). The insulin receptor is most highly expressed in anterior thalamic and hypothalamic nuclei, including the periventricular, reticular, and anterior thalamic nuclear complex and the paraventricular and supraoptic nuclei (*Figure 7-3*) (Bondy et al., 1992a, b), consistent with insulin's neuroendocrine role in connecting peripheral metabolic signals to central control of appetite and metabolic activity (reviewed in Porte et al., 2005). The significance of insulin receptor expression throughout the brain is unclear, given that little insulin is found in brain outside the hypothalamus. One explanation is that the ancestral insulin/IGF receptor was heavily expressed in the nervous system, and that regulation of "offspring" receptor gene expression continued this pattern, despite the evolutionary specialization of the ligand insulin as regulator of peripheral metabolism.

Circulating insulin and IGF-1 may influence hypothalamic and other periventricular regions by interacting with receptors localized in the median eminence and circumventricular structures outside the blood–brain barrier (BBB). Both insulin and IGF1Rs are expressed on brain capillaries, but IGF-1 crosses the BBB with greater efficiency than insulin (Reinhardt and Bondy, 1994). A number of factors may explain IGF-1's relative facility in crossing the BBB. The coexpression of insulin and IGF1Rs in brain capillary endothelium may result in formation of hybrid receptors, which bind IGF-1 with substantially greater affinity than insulin (Soos et al., 1993). In addition, IGFBP2 is abundant in capillary endothelium, median eminence, and other circumventricular sites (Lee et al., 1993), suggesting possible carrier-mediated IGF transport across the BBB. While IGF-1 is abundant, very little insulin is detected within the murine brain (Coker et al., 1990), although small foci of insulin mRNA have been detected in the anterior hypothalamus (Young, 1986). A very recent study reported detection of insulin, IGF-1, and IGF-2 mRNA in postmortem human brain tissue using quantitative polymerase chain reaction (PCR) (Steen et al., 2005), but this novel report awaits confirmation.

Given IGF-1's abundant expression within the brain, and its apparent facility in crossing the BBB, both in contrast to insulin, it seems unlikely that insulin is required as an additional trophic factor for brain. The brain requires trophic support for developmental needs and responses to new learning or injury, but insulin secretion is tightly coupled to the timing and composition of meals. It seems unlikely that a gastrointestinal peptide, for which synthesis and secretion are tightly coupled to the contents of the duodenum, should be critically involved in brain development, function, or protection from degeneration. If this notion were true, then lean vegetarian individuals with very low levels of insulin secretion would be mentally deficient and at risk for premature neurodegeneration, while consumers of refined carbohydrates, which is associated with a high level of insulin secretion, would be intellectually superior and spared from senility. Further

evidence that insulin is not involved in brain development or function is the finding that brain-specific insulin receptor knockouts have normal brains and brain function, although neuroendocrine regulation of appetite is disturbed (Bruning et al., 2000).

## 5 Regulation of Brain IGF System

While it has been known for many years that IGF-1 produced in the liver is closely regulated by GH, there is limited understanding of factors that regulate brain IGF-1 gene expression. Certainly, expression of brain IGF-1 and IGF1R are developmentally controlled (Bondy et al., 1990; Bondy, 1991). Expression of IGF-1 is also elevated dramatically after hypoxia, ischemia, and other brain injury (see Section 8 for details). Thus, high-level expression of IGF-1 appears in situations where extraordinary energy needs for brain cell growth or repair processes are engaged. However, the molecular mechanisms underlying IGF-1's developmental stage and cell-specific expression in brain are still unclear. Recent evidence shows that modest caloric restriction significantly reduces brain IGF-1 and IGF1R mRNA levels in rats on a carbohydrate-dominant diet. A diet with the same calorie content composed primarily of lipid, however, "increased" brain IGF1R expression (Cheng et al., 2003a). Additional studies in rats (Chowen et al., 2002) and *Drosophila* (Ikeya et al., 2002) support the view that nutrient supply has important and complex effects on brain IGF system gene expression. Further study is required to elucidate the specific mechanisms regulating brain IGF system expression and the functional consequences of these changes.

## 6 IGF-1 and Normal Brain Growth

IGF-1 deletion or inhibition during brain development attenuates brain growth, with reductions in both cell number and cell size (Beck et al., 1995; Cheng et al., 2000). This effect is more profound in the nullizygous state, but even partial IGF-1 deficiency, as in IGF-1(+/−) mice, results in significantly diminished brain growth (Cheng et al., 2000). Cell numbers are notably reduced in the olfactory system, the dentate gyrus of the hippocampus, and the striatum. Brain volume is globally decreased due to a loss of neuropil, with significant reductions in neuronal soma volume, dendritic length and complexity, and synapse number. A fundamental requirement for cell, organ, and organism growth is nutrient acquisition and utilization. This most basic of functions is a prerequisite for cell division as well as somatic and process growth. IGF-1's fundamental role in the brain, as in peripheral tissues, is to promote nutrient acquisition and thus enhance cell proliferation, growth of the cell soma and processes, and more differentiated functions at later stages of development. Depending on where and when IGF-1 and its receptor are expressed during brain development, it may predominantly impact cell proliferation, or postmitotic growth processes.

IGF-1's anabolic functions on brain growth involve IRS2, PI3K, and Akt, as demonstrated by the growth phenotypes in genetic models with altered expression of each of these signaling molecules. Overexpression of PI3K/Akt or deletion of PTEN leads to increased brain size (Backman et al., 2001; Kwon et al., 2001). Activation of this pathway early in brain development is associated with augmented proliferation of neural stem cells (Groszer et al., 2001), while activation later, when more cells are in a more differentiated, postmitotic state, results in increased soma size (Kwon et al., 2003). Deletion of Akt3 results in a major brain growth deficit, due to both decreased cell numbers and decreased cell size, suggesting that this specific Akt isoform mediates IGF-1 effects on brain growth (Easton et al., 2005). Deletion of IRS2 results in significant reduction in brain growth, largely due to reduced cell proliferation (Schubert et al., 2003), thus implicating IGF-1 as the driver of this pathway in brain development.

### 6.1 Neurogenesis

The IGF1R is expressed at high levels in the developing nervous system, with highest expression concentrated in the germinal and subventricular zones that give rise to new neurons (Bondy, 1991; Bondy et al., 1992b). IGF-1 is coexpressed with the IGF1R in the subventricular zone of the anterior lateral ventricles that

give rise to olfactory system neurons (Bartlett et al., 1991) where it most likely acts as an autocrine factor to stimulate, alone or together with other neurotrophic factors, the proliferation of neural stem and precursor cells. For example, epidermal growth factors (EGFs) or fibroblast growth factor-2 (FGF-2) is known to stimulate neural stem cells to renew, expand, and differentiate into neural precursors, but they are effective only in the presence of IGF-1 (Arsenijevic et al., 2001). Given what is known about IGF-1's anabolic signaling in neural cells, it seems likely that IGF-1 supports proliferation triggered by the other growth factors by providing essential anabolic support through nutrient acquisition and protein synthesis.

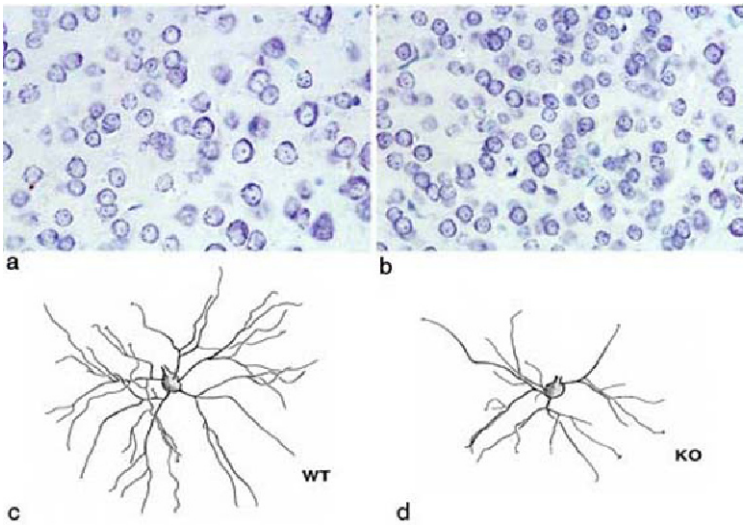
IGF-1 normally has a very selective, cell-specific, and developmentally timed pattern of expression in normal brain maturation. Elucidation of the phenotype of these IGF-1-expressing and neighboring cells in the IGF-1-null mouse provides insight into IGF-1's role in normal brain development. The study of transgenic mice that overexpress IGF-1 ectopically under transgene control reveals what may happen when IGF-1 is expressed at abnormally high levels in various different cell types at various developmental stages. Depending on the cellular pattern and developmental timing of IGF-1 transgene expression, neuron numbers are increased in the cerebral cortex (Gutierrez-Ospina et al., 1996), cerebellar cortex (Ye et al., 1996), hippocampus (O'Kusky et al., 2000), and brainstem (Dentremont et al., 1999). This increase in neuron numbers is due to, at least partially, increased neurogenesis (Ye et al., 1996; O'Kusky et al., 2000). These observations reveal what happens as a result of abnormal IGF-1 expression in brain, but do not reveal the nature of IGF-1's role in normal brain development. In fact, the high-level IGF-1 expression under transgene control may suppress normal IGF-1 production, and alter IGF1R and IGFBP expression as well, thus distorting normal developmental patterns.

## 6.2 Neuronal Somatic Growth and Dendritogenesis

The 30–40% reduction in brain size in adult IGF-1-null mice is due to a reduction in cell size and neuropil, or neuronal processes. Cell density is significantly increased throughout the IGF-1-null brain (❯ *Figure 7-4*)

**❑ Figure 7-4**

**Cortical neurons are smaller with hypotrophic dendritic trees in the IGF-1-null brain. Panels a (wild type, WT) and b (IGF-1-null) are micrographs of Nissl-stained cortical sections. The soma size is distinctly smaller and the cell density increased in IGF-1-null brains. Camera lucida drawings of Golgi-stained cortical pyramids (layers II–III) reveal dramatically reduced dendritic profiles of the IGF-null neurons (c and d). Adapted from Cheng et al. 2003. J Neurosci Res 73: 3**

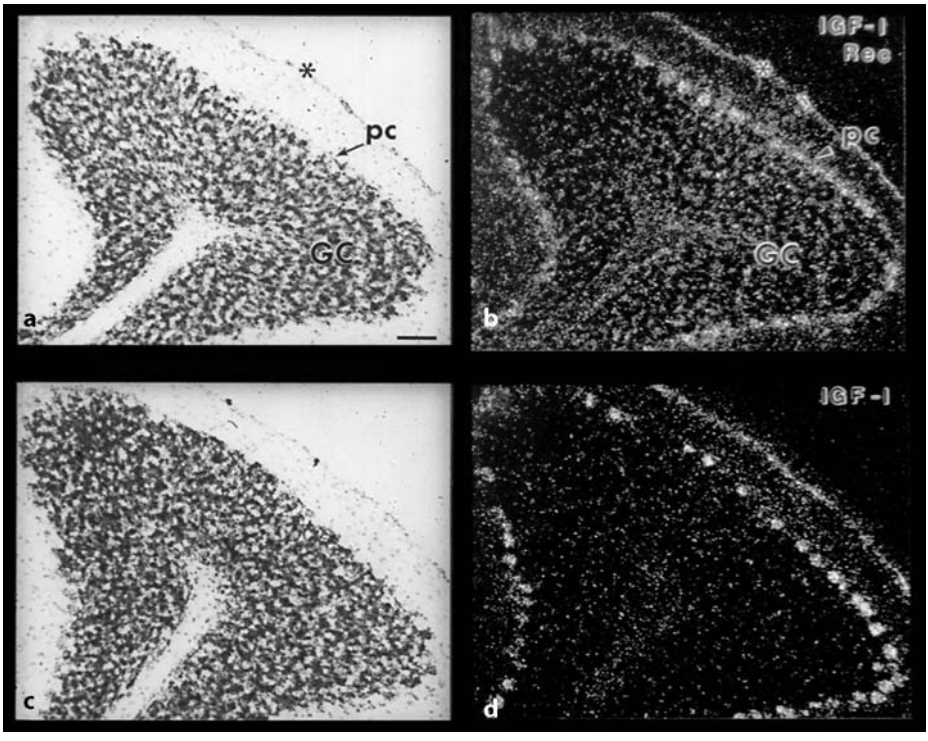


(Beck et al., 1995; Cheng et al., 1998; Cheng et al., 2003b). This observation is explained by reduced process growth, since the space between neurons is normally occupied by extensively branched neuronal processes. Soma size of projection neurons in the IGF-1-null brain is reduced by ~25%, and dendritic length, branching, and synapses are reduced by a similar amount (*Figure 7-4*). IGF-1 mRNA is most abundant in growing projection neurons in sensory and cerebellar relay systems (Bondy, 1991). Interestingly, very high-level IGF-1 expression is concentrated in all the auditory system nuclei: the medial geniculate, inferior colliculus, inferior olives, and cochlear nuclei (Bondy, 1991). These auditory system way stations are known to exhibit extremely high levels of glucose utilization. Humans with IGF-1 deficiency suffer sensory-neural hearing loss, in addition to mental retardation (Woods et al., 1997; Bonapace et al., 2003; Walenkamp et al., 2005). A recent study using auditory brainstem response testing in IGF-1-null mice has shown that the hearing loss is composed of both peripheral and central defects, consistent with IGF-1's extensive expression throughout the auditory neural system (Cediel et al., 2006). The IGF-1-expressing neurons attain exceptionally large soma size and develop extraordinarily extensive and complex dendritic formations. For example, the Purkinje cell expresses the highest level of IGF-1 mRNA of any cell type in the brain and at maturity has the largest soma and most elaborate dendritic arbor of any brain cell (*Figure 7-5*).

Thus it appears that impaired neuronal somatic growth and process formation accounts in large part for the reduction in IGF-1-null brain size. In the transgenic IGF-1-overexpressing brain, neuropil and synapses are significantly increased (O'Kusky et al., 2000). IGF-1 treatment significantly increased dendritic growth in cortical slices, which supports these in vivo findings (Niblock et al., 2000). In addition, IGF-1 has been shown to stimulate neuritic outgrowth in rat embryonic day 16–17 cortical neurons, rat hypothalamic

**Figure 7-5**

**Coexpression of IGF1 receptor (IGF1R) (a and b) and IGF-1 (c and d) mRNAs by Purkinje cells of the cerebellar cortex in the early postnatal mouse brain. Bright and dark-field photomicrographs of in situ hybridization are shown in pairs (a and b, c and d). In the dark field, white sliver grains are hybridized mRNA signals. Arrowheads point to Purkinje cells (*PC*). *GC* granule cells**



neurons (Torres-Aleman et al., 1990), chicken sympathetic neurons (Zackenfels et al., 1995), and mouse Purkinje cells (Fukudome et al., 2003).

## 6.3 Neuronal Survival

The dentate gyrus is selectively reduced in size and cell number in the adult IGF-1 knockout mouse (Beck et al., 1995; Cheng et al., 2001). IGF-1, however, is not expressed in the germinal zone supplying progenitors for the dentate gyrus, though it is expressed by unidentified cells scattered throughout the hippocampal formation. Unexpectedly, bromodeoxyuridine (BRDU) incorporation was actually increased in the IGF-1-null subventricular zone (Cheng et al., 2001). Apoptotic cells were also increased throughout the dentate gyrus in the IGF-1-null brain, however, indicating that IGF-1 normally promotes neuronal survival in this structure. There is abundant evidence from in vitro models supporting IGF-1's role in promoting neuronal survival.

IGF-1 promotes the in vitro survival of many different types of cultured neurons derived from many regions of the nervous system, including cortical neurons (Aizenman and de Vellis, 1987; Harper et al., 1996) and hippocampal neurons (Zheng and Quirion, 2004) in situations of serum or glucose deprivation. IGF-1 also protects hippocampal neurons from toxic effects of corticosterone (Nitta et al., 2004), nitric oxide, hypoxia (Tamatani et al., 1998; Yamaguchi et al., 2001), and amyloid (Dore et al., 1997). In primary cerebellar neuronal cultures, IGF-1 increased the survival of Purkinje cells (Torres-Aleman et al., 1992) and granule neurons in situations of serum, potassium (D'Mello et al., 1993), or glucose deprivation (Harper et al., 1996). IGF-1 also protects granule neurons against toxicity induced by dopamine (Offen et al., 2001) and polyQ-huntingtin (Humbert et al., 2002), which is involved in the pathogenesis of Huntington's disease. Moreover, IGF-1 partially prevents apoptosis of granule neurons isolated from Weaver mutant mice (Zhong et al., 2002), a mouse model of hereditary cerebellar ataxia. Finally, IGF-1 also enhances the survival of spinal cord motoneurons (Ang et al., 1992), parasympathetic neurons (Crouch and Hendry, 1991), hypothalamic neurons (Torres-Aleman et al., 1990), and striatal neurons (Nakao et al., 1996) in culture.

The neuronal survival signaling of IGF-1 has been investigated in primary cerebellar granule neuron cultures (D'Mello et al., 1993). Upon IGF-1's binding to its cognate receptor, both MAPK and PI3K are normally activated (❯ *Figure 7-2*). Under serum and potassium deprivation, most cerebellar granule neurons die unless IGF-1 is added in culture media. IGF-1's survival effect on cerebellar granule neurons is mediated by the PI3 kinase/Akt signaling pathway, since specific PI3 kinase inhibitors block IGF-1's survival-promoting activity, while the MAP kinase specific inhibitor PD98059 had no effect (D'Mello et al., 1997; Miller et al., 1997). The involvement of PI3K/Akt in this pathway has been confirmed by expressing WT or dominant-negative forms of Akt (Dudek et al., 1997). In fact, activation of PI3 kinase/Akt cascade is a common mechanism that mediates IGF-1's actions not only on cerebellar granule neurons, but also on other neuronal culture models (Matsuzaki et al., 1999; Yamaguchi et al., 2001; Zhong et al., 2002; Rangone et al., 2005). Downstream from Akt, different substrates are required to mediate IGF-1's survival effects depending on the type of neurons and the kind of adverse stimuli, e.g., potentiation of L calcium channels (Blair et al., 1999), NF-κB activation (Koulich et al., 2001), and Bim induction (Linseman et al., 2002). In hippocampal neurons, Akt mediates IGF-1's survival action against hypoxia or NO by inhibiting p53 transcriptional activity (Yamaguchi et al., 2001), but mediates IGF-1's rescue action from dehydroepiandrosterone-induced apoptosis by inactivation of GSK3 (Lin et al., 2004). Overall, IGF-1 promotes the in vitro survival of many types of differentiated neurons through the PI3/Akt pathway and multiple downstream signaling molecules that are specific for particular neuronal types.

Given all these data from in vitro studies, one might expect to see massive, widespread apoptosis in the IGF-1-null mouse brain. However, cell numbers are normal in the cerebellar and cerebral cortices and other brain regions, except for the dentate gyrus, olfactory bulb, and striatum, as documented in two independent targeted deletions in different outbred mouse lines (Beck et al., 1995; Cheng et al., 1998). While there were increased apoptotic figures in the dentate gyrus, there were few apoptotic cells in other regions (Cheng et al., 2001). Moreover, the IRS2 deletion model does not demonstrate increased brain cell death, despite a 30–40% reduction in brain size (Schubert et al., 2003). Thus, it seems likely that IGF-1 is essential for cell

survival under the stress of in vitro conditions and in response to brain insult, more than in normal brain development.

## 6.4 Myelination

IGF-1 promotes the survival and production of myelin by cultured oligodenrdocytes (Mozell and McMorris, 1991; McMorris and McKinnon, 1996). These in vitro observations led to the view that IGF-1 has a role in oligodendrocyte generation or differentiation and myelin synthesis. However, IGF-1 and IGF1R expression are lowest in white matter (Bondy and Lee, 1993b) and there is no apparent sign of central or peripheral myelinopathy in IGF-1-null mice (Cheng et al., 1998; Gao et al., 1999). Myelin concentration, normalized to brain weight or protein, is equal in IGF-1-null and WT littermate mice. Likewise, concentrations of myelin-specific proteins (MBP, PLP, MAG, and CNPase) are equal in IGF-1 null and WT littermate mice. Oligodendrocyte numbers and myelin are reduced in the IGF-1-null olfactory system, which is profoundly reduced in size and depleted of neurons, with efferent tracts correspondingly diminished, associated with decreased myelin in anterior white matter tracts that include a large olfactory component (Beck et al., 1995).

In brain structures where neurons are preserved, however, such as the cerebellum, myelination appears normal. This observation suggests that if the system projection neurons survive despite the lack of IGF-1, as in the cerebellum, oligodendrocytes prosper and appropriate myelination occurs. The PNS of IGF-1-null mice demonstrates reduced axonal diameter and proportionately reduced myelin sheath thickness, with no evidence of peripheral myelinopathy (Gao et al., 1999). The IGF-1-null mice show no neurological signs of myelinopathy, with normal motor function, coordination, and gait (Cheng et al., 1998), all functions normally impaired by myelin defects. Finally, the mentally retarded individual with IGF-1 gene deletions shows no evidence of dysmyelination or myelinopathy (Woods et al., 1996, 1997).

Observations of increased myelin content in the brains of transgenic mice overexpressing IGF-1 have been invoked to support a primary role for IGF-1 in myelination (Carson et al., 1993). The study reported that both brain size and myelin content, but not DNA content and oligodendrocyte numbers, are increased in the transgenic mice, suggesting that the increased brain mass is primarily due to increased cell size and/or process growth. Further investigation showed that myelin sheath thickness was increased in proportion to increased axonal diameter in this transgenic model (Ye et al., 1995). These findings in IGF-1-null and overexpressing brains are consistent with the current view (Barres and Raff, 1999) that myelination is induced by neuronal fiber growth and/or activity. IGF-1 overexpression stimulates excessive growth in size and number of neuronal processes and possibly also the survival of additional neurons, which, in turn, stimulates additional oligodendrocyte biosynthetic activity and myelination. The findings that myelination in IGF-1-null and IGF-1-overexpressing mice is essentially matched to neuroaxonal mass is best explained by the simple hypothesis that IGF-1 stimulates neuronal process growth, which in turn stimulates myelin formation.

The fact that IGF-1 does not seem to have an essential role in developmental myelination does not mean that it is not important in repair processes after nervous system injury. IGF-1 expression is induced in reactive astrocytes responding to demyelinating insults and IGF1R expression is enhanced in injured oligodendrocytes (Komoly et al., 1992). When cuprizone induced demyelination in the CNS of mice whose IGF1R was selectively mutated, oligodendrocyte progenitors did not accumulate, proliferate, or survive, indicating that signaling through IGF1R plays a critical role in remyelination (Mason et al., 2003). Administration of exogenous IGF-1 improves remyelination after injury, which supports the significance of these expression patterns (Yao et al., 1995). IGF-1's prominent effects on oligodendrocytes in vitro may actually reflect the fact that cell culture is essentially an injury model system.

# 7 IGF-1 and Neurogenesis in the Mature Brain

Adulthood neurogenesis occurs continuously within the subventricular zone of the hippocampal dentate gyrus and is important in learning and memory (Nilsson et al., 1999; Shors et al., 2001). This neurogenesis

can be enhanced by exercise (Neeper et al., 1995; Trejo et al., 2001), an enriched environment (Nilsson et al., 1999; Shors et al., 2001), and by IGF-1 (Aberg et al., 2000). Subcutaneous infusion of IGF-1 significantly increased the proliferation of neural progenitors in the hippocampal dentate gyrus in adult hypophysectomized rats (Aberg et al., 2000) or in rats after cerebral ischemia insults (Dempsey et al., 2003). Interestingly, exercise-induced neurogenesis in the adult hippocampus appears to be mediated by uptake of IGF-1 into the brain. This is because exercise-induced increases in the number of new neurons in the hippocampus were blocked by the administration of an antibody that prevents passage of systemic IGF-1 into the brain (Trejo et al., 2001). On the other hand, neurogenesis was significantly increased in the dentate of dwarf mice that have virtually no circulating IGF-1, although IGF-1 production is normal in the brain (Sun et al., 2005).

Interestingly, cerebral ischemia appears to increase the proliferation of progenitor cells in the cortex and subventricular zone of adult rats (Zhang et al., 2001). Since ischemia also activates astrocytic IGF-1 expression at a late stage, it may be that this ischemia-induced neurogenesis is partially mediated by IGF-1 released from astrocytes. This hypothesis is supported by the increase in neuron numbers in astrocyte-specific IGF-1 transgenic mice upon induction (Ye et al., 2004).

## 8 IGF-1 and Brain Injury

There has been a great deal of interest in the idea of treating brain injury with trophic agents including IGF-1. This was prompted in part by the finding that many components of the IGF system are induced in response to diverse types of brain injury. In contrast to the predominantly neuronal pattern of IGF-1 expression during normal brain development, this injury-invoked IGF-1 expression is generally observed in astrocytes (Komoly et al., 1992; Lee et al., 1992a; Gehrmann et al., 1994; Li et al., 1998). Interestingly, local IGF-1 expression at brain injury sites is also strongly correlated with local [$^{14}$C]-2-deoxyglucose uptake (Cheng et al., 2000). Potential roles for IGF-1 in response to brain injury have been studied in animal models, such as hypoxia and/or ischemia (Tagami et al., 1997a, b; Guan et al., 2003) and various models of traumatic brain injury (Saatman et al., 1997; Walter et al., 1997; Li et al., 1998; Kazanis et al., 2004). Regardless of primary insult, IGF-1 expression decreases in the early phases of the injury (Lee et al., 1992a, 1996; Clawson et al., 1999). This immediate suppression of neuronal IGF-1 gene expression is best characterized in an animal model of hypoxic–ischemic encephalopathy, where neuronal IGF-1 expression decreased within the hypoxic–ischemic hemisphere as early as 1 h (the earliest time studied) following the insult. IGF-1 mRNA levels are inversely correlated with the length of the hypoxia and the number of apoptotic cells (Clawson et al., 1999). IGF-1 mRNA levels continued to decrease with a nadir at 24 h of recovery (Lee et al., 1996), when the number of apoptotic cells was also at the maximum (Clawson et al., 1999). This correlation would indicate that the early decrease in neuronal IGF-1 expression likely contributes to hypoxia–ischemia-induced neuronal death. At a delayed phase of the recovery, endogenous IGF-1 genes become activated in astrocytes as they react to the injury (Lee et al., 1992a, 1996; Clawson et al., 1999). These observations provide a rationale for restoring IGF-1 during the early phase of hypoxia–ischemia.

Encouraging results have been obtained in adult rats (Guan et al., 1993) and fetal sheep (Johnston et al., 1996; Guan et al., 2000a). In these animal models, supplying IGF-1 to the injured brain intraventricularly within 2 h of hypoxia–ischemia promoted neuronal survival (Guan et al., 2000b). In both these models, IGF-1 treatment reduced infarct size and, more impressively, improved somatosensory function as evaluated by bilateral tactile test (Guan et al., 2001). In another injury model, IGF-1's effect on long-term recovery can be attributed to specific effects on oligodendrocytes. During myelinogenesis, younger rats are more sensitive to hypoxic–ischemic insult, manifested as ipsilateral necrosis originating in and spreading from myelinogenic foci (Rice et al., 1981). This hypoxia–ischemia-induced injury to immature oligodendrocytes may be alleviated by IGF-1. In fact, infusion of IGF-1 (3 μg over 1 h) into the cerebroventricles of fetal sheep at 90 min after recovery from hypoxia–ischemia prevented the delayed oligodendrocyte loss and associated demyelination (Guan et al., 2001).

## 9 IGF-1, Brain Aging, and Neurodegeneration

Normal aging is often accompanied by cognitive decline associated with reduced glucose utilization, altered synaptic plasticity, decreased hippocampal neurogenesis, impaired brain angiogenesis, and in more severe cases, accumulation of amyloid plaques and neurofibrillary tau-containing tangles as well as neuronal cell death (Hof and Mobb, 2001). Both circulating and endogenous brain IGF-1 are reduced with aging (Breese et al., 1991). IGF-1 mRNA is significantly decreased in hippocampal neurons (Lai et al., 2000) and IGF-1 and IGF1R levels are decreased in the cerebral cortex of aged rats (Sonntag et al., 1999). In addition, IGF-1 and IGFBPs are altered in many types of neural disease, including Alzheimer's disease (AD), amyotrophic lateral sclerosis, and inherited neurodegenerative conditions (Torres-Aleman et al., 1996, 1998; Busiguina et al., 2000). Serum IGF-1 is positively correlated with cognitive performance in older men (Aleman et al., 1999). It is unknown, however, if reduced circulating or brain IGF-1 is a cause or effect of brain disease.

Although IGF-1's effects on synaptogenesis and dendritic growth are most profound during brain development, recent findings indicate that IGF-1 and other neurotrophic factors may continually modulate neuronal circuits where the reshaping of the synapse contacts continues throughout life (Caroni, 1993; Schuman, 1999). For example, IGF-1 infusion increases synaptic density and number in the hippocampus in aged rats (Shi et al., 2005). Moreover, IGF-1 infusion improves memory and some age-related behavioral deficits in aged rats (Markowska et al., 1998). In summary, IGF-1 treatment seems to enhance neurogenesis and synaptogenesis and possible cognitive function in rodents, suggesting a role of IGF-1 in ameliorating age-related cognitive impairment. This view contrasts with the finding that IGF-1-deficient and IGF-1-receptor-deficient mice have longer life spans (Katic and Kahn, 2005) and maintain physiological functions, including cognitive function, at youthful levels into old age (Kinney et al., 2001).

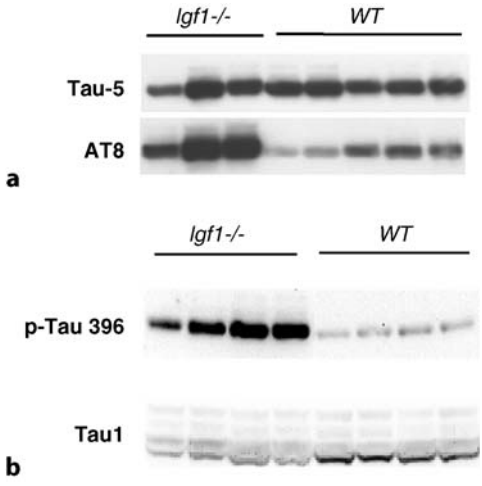
### 9.1 IGF-1 and Alzheimer's disease

Alzheimer's disease (AD) has two pathological hallmarks, the accumulation of neurofibrillary tangles (NFTs) and deposition of β-amyloid plaques (Dani, 1997). Recent evidence suggests that IGF-1 deficiency may contribute to the development of both these pathological features. First, IGF-1 regulates the phosphorylation of tau (Hong and Lee, 1997; Bondy and Cheng, 2004; Cheng et al., 2005), a microtubule-associated protein involved in microtubule assembly and stabilization (Barghorn et al., 2000). Hyperphosphorylated tau disrupts normal microtubule-dependent processes (Lee et al., 2001) and is resistant to degradation and prone to aggregation, culminating in the formation of NFT (Spillantini and Goedert, 1998; Lee et al., 2001). Hyperphosphorylated tau is associated with cognitive dysfunction in normal aged and disease brains. Tau is hyperphosphorylated in the IGF-1-null mouse brain (*Figure 7-6*) (Bondy and Cheng, 2004; Cheng et al., 2005). In addition, inhibition of IGF-1 signaling in IRS2 knockout mice increased tau phosphorylation and led to NFT accumulation (Schubert et al., 2003). Tau hyperphosphorylation in IGF-1-null mice appears due to overactivity of GSK3. In addition to regulating glycogen synthesis, GSK3 is also involved in tau phosphorylation in brain (Ishiguro et al., 1993; Jope and Johnson, 2004); thus reduced inhibition of GSK3 in IGF-1-deficient brains (Cheng et al., 2000) is associated with tau hyperphosphorylation (Bondy and Cheng, 2004; Cheng et al., 2005). In AD, NFTs consistently contain tau phosphorylated on GSK3 target residues, and GSK3 is physically associated with pretangle and tangle-bearing neurons in human brains (Pei et al., 1999).

IGF-1 protects neurons from β-amyloid toxicity (Dore et al., 1997) and appears to promote clearance of brain β amyloid (Gasparini et al., 2001; Carro et al., 2002). Increased serum IGF-1 levels are associated with reduced brain β-amyloid burden (Carro et al., 2002). Insulin, while its structure and function are closely related to IGF-1, may have distinct mechanisms in modulating brain amyloid levels (for a review see Carro and Torres-Aleman, 2004). It can directly stimulate the release of β amyloid from neurons and also increase extraneuronal accumulation of β amyloid by competing with β amyloid for insulin-degrading enzyme (Gasparini et al., 2001; Watson and Craft, 2003). Therefore, insulin seems to increase brain β-amyloid

**Figure 7-6**

**Hyperphosphorylated tau in the IGF-1-null brain. Total and phospho-tau were examined in IGF-1-null (*Igf1*(−/−) and wild-type (WT) brains by immunoblots. (a) Total tau was detected by anti-Tau 5, which recognizes tau protein irrespective of its phosphorylation status. This blot was striped and reprobed with antibody AT-8 that recognizes PHT-tau with phosphorylated ser202 residue. (b) Phospho-tau was detected by antibody p-Tau 396, which specifically recognizes tau phosphorylation on serine 396, a site prominently phosphorylated in PHF-tau. The same blot was then stripped and reprobed with tau-1 antibody, which detects dephosphorylated tau. These blots show that total tau protein is preserved in *Igf1*(−/−) brain, while tau phosphorylation is dramatically increased in specific epitope in aged *Igf1*(−/−) brains as compared with wild types (WTs). Adapted from Cheng et al. 2005. Endocrinology**




release. Taken together, dysfunction of insulin/IGF-1 signaling contributes to the major pathological events occurring in the brains of patients with AD.

## 10 Summary

The brain requires enormous supplies of fuel and substrate to support neuroglial growth and process formation during early postnatal development. Murine and human brains consume over half the energy available to the organism as a whole during this critical period that is characterized more by synapse formation than by synaptic activity. Purkinje cells grow into giant cells with surface areas exceeding all other cells in the body. How this remarkable anabolic feat is achieved when all brain cells are exposed to the same extracellular nutrient supply is unclear. Evidence from in vivo studies of murine brain development suggests that IGF-1's role in normal brain development is to promote these extraordinary growth processes via PI3K/Akt/GSK3β pathways that are similar to insulin signaling pathways in peripheral tissues. IGF-1 promotes hypertrophy of muscle cells using these same molecular signals, including GSK3 (Rommel et al., 2001). These observations in the mouse are supported by data from *Drososphila*, in which inactivation of paralogs of the insulin/IGF receptor, IRS, PI3K, and Akt all result in globally reduced cell size, which results in proportionate dwarfism, while overexpression of any of these molecules results in increased cell size and gigantism (Potter and Xu, 2001). As a further comment on IGF-1 action in general, all of these studies in mice and in *Drosophila* suggest that IGF-1 effects are neutral with respect to cellular differentiation.

This is not to suggest that IGF-1's only role in brain is to promote neuronal growth. Reduced cell numbers in specific brain regions of the IGF-1-null mouse support an IGF-1 role in developmental neurogenesis, through either increased proliferation or increased survival of nascent neurons. However,

further investigation is required to elucidate whether IGF-1 mainly promotes faster rates of mitosis or increases the number of cells entering the mitotic cycle, what signaling pathways are involved, and whether IGF-1 acts in an autocrine, paracrine, or even endocrine fashion to promote neurogenesis. In addition, further work is necessary to identify cell populations where IGF-1 promotes survival, and to discover the cellular interactions and signaling molecules active in this function. A major area for future investigation is the role of IGF-1 in brain aging. As we have described, there are opposing views as to potential positive versus negative effects of insulin/IGF action on aging. Evidence from diverse organisms shows that these growth peptides are important for somatic growth and reproduction, but at a cost of accelerated aging. Suppression of insulin/IGF signaling results in smaller size and reduced fecundity, but prolongation of life span. The natural reduction in IGF-1 levels with aging could be protective, allowing cells to devote more resources to repair processes, thus preventing cell death from oxidative damage, and retarding abnormal growths. However, since IGF-1 signaling clearly represses tau phosphorylation, a major feature of degenerative aging in brain, the story could be different in this unique organ. This should be a very productive area for future research.

## References


Aberg MA, Aberg ND, Hedbacker H, Oscarsson J, Eriksson PS. 2000. Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. J Neurosci 20: 2896-2903.

Aizenman Y, de Vellis J. 1987. Brain neurons develop in a serum and glial free environment: effects of transferrin, insulin, insulin-like growth factor-I and thyroid hormone on neuronal survival, growth, and differentiation. Brain Res 406: 32-42.

Aleman A, Verhaar HJ, De Haan EH, De Vries WR, Samson MM, et al. 1999. Insulin-like growth factor-I and cognitive function in healthy older men. J Clin Endocrinol Metab 84: 471-475.

Ang LC, Bhaumick B, Munoz DG, Sass J, Juurlink BH. 1992. Effects of astrocytes, insulin, and insulin-like growth factor I on the survival of motoneurons in vitro. J Neurol Sci 109: 168-172.

Arsenijevic Y, Weiss S, Schneider B, Aebischer P. 2001. Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. J Neurosci 21: 7194-7202.

Backman SA, Stambolic V, Suzuki A, Haight J, Elia A, et al. 2001. Deletion of Pten in mouse brain causes seizures, ataxia, and defects in soma size resembling Lhermitte-Duclos disease. Nat Genet 29: 396-403.

Baker J, Liu JP, Robertson EJ, Efstratiadis A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75: 73-82.

Barghorn S, Zheng-Fischhofer Q, Ackmann M, Biernat J, von Bergen M, et al. 2000. Structure, microtubule interactions, and paired helical filament aggregation by tau mutants of frontotemporal dementias. Biochemistry 39: 11714-11721.

Barres BA, Raff MC. 1999. Axonal control of oligodendrocyte development. J Cell Biol 147: 1123-1128.

Bartlett WP, Li XS, Williams M, Benkovic S. 1991. Localization of insulin-like growth factor-1 mRNA in murine central nervous system during postnatal development. Dev Biol 147: 239-250.

Beck KD, Powell-Braxton L, Widmer HR, Valverde J, Hefti F. 1995. Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. Neuron 14: 717-730.

Blair LA, Bence-Hanulec KK, Mehta S, Franke T, Kaplan D, et al. 1999. Akt-dependent potentiation of L channels by insulin-like growth factor-1 is required for neuronal survival. J Neurosci 19: 1940-1951.

Bohannon NJ, Corp ES, Wilcox BJ, Figlewicz DP, Dorsa DM, et al. 1988. Localization of binding sites for insulin-like growth factor-I (IGF-I) in the rat brain by quantitative autoradiography. Brain Res 444: 205-213.

Bonapace G, Concolino D, Formicola S, Strisciuglio P. 2003. A novel mutation in a patient with insulin-like growth factor 1 (IGF1) deficiency. J Med Genet 40: 913-917.

Bondy C, Bach M, Lee W. 1992a. Mapping of brain insulin and insulin-like growth factor receptor gene expression by in situ hybridization. Neuroprotocols 1: 240-249.

Bondy C, Lee WH. 1993a. Correlation between insulin-like growth factor (IGF)-binding protein 5 and IGF-I gene expression during brain development. J Neurosci 13: 5092-5104.

Bondy C, Werner H, Roberts CT Jr, LeRoith D. 1992b. Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II. Neuroscience 46: 909-923.

Bondy CA. 1991. Transient IGF-I gene expression during the maturation of functionally related central projection neurons. J Neurosci 11: 3442-3455.

Bondy CA, Cheng CM. 2004. Signaling by insulin-like growth factor 1 in brain. Eur J Pharmacol 490: 25-31.

Bondy CA, Lee WH. 1993b. Patterns of insulin-like growth factor and IGF receptor gene expression in the brain: functional implications. Ann N Y Acad Sci 692: 33-43.

Bondy CA, Werner H, Roberts CT, Leroith D. 1990. Cellular pattern of insulin-like growth factor-I and type-I IGF receptor gene expression in early organogenesis: comparison with IGF-II gene expression. Mol Endocrinol 4: 1386-1398.

Brar AK, Chernausek SD. 1993. Localization of insulin-like growth factor binding protein-4 expression in the developing and adult rat brain: analysis by in situ hybridization. J Neurosci Res 35: 103-114.

Breese CR, Ingram RL, Sonntag WE. 1991. Influence of age and long-term dietary restriction on plasma insulin-like growth factor-1 (IGF-1), IGF-1 gene expression, and IGF-1 binding proteins. J Gerontol 46: B180-B187.

Bruning JC, Gautam D, Burks DJ, Gillette J, Schubert M, et al. 2000. Role of brain insulin receptor in control of body weight and reproduction. Science 289: 2122-2125.

Busiguina S, Fernandez AM, Barrios V, Clark R, Tolbert DL, et al. 2000. Neurodegeneration is associated to changes in serum insulin-like growth factors. Neurobiol Dis 7: 657-665.

Caroni P. 1993. Activity-sensitive signaling by muscle-derived insulin-like growth factors in the developing and regenerating neuromuscular system. Ann N Y Acad Sci 692: 209-222.

Carro E, Torres-Aleman I. 2004. The role of insulin and insulin-like growth factor I in the molecular and cellular mechanisms underlying the pathology of Alzheimer's disease. Eur J Pharmacol 490: 127-133.

Carro E, Trejo JL, Gomez-Isla T, Le Roith D, Torres-Aleman I. 2002. Serum insulin-like growth factor I regulates brain amyloid-beta levels. Nat Med 8: 1390-1397.

Carson MJ, Behringer RR, Brinster RL, McMorris FA. 1993. Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. Neuron 10: 729-740.

Cediel R, Riquelme R, Contreras J, Diez A, Varela-Neito I. 2006. Sensorineural hearing loss in insulin-like growth factor 1-null mice: a new model of human deafness. Eur J Neurosci 23: 587-590.

Cheng CM, Cohen M, Tseng V, Bondy CA. 2001. Endogenous IGF1 enhances cell survival in the postnatal dentate gyrus. J Neurosci Res 64: 341-347.

Cheng CM, Joncas G, Reinhardt RR, Farrer R, Quarles R, et al. 1998. Biochemical and morphometric analyses show that myelination in the insulin-like growth factor 1 null brain is proportionate to its neuronal composition. J Neurosci 18: 5673–5681.

Cheng CM, Kelley B, Wang J, Strauss D, Eagles DA, et al. 2003a. A ketogenic diet increases brain insulin-like growth factor receptor and glucose transporter gene expression. Endocrinology 144: 2676-2682.

Cheng CM, Mervis RF, Niu SL, Salem N Jr, Witters LA, et al. 2003b. Insulin-like growth factor 1 is essential for normal dendritic growth. J Neurosci Res 73: 1-9.

Cheng CM, Reinhardt RR, Lee WH, Joncas GS, Patel C, et al. 2000. Insulin-like growth factor 1 regulates developing brain glucose metabolism. Proc Natl Acad Sci USA 97: 10236-10241.

Cheng CM, Tseng V, Wang J, Wang D, Matyakhina L, Bondy, CA. 2005. Tau is hyperphosphorylated in the insulin-like growth factor-1 null brain. Endocrinology 146: 5086-5091.

Chowen JA, Goya L, Ramos S, Busiguina S, Garcia-Segura LM, et al. 2002. Effects of early undernutrition on the brain insulin-like growth factor-I system. J Neuroendocrinol 14: 163-169.

Clawson TF, Vannucci SJ, Wang GM, Seaman LB, Yang XL, et al. 1999. Hypoxia–ischemia-induced apoptotic cell death correlates with IGF-I mRNA decrease in neonatal rat brain. Biol Signals Recept 8: 281-293.

Clemmons DR. 1989. Structural and functional analysis of insulin-like growth factors. Br Med Bull 45: 465-480.

Clemmons DR. 1998. Role of insulin-like growth factor binding proteins in controlling IGF actions. Mol Cell Endocrinol 140: 19-24.

Coker GT, 3rd, Studelska D, Harmon S, Burke W, O'Malley KL. 1990. Analysis of tyrosine hydroxylase and insulin transcripts in human neuroendocrine tissues. Brain Res Mol Brain Res 8: 93-98.

Crouch MF, Hendry IA. 1991. Co-activation of insulin-like growth factor-I receptors and protein kinase C results in parasympathetic neuronal survival. J Neurosci Res 28: 115-120.

Dani S. 1997. Histological markers of neuronal aging and their meaning. Dani S, Hori A, Walter HJ, editors. Amsterdam: Elsevier; Oxford, p. 263.

Daughaday WH, Rotwein P. 1989. Insulin-like growth factors I and II. Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocr Rev 10: 68-91.

DeChiara TM, Efstratiadis A, Robertson EJ. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 345: 78-80.

Dempsey RJ, Sailor KA, Bowen KK, Tureyen K, Vemuganti R. 2003. Stroke-induced progenitor cell proliferation in adult

spontaneously hypertensive rat brain: effect of exogenous IGF-1 and GDNF. J Neurochem 87: 586-597.

Dentremont KD, Ye P, D'Ercole AJ, O'Kusky JR. 1999. Increased insulin-like growth factor-I (IGF-I) expression during early postnatal development differentially increases neuron number and growth in medullary nuclei of the mouse. Brain Res Dev Brain Res 114: 135-141.

D'Mello SR, Borodezt K, Soltoff SP. 1997. Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-1 signaling. J Neurosci 17: 1548-1560.

D'Mello SR, Galli C, Ciotti T, Calissano P. 1993. Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. Proc Natl Acad Sci USA 90: 10989-10993.

Dore S, Kar S, Quirion R. 1997. Insulin-like growth factor I protects and rescues hippocampal neurons against beta-amyloid- and human amylin-induced toxicity. Proc Natl Acad Sci USA 94: 4772-4777.

Duan C. 2002. Specifying the cellular responses to IGF signals: roles of IGF-binding proteins. J Endocrinol 175: 41-54.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, et al. 1997. Regulation of neuronal survival by the serine–threonine protein kinase Akt. Science 275: 661-665.

Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, et al. 2005. Role for Akt3/protein kinase Bγ in attainment of normal brain size. Mol Cell Biol 25: 1869-1878.

Favelyukis S, Till JH, Hubbard SR, Miller WT. 2001. Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nat Struct Biol 8: 1058-1063.

Finch CE, Ruvkun G. 2001. The genetics of aging. Annu Rev Genomics Hum Genet 2: 435-462.

Foulstone E, Prince S, Zaccheo O, Burns JL, Harper J, et al. 2005. Insulin-like growth factor ligands, receptors, and binding proteins in cancer. J Pathol 205: 145-153.

Fukudome Y, Tabata T, Miyoshi T, Haruki S, Araishi K, et al. 2003. Insulin-like growth factor-I as a promoting factor for cerebellar Purkinje cell development. Eur J Neurosci 17: 2006-2016.

Gao WQ, Shinsky N, Ingle G, Beck K, Elias KA, et al. 1999. IGF-I deficient mice show reduced peripheral nerve conduction velocities and decreased axonal diameters and respond to exogenous IGF-I treatment. J Neurobiol 39: 142-152.

Gasparini L, Gouras GK, Wang R, Gross RS, Beal MF, et al. 2001. Stimulation of beta-amyloid precursor protein trafficking by insulin reduces intraneuronal beta-amyloid and requires mitogen-activated protein kinase signaling. J Neurosci 21: 2561-2570.

Gehrmann J, Yao DL, Bonetti B, Bondy CA, Brenner M, et al. 1994. Expression of insulin-like growth factor-I and related peptides during motoneuron regeneration. Exp Neurol 128: 202-210.

Groszer M, Erickson R, Scripture-Adams DD, Lesche R, Trumpp A, et al. 2001. Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. Science 294: 2186-2189.

Guan J, Bennet L, Gluckman PD, Gunn AJ. 2003. Insulin-like growth factor-1 and post-ischemic brain injury. Prog Neurobiol 70: 443-462.

Guan J, Bennet TL, George S, Waldvogel HJ, Faull RL, et al. 2000a. Selective neuroprotective effects with insulin-like growth factor-1 in phenotypic striatal neurons following ischemic brain injury in fetal sheep. Neuroscience 95: 831-839.

Guan J, Gunn AJ, Sirimanne ES, Tuffin J, Gunning MI, et al. 2000b. The window of opportunity for neuronal rescue with insulin-like growth factor-1 after hypoxia–ischemia in rats is critically modulated by cerebral temperature during recovery. J Cereb Blood Flow Metab 20: 513-519.

Guan J, Miller OT, Waugh KM, McCarthy DC, Gluckman PD. 2001. Insulin-like growth factor-1 improves somatosensory function and reduces the extent of cortical infarction and ongoing neuronal loss after hypoxia–ischemia in rats. Neuroscience 105: 299-306.

Guan J, Williams C, Gunning M, Mallard C, Gluckman P. 1993. The effects of IGF-1 treatment after hypoxic–ischemic brain injury in adult rats. J Cereb Blood Flow Metab 13: 609-616.

Gutierrez-Ospina G, Calikoglu AS, Ye P, D'Ercole AJ. 1996. In vivo effects of insulin-like growth factor-I on the development of sensory pathways: analysis of the primary somatic sensory cortex (S1) of transgenic mice. Endocrinology 137: 5484-5492.

Gutierrez-Ospina G, Saum L, Calikoglu AS, Diaz-Cintra S, Barrios FA, et al. 1997. Increased neural activity in transgenic mice with brain IGF-I overexpression: a [3H]2DG study. Neuroreport 8: 2907-2911.

Harper SJ, Macaulay AJ, Hill RG, Priestley T. 1996. The effects of insulin-like growth factor analogues on survival of cultured cerebral cortex and cerebellar granule neurones. Brain Res 709: 303-310.

Haspel HC, Stephenson KN, Davies-Hill T, El-Barbary A, Lobo JF, et al. 1999. Effects of barbiturates on facilitative glucose transporters are pharmacologically specific and isoform selective. J Membr Biol 169: 45-53.

Hawkes C, Kar S. 2004. The insulin-like growth factor-II/mannose-6-phosphate receptor: structure, distribution, and function in the central nervous system. Brain Res Rev 44: 117-140.

Hill JM, Lesniak MA, Pert CB, Roth J. 1986. Autoradiographic localization of insulin receptors in rat brain: prominence in olfactory and limbic areas. Neuroscience 17: 1127-1138.

Hof P, Mobb C. 2001. Functional neurobiology of aging. San Diego: Academic Press.

Hong M, Lee VM. 1997. Insulin and insulin-like growth factor-1 regulate tau phosphorylation in cultured human neurons. J Biol Chem 272: 19547-19553.

Hoyer S. 2004. Glucose metabolism and insulin receptor signal transduction in Alzheimer's disease. Eur J Pharmacol 490: 115-125.

Hubbard SR. 1999. Structural analysis of receptor tyrosine kinases. Prog Biophys Mol Biol 71: 343-358.

Humbert S, Bryson EA, Cordelieres FP, Connors NC, Datta SR, et al. 2002. The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. Dev Cell 2: 831-837.

Ikeya T, Galic M, Belawat P, Nairz K, Hafen E. 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr Biol 12: 1293-1300.

Ishiguro K, Shiratsuchi A, Sato S, Omori A, Arioka M, et al. 1993. Glycogen synthase kinase 3 beta is identical to tau protein kinase I generating several epitopes of paired helical filaments. FEBS Lett 325: 167-172.

Johnston BM, Mallard EC, Williams CE, Gluckman PD. 1996. Insulin-like growth factor-1 is a potent neuronal rescue agent after hypoxic–ischemic injury in fetal lambs. J Clin Invest 97: 300-308.

Jope RS, Johnson GV. 2004. The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 29: 95-102.

Katic M, Kahn CR. 2005. The role of insulin and IGF-1 signaling in longevity. Cell Mol Life Sci 62: 320-343.

Kazanis I, Giannakopoulou M, Philippidis H, Stylianopoulou F. 2004. Alterations in IGF-I, BDNF, and NT-3 levels following experimental brain trauma and the effect of IGF-I administration. Exp Neurol 186: 221-234.

Kiess W, Blickenstaff G, Sklar M, Thomas C, Nissley S, et al. 1988. Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. J Biol Chem 263: 9339-9344.

Kinney BA, Meliska CJ, Steger RW, Bartke A. 2001. Evidence that Ames dwarf mice age differently from their normal siblings in behavioral and learning and memory parameters. Horm Behav 39: 277-284.

Kohn AD, Summers SA, Birnbaum MJ, Roth RA. 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem 271: 31372-31378.

Komoly S, Hudson LD, Webster HD, Bondy CA. 1992. Insulin-like growth factor I gene expression is induced in astrocytes during experimental demyelination. Proc Natl Acad Sci USA 89: 1894-1898.

Koulich E, Nguyen T, Johnson K, Giardina C, D'Mello S. 2001. NF-κB is involved in the survival of cerebellar granule neurons: association of Iκβ phosphorylation with cell survival. J Neurochem 76: 1188-1198.

Kwon C-H, Zhu X, Zhang J, Baker SJ. 2003. mTor is required for hypertrophy of Pten-deficient neuronal soma in vivo. Proc Natl Acad Sci USA 100: 12923-12928.

Kwon CH, Zhu X, Zhang J, Knoop LL, Tharp R, et al. 2001. Pten regulates neuronal soma size: a mouse model of Lhermitte-Duclos disease. Nat Genet 29: 404-411.

Lai M, Hibberd CJ, Gluckman PD, Seckl JR. 2000. Reduced expression of insulin-like growth factor 1 messenger RNA in the hippocampus of aged rats. Neurosci Lett 288: 66-70.

Laron Z. 2001. Insulin-like growth factor 1 (IGF-1): a growth hormone. Mol Pathol 54: 311-316.

Lee VM, Goedert M, Trojanowski JQ. 2001. Neurodegenerative tauopathies. Annu Rev Neurosci 24: 1121-1159.

Lee W, Clemens J, Bondy C. 1992a. Insulin-like growth factors in the response to cerebral ischemia. Mol Cell Neurosci 3: 36-43.

Lee WH, Bondy C. 1993. Insulin-like growth factors and cerebral ischemia. Ann N Y Acad Sci 679: 418-422.

Lee WH, Javedan S, Bondy CA. 1992b. Coordinate expression of insulin-like growth factor system components by neurons and neuroglia during retinal and cerebellar development. J Neurosci 12: 4737-4744.

Lee WH, Michels KM, Bondy CA. 1993. Localization of insulin-like growth factor binding protein-2 messenger RNA during postnatal brain development: correlation with insulin-like growth factors I and II. Neuroscience 53: 251-265.

Lee WH, Wang GM, Seaman LB, Vannucci SJ. 1996. Coordinate IGF-I and IGFBP5 gene expression in perinatal rat brain after hypoxia–ischemia. J Cereb Blood Flow Metab 16: 227-236.

Le Roith D. 1996. Insulin-like growth factor receptors and binding proteins. Baillieres Clin Endocrinol Metab 10: 49-73.

Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. 2001. The somatomedin hypothesis: 2001. Endocr Rev 22: 53-74.

Le Roith D, Delahunty G, Wilson GL, Roberts CT Jr, Shemer J, et al. 1986. Evolutionary aspects of the endocrine and nervous systems. Recent Prog Horm Res 42: 549-587.

Le Roith D, Kavsan VM, Koval AP, Roberts CT Jr. 1993. Phylogeny of the insulin-like growth factors (IGFs) and receptors: a molecular approach. Mol Reprod Dev 35: 332-6; discussion 337–338.

Li XS, Williams M, Bartlett WP. 1998. Induction of IGF-1 mRNA expression following traumatic injury to the postnatal brain. Brain Res Mol Brain Res 57: 92-96.

Lin SY, Cui H, Yusta B, Belsham DD. 2004. IGF-I signaling prevents dehydroepiandrosterone (DHEA)-induced

apoptosis in hypothalamic neurons. Mol Cell Endocrinol 214: 127-135.

Linseman DA, Phelps RA, Bouchard RJ, Le SS, Laessig TA, et al. 2002. Insulin-like growth factor-I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons. J Neurosci 22: 9287-9297.

Logan A, Gonzalez AM, Hill DJ, Berry M, Gregson NA, et al. 1994. Coordinated pattern of expression and localization of insulin-like growth factor-II (IGF-II) and IGF-binding protein-2 in the adult rat brain. Endocrinology 135: 2255-2264.

Luo RZ-T, Beniac DR, Fernandes A, Yip CC, Ottensmeyer FP. 1999. Quaternary structure of the insulin–insulin receptor complex. Science 285: 1077-1080.

Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A. 2001. Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. Dev Biol 229: 141-162.

Markowska AL, Mooney M, Sonntag WE. 1998. Insulin-like growth factor-1 ameliorates age-related behavioral deficits. Neuroscience 87: 559-569.

Mason JL, Xuan S, Dragatsis I, Efstratiadis A, Goldman JE. 2003. Insulin-like growth factor (IGF) signaling through type 1 IGF receptor plays an important role in remyelination. J Neurosci 23: 7710-7718.

Matsuzaki H, Tamatani M, Mitsuda N, Namikawa K, Kiyama H, et al. 1999. Activation of Akt kinase inhibits apoptosis and changes in Bcl-2 and Bax expression induced by nitric oxide in primary hippocampal neurons. J Neurochem 73: 2037-2046.

McMorris FA, McKinnon RD. 1996. Regulation of oligodendrocyte development and CNS myelination by growth factors: prospects for therapy of demyelinating disease. Brain Pathol 6: 313-329.

Miller TM, Tansey MG, Johnson EM Jr, Creedon DJ. 1997. Inhibition of phosphatidylinositol 3-kinase activity blocks depolarization- and insulin-like growth factor I-mediated survival of cerebellar granule cells. J Biol Chem 272: 9847-9853.

Mozell RL, McMorris FA. 1991. Insulin-like growth factor I stimulates oligodendrocyte development and myelination in rat brain aggregate cultures. J Neurosci Res 30: 382-390.

Nakao N, Odin P, Lindvall O, Brundin P. 1996. Differential trophic effects of basic fibroblast growth factor, insulin-like growth factor-1, and neurotrophin-3 on striatal neurons in culture. Exp Neurol 138: 144-157.

Navarro I, Leibush B, Moon TW, Plisetskaya EM, Banos N, et al. 1999. Insulin, insulin-like growth factor-I (IGF-I) and glucagon: the evolution of their receptors. Comp Biochem Physiol B Biochem Mol Biol 122: 137-153.

Neeper SA, Gomez-Pinilla F, Choi J, Cotman C. 1995. Exercise and brain neurotrophins. Nature 373: 109.

Niblock MM, Brunso-Bechtold JK, Riddle DR. 2000. Insulin-like growth factor I stimulates dendritic growth in primary somatosensory cortex. J Neurosci 20: 4165-4176.

Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS. 1999. Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. J Neurobiol 39: 569-578.

Nitta A, Zheng WH, Quirion R. 2004. Insulin-like growth factor 1 prevents neuronal cell death induced by corticosterone through activation of the PI3k/Akt pathway. J Neurosci Res 76: 98-103.

Offen D, Shtaif B, Hadad D, Weizman A, Melamed E, et al. 2001. Protective effect of insulin-like-growth-factor-1 against dopamine-induced neurotoxicity in human and rodent neuronal cultures: possible implications for Parkinson's disease. Neurosci Lett 316: 129-132.

O'Kusky JR, Ye P, D'Ercole AJ. 2000. Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. J Neurosci 20: 8435-8442.

Pei J-J, Braak E, Braak H, Grundke-Iqbal I, Iqbal K, et al. 1999. Distribution of active glycogen synthase kinase 3β (GSK-3β) in brains staged for Alzheimer disease neurofibrillary changes. J Neuropathol Exp Neurol 58: 1010-1019.

Porte D, Jr., Baskin DG, Schwartz MW. 2005. Insulin signaling in the central nervous system: a critical role in metabolic homeostasis and disease from C. elegans to humans. Diabetes 54: 1264-1276.

Potter CJ, Xu T. 2001. Mechanisms of size control. Curr Opin Genet Dev 11: 279-286.

Rangone H, Pardo R, Colin E, Girault JA, Saudou F, Humbert S. 2005. Phosphorylation of arfaptin 2 at Ser260 by Akt Inhibits PolyQ-huntingtin-induced toxicity by rescuing proteasome impairment. J Biol Chem 280: 22021-22028.

Reijnders C, Koster J, van Buul-Offers S. 2004. Overexpression of human IGF-II mRNA in the brain of transgenic mice modulates IGFBP-2 gene expression in the medulla oblongata. J Endocrinol 182: 445-455.

Reinecke M, Collet C. 1998. The phylogeny of the insulin-like growth factors. Int Rev Cytol 183: 1-94.

Reinhardt RR, Bondy CA. 1994. Insulin-like growth factors cross the blood–brain barrier. Endocrinology 135: 1753-1761.

Rice JE 3rd, Vannucci RC, Brierley JB. 1981. The influence of immaturity on hypoxic–ischemic brain damage in the rat. Ann Neurol 9: 131-141.

Richardson A, Liu F, Adamo ML, Remmen HV, Nelson JF. 2004. The role of insulin and insulin-like growth factor-I in

mammalian ageing. Best Prac Res Clin Endo Metab 18: 393-406.

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, et al. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 3: 1009-1013.

Rotwein P, Burgess SK, Milbrandt JD, Krause JE. 1988. Differential expression of insulin-like growth factor genes in rat central nervous system. Proc Nat Acad Sci USA 85: 265-269.

Rulifson EJ, Kim SK, Nusse R. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science 296: 1118-1120.

Saatman KE, Contreras PC, Smith DH, Raghupathi R, McDermott KL, et al. 1997. Insulin-like growth factor-1 (IGF-1) improves both neurological motor and cognitive outcome following experimental brain injury. Exp Neurol 147: 418-427.

Saltiel AR, Kahn CR. 2001. Insulin signaling and the regulation of glucose and lipid metabolism. Nature 414: 799-806.

Sara VR, Carlsson-Skwirut C. 1986. The biosynthesis of somatomedins and their role in the fetus. Acta Endocrinol Suppl (Copenhagen) 279: 82-85.

Schubert M, Brazil DP, Burks DJ, Kushner JA, Ye J, et al. 2003. Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. J Neurosci 23: 7084-7092.

Schuman EM. 1999. Neurotrophin regulation of synaptic transmission. Curr Opin Neurobiol 9: 105-109.

Shi L, Linville MC, Tucker EW, Sonntag WE, Brunso-Bechtold JK. 2005. Differential effects of aging and insulin-like growth factor-1 on synapses in CA1 of rat hippocampus. Cereb Cortex 15: 571-577.

Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, et al. 2001. Neurogenesis in the adult is involved in the formation of trace memories. Nature 410: 372-376.

Sonntag WE, Lynch CD, Bennett SA, Khan AS, Thornton PL, et al. 1999. Alterations in insulin-like growth factor-1 gene and protein expression and type 1 insulin-like growth factor receptors in the brains of ageing rats. Neuroscience 88: 269-279.

Soos MA, Field CE, Siddle K. 1993. Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. Biochem J 290: 419-426.

Spillantini MG, Goedert M. 1998. Tau protein pathology in neurodegenerative diseases. Trends Neurosci 21: 428-433.

Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, et al. 2005. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease—is this type 3 diabetes? J Alzheimers Dis 7: 63-80.

Sullivan KA, Feldman EL. 1994. Immunohistochemical localization of insulin-like growth factor-II (IGF-II) and IGF-binding protein-2 during development in the rat brain. Endocrinology 135: 540-547.

Summers SA, Birnbaum MJ. 1997. A role for the serine/threonine kinase, Akt, in insulin-stimulated glucose uptake. Biochem Soc Trans 25: 981-988.

Summers SA, Kao AW, Kohn AD, Backus GS, Roth RA, et al. 1999. The role of glycogen synthase kinase 3β in insulin-stimulated glucose metabolism. J Biol Chem 274: 17934-17940.

Sun LY, Evans MS, Hsieh J, Panici J, Bartke A. 2005. Increased neurogenesis in dentate gyrus of long-lived Ames dwarf mice. Endocrinology 146: 1138-1144.

Sussenbach JS. 1989. The gene structure of the insulin-like growth factor family. Prog Growth Factor Res 1: 33-48.

Tagami M, Ikeda K, Nara Y, Fujino H, Kubota A, et al. 1997a. Insulin-like growth factor-1 attenuates apoptosis in hippocampal neurons caused by cerebral ischemia and reperfusion in stroke-prone spontaneously hypertensive rats. Lab Invest 76: 613-617.

Tagami M, Yamagata K, Nara Y, Fujino H, Kubota A, et al. 1997b. Insulin-like growth factors prevent apoptosis in cortical neurons isolated from stroke-prone spontaneously hypertensive rats. Lab Invest 76: 603-612.

Tager HS, Steiner DF, Patzelt C. 1981. Biosynthesis of insulin and glucagon. Methods Cell Biol 23: 73-88.

Tamatani M, Ogawa S, Nunez G, Tohyama M. 1998. Growth factors prevent changes in Bcl-2 and Bax expression and neuronal apoptosis induced by nitric oxide. Cell Death Differ 5: 911-919.

Torres-Aleman I, Barrios V, Berciano J. 1998. The peripheral insulin-like growth factor system in amyotrophic lateral sclerosis and in multiple sclerosis. Neurology 50: 772-776.

Torres-Aleman I, Barrios V, Lledo A, Berciano J. 1996. The insulin-like growth factor I system in cerebellar degeneration. Ann Neurol 39: 335-342.

Torres-Aleman I, Naftolin F, Robbins RJ. 1990. Trophic effects of insulin-like growth factor-I on fetal rat hypothalamic cells in culture. Neuroscience 35: 601-608.

Torres-Aleman I, Pons S, Santos-Benito FF. 1992. Survival of Purkinje cells in cerebellar cultures is increased by insulin-like growth factor I. Eur J Neurosci 4: 864-869.

Trejo JL, Carro E, Torres-Aleman I. 2001. Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. J Neurosci 21: 1628-1634.

Walenkamp MJ, Karperien M, Pereira AM, Hilhorst-Hofstee Y, van Doorn J, et al. 2005. Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 90: 2855-2864.

Walter HJ, Berry M, Hill DJ, Logan A. 1997. Spatial and temporal changes in the insulin-like growth factor (IGF) axis indicate autocrine/paracrine actions of IGF-I within wounds of the rat brain. Endocrinology 138: 3024-3034.

Wang J, Zhou J, Powell-Braxton L, Bondy C. 1999. Effects of Igf1 gene deletion on postnatal growth patterns. Endocrinology 140: 3391-3394.

Watson GS, Craft S. 2003. The role of insulin resistance in the pathogenesis of Alzheimer's disease: implications for treatment. CNS Drugs 17: 27-45.

Woods KA, Camacho-Hubner C, Barter D, Clark AJ, Savage MO. 1997. Insulin-like growth factor I gene deletion causing intrauterine growth retardation and severe short stature. Acta Paediatr Suppl 423: 39-45.

Woods KA, Camacho-Hubner C, Savage MO, Clark AJ. 1996. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. N Engl J Med 335: 1363-1367.

Wylie AA, Pulford DJ, McVie-Wylie AJ, Waterland RA, Evans HK, et al. 2003. Tissue-specific inactivation of murine M6P/IGF2R. Am J Pathol 162: 321-328.

Yamaguchi A, Tamatani M, Matsuzaki H, Namikawa K, Kiyama H, et al. 2001. Akt activation protects hippocampal neurons from apoptosis by inhibiting transcriptional activity of p53. J Biol Chem 276: 5256-5264.

Yao DL, West NR, Bondy CA, Brenner M, Hudson LD, et al. 1995. Cryogenic spinal cord injury induces astrocytic gene expression of insulin-like growth factor I and insulin-like growth factor binding protein 2 during myelin regeneration. J Neurosci Res 40: 647-659.

Ye P, Carson J, D'Ercole AJ. 1995. In vivo actions of insulin-like growth factor-I (IGF-I) on brain myelination: studies of IGF-I and IGF binding protein-1 (IGFBP-1) transgenic mice. J Neurosci 15: 7344-7356.

Ye P, D'Ercole J. 1998. Insulin-like growth factor I (IGF-I) regulates IGF binding protein-5 gene expression in the brain. Endocrinology 139: 65-71.

Ye P, Popken GJ, Kemper A, McCarthy K, Popko B, et al. 2004. Astrocyte-specific overexpression of insulin-like growth factor-I promotes brain overgrowth and glial fibrillary acidic protein expression. J Neurosci Res 78: 472-484.

Ye P, Xing Y, Dai Z, D'Ercole AJ. 1996. In vivo actions of insulin-like growth factor-I (IGF-I) on cerebellum development in transgenic mice: evidence that IGF-I increases proliferation of granule cell progenitors. Brain Res Dev Brain Res 95: 44-54.

Young WS. 1986. Periventricular hypothalamic cells in rat brain contain insulin mRNA. Neuropeptides 8: 93-97.

Zackenfels K, Oppenheim RW, Rohrer H. 1995. Evidence for an important role of IGF-I and IGF-II for the early development of chick sympathetic neurons. Neuron 14: 731-741.

Zhang RL, Zhang ZG, Zhang L, Chopp M. 2001. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. Neuroscience 105: 33-41.

Zheng WH, Quirion R. 2004. Comparative signaling pathways of insulin-like growth factor-1 and brain-derived neurotrophic factor in hippocampal neurons and the role of the PI3 kinase pathway in cell survival. J Neurochem 89: 844-852.

Zhong J, Deng J, Ghetti B, Lee WH. 2002. Inhibition of insulin-like growth factor I activity contributes to the premature apoptosis of cerebellar granule neuron in weaver mutant mice: in vitro analysis. J Neurosci Res 70: 36-45.

# 8 Erythropoietin Signaling Pathways in Neuroprotection

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**Abstract:** Erythropoietin (EPO) is the principal regulator of red blood cell production and is synthesized by the adult kidney. Insulin-like growth factor-I (IGF-I) is a neuroprotective cytokine that supports neuronal development and survival. As neuroprotectants, EPO and IGF-I have synergistic effects when combined. Both EPO and IGF-I and their receptor are expressed in the mammalian central nervous system (CNS) where they have been shown to play a neuroprotective role. The aim of this chapter is to identify and discuss the key signaling molecules and events published in numerous reports that are involved in EPO- and IGF-I-mediated neuroprotection. Better understanding of the intricacies of EPO signaling and EPO/IGF-I synergy in the central and peripheral nervous system provide new strategies for novel therapies.

**List of Abbreviations:** BAD, Bcl2-antagonist of cell death; c-myc, myc protooncogene protein; casp-3, caspain-3; CNS, central nervous system; EPO, erythropoietin; EPO-R, erythropoietin receptor; GSK-3β, glycogen synthase kinase-3β; IAP, inhibitors of apoptosis; IGF-I, insulin-like growth factor-I; IGF-IR, IGF-I receptor; IKK, inhibitor of nuclear factor kappa-B kinase; JAK2, janus family of protein tyrosine kinase-2; MnSOD, Mn-superoxide dismutase; NF-κB, nuclear factor kappa-B; PI-3 kinase, phosphatidylinositol-3 kinase; PI-3,4-$P_2$, phosphatidylinositol-3,4,-diphosphate; PI-3,4,5-$P_3$, phosphatidylinositol-3,4,5-trisphosphate; STAT5, nuclear translocation of the signal transducer and activator of transcription-5; XIAP, X-linked IAP

## 1 Introduction

Erythropoietin (EPO) is the principal regulator of red blood cell production and is synthesized by the adult kidney (Koury and Bondurant, 1990; Jelkmann, 1992; Koury, 1992). EPO synthesis is upregulated under hypoxic conditions. The EPO receptor (EPO-R) is expressed in the bone marrow and prevents apoptosis in immature erythrocytes (D'Andrea et al., 1989; Wilson and Jolliffe, 1999). As we and others have shown, both EPO and EPO-R are expressed in the mammalian central nervous system (CNS) (Masuda, 1993; Digicaylioglu et al., 1995; Marti et al., 1996; Morishita et al., 1997; Chin et al., 2000; Weishaupt et al., 2004). In recent years, it has been reported in numerous publications that EPO and its receptor have a neuroprotective role within the CNS. Exogenous EPO has been shown to be neuroprotective in animal models of cerebral hypoxia/ischemia (stroke), neurodegenerative diseases, retinal degeneration, experimental spinal cord injuries, and gp120/HIV dementia. (Sakanaka et al., 1998; Brines et al., 2000; Sinor and Greenberg, 2000; Siren et al., 2001; Celik et al., 2002; Gorio et al., 2002; Grimm et al., 2002; Junk et al., 2002; Kumral et al., 2003; Prass, 2003; Solaroglu et al., 2003; Villa et al., 2003; Digicaylioglu et al., 2004a, b; Weishaupt et al., 2004). The focus of the present chapter is to introduce the intracellular signaling pathways involved in EPO neuroprotection in the CNS.

## 2 Intracellular Signaling Pathways

### 2.1 JAK2 and STAT

In nonneuronal cells, ligand binding to the EPO-R is known to induce activation of the Janus family of protein tyrosine kinase-2 (JAK2) and nuclear translocation of the signal transducer and activator of transcription-5 (STAT5) (Yousouffian et al., 1993; Ihle et al., 1995; Kirito et al., 1997; Oda et al., 1998; Verdier et al., 1998; Uddin et al., 2000; Gorio et al., 2002; Yu et al., 2002). JAK2 phosphorylates intracellular tyrosine residues on the EPO-R, which are thought to provide docking sites for intracellular signaling molecules (Ihle and Kerr, 1995).

Previously we reported the association of JAK2 with the EPO-R complex in cortical neurons (Digicaylioglu and Lipton, 2001). Although direct functional evidence is lacking, ligand binding to the neuronal EPO-R promoted the association of p85 and JAK2 with the EPO-R and activation of phosphatidylinositol-3 kinases (Digicaylioglu et al., 2004a, b).

STAT5 is also required in nonneuronal EPO/EPO-R signaling. JAK phosphorylation of STAT results in their dimerization and translocation into the nucleus, where they bind to specific sequences in the promoter of STAT-regulated genes (Ihle and Kerr, 1995). In embryonic STAT−/− mice, severe anemia is caused by the disruption of the EPO/JAK2/STAT5 signaling pathway (Socolovsky et al., 1999).

STAT proteins are constitutively expressed, present in the cytosol of neuronal cells, and recruited to the phosphorylated tyrosine residues Y343 and Y401 of the activated EPO-R complex (Damen et al., 1995a, b; Quelle et al., 1996). Although disruption of STAT 5a/b function in EPO-dependent cells results in higher levels of apoptosis (Socolovsky et al., 1999), the participation of STAT5 in EPO signaling in neurons is disputed. In our experiments we have not observed any loss in EPO-mediated protection in neurons expressing the dominant negative nonphosphorylatable form of STAT5a/b (Digicaylioglu and Lipton, 2001). Supporting our results, Ruscher et al. also could not detect any functional role for STAT in hippocampal neurons under ischemic conditions (Ruscher et al., 2002). However, other groups have reported activation of neural STAT5 by JAK2 (De-Fraja et al., 1998; Chong et al., 2002). Moreover, Bittorf et al. showed that in presence of EPO, cell lines expressing the truncated form of STAT5 with sustained binding to specific DNA sequences underwent less apoptosis than cells expressing the wild-type STAT5 (Bittorf et al., 2001). Interestingly, one report indicates that STAT5 is activated in axotomized neurons in the peripheral nervous system but not in the CNS (Schwaiger et al., 2000; Liu and Snider, 2001). At present, there is some evidence that STAT5 might be a downstream target of JAK2 in regeneration but not in development of peripheral sensory neurons. It is conceivable that different signaling molecules are involved in EPO-mediated neuroprotection and in development or regeneration. Furthermore, downstream targets of EPO-activated JAK2 might be cell-specific and may not always utilize STAT5.

## 2.2 PI-3 Kinase, Akt, and GSK-3β

Phosphatidylinositol-3 kinases (PI-3 kinases) are members of a lipid kinase family (Fry and Waterfield, 1993). PI-3 kinases consist of the catalytic subunit p110 and the regulatory subunit p85 and are activated by receptor tyrosine kinases. PI-3 kinases phosphorylate and activate the inositol 3′-OH group in inositol phospholipids, resulting in the second messengers phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-$P_3$) and -diphosphate (PI-3,4-$P_2$). Interaction with these phospholipids results in activation of Akt.

In nonneuronal cells EPO-mediated phosphorylation and activation of the PI-3 kinases has been reported extensively (LeRoith and Roberts, 1993; Mayeux et al., 1993; Pleiman et al., 1994; Klingmuller et al., 1997; Park et al., 2001). As in nonneuronal cells, activated EPO-R in neurons provides binding sites for the regulatory subunit of PI-3 kinases, p85 (Nguyen et al., 2001; Digicaylioglu et al., 2004a, b). Phosphorylation and activation of p85 results in association of its Src2 homology domains with the EPO-R and generation of second messengers (Damen et al., 1993). Subsequent activation of Akt by PI-3 kinases is required for neuroprotection (Ruscher et al., 2002; Chong and Maiese, 2003; Weishaupt et al., 2004).

In neurons, Akt plays a crucial role in mediating EPO/JAK2/PI-3 kinase signaling (Digicaylioglu et al., 2004a, b). As a serine-threonine kinase, activated Akt phosphorylates its downstream targets, which also include Bcl2-antagonist of cell death (BAD) (Datta et al., 1997), caspase-9 (Zhou et al., 2000), and GSK-3β (Noshita et al., 2002), thereby decreasing or blocking neuronal apoptosis. Under apoptotic conditions, caspase-9 activates caspase-3, which directly induces DNA fragmentation by activating DNases (Krajewski et al., 1999; Okamoto et al., 2002; Bossy-Wetzel et al., 2004). Akt blocks caspase-3 activation by phosphorylating the proapoptotic molecule BAD, which suppresses expression and activation of the antiapoptotic factor Bcl-$X_L$.

Glycogen synthase kinase-3β (GSK-3β), another downstream target of Akt, promotes apoptosis in neurons possibly through hyperphosphorylation of tau and beta-catenin (Crowder and Freeman, 2000; Hetman et al., 2000; Higuchi et al., 2003; Jones et al., 2003; Lucas et al., 2001; Mora et al., 2001; Schubert et al., 2004). Overexpression of GSK-3β results in increased neuronal apoptosis (Bhat et al., 2000). Similarly, expression of noninducible form of Akt, which lacks serine phosphorylation ability, results in activation of GSK-3β by dephophorylation and by higher neuronal apoptosis (Fujio and Walsh, 1999; Crowder and Freeman, 2000; Noshita et al., 2002; Stoica et al., 2003).

### 2.3 NF-κB and XIAP

Nuclear factor kappa-Bs (NF-κB) are a family of transcription factors that have been implicated to play a role in survival and apoptotic signaling pathways. These factors are sequestered in the cytoplasm by IκBs, which lose their ability to bind NF-κB when their ubiquitin-dependent degradation is initiated via phosphorylation, usually by inhibitor of nuclear factor kappa-B kinase (IKK). NF-κB is then free to translocate to the nucleus and bind to DNA (Karin and Ben-Neriah, 2000). NF-κB targets a number of genes that are both proapoptotic and antiapoptotic, including p53, c-myc, Fas, Bcl-x, Bcl-2, XIAP, cIAP2, and MnSOD.

Classically, NF-κB is known to operate in immune cells, mediating the inflammatory response, but was later found to be expressed continuously in both neurons and glia (Kaltschmidt et al., 1994a, b). Activation of NF-κB in glial cells leads to expression of inflammatory proteins that cause apoptosis of neurons in mixed cultures. The role of NF-κB in neurons is complex; both pro-and antiapoptotic functions have been described in a number of varied treatments and models. Several lines of evidence support the hypothesis that acute increases in NF-κB activate an apoptotic signaling pathway, whereas stimuli that lead to large increases in steady-state NF-κB activity provide neuroprotection (Lin et al., 1998; Shen et al., 2002; Aleyasin et al., 2004).

Proapoptotic roles for NF-κB in neuronal cells in vivo have been described in several animal models. Excitotoxicity resulted in increased nuclear translocation of NF-κB and consequent upregulation of apoptotic genes and cell death, but these effects were abrogated by interfering with translocation of NF-κ B to the nucleus (Qin et al., 1999). Ischemia in a transgenic model provided additional support for the proapoptotic role of NF-κB in acute cellular trauma (Schneider et al., 1999). However, similar methods have discerned protective roles for the protein in neurons (Botchkina et al., 1999). In vitro studies have also found both protective and degenerative roles for NF-κB in the nervous system. A basal rate of activity is critical for the survival of primary cortical neurons in culture but numerous in vitro insults lead to acute activation and subsequent apoptosis (Barkett and Gilmore, 1999). Pharmacologic, functional, and genetic inhibitors of NF-κB can increase neuronal death upon prolonged exposure (Natarajan et al., 1998; Aleyasin et al., 2004). However, these same methods of inhibition can provide protection to cultured neurons against death from acute insults, such as excitotoxicity and DNA damage (Rothman and Olney, 1986; Grilli et al., 1996; Aleyasin et al., 2004).

We investigated whether EPO was able to act through NF-κB to protect neurons from excitotoxic and nitrosative stress (Digicaylioglu and Lipton, 2001). Treatment of cerebrocortical cultures with EPO resulted in nuclear translocation, and a large, sustained increase in DNA-binding activity of NF-κB in neurons (but not astrocytes). Expression of a reporter gene confirmed the transcriptional activity of NF-κB in this model. EPO treatment caused JAK2 to become phosphorylated, and, in turn, to directly phosphorylate IκB. Inhibition of this pathway, either by pharmacological inhibition of JAK2, or expression of a dominant interfering form of JAK2, or an IκB super-repressor construct, significantly attenuated NF-κB DNA-binding and reporter gene transcription in response to EPO and abrogated EPO-mediated neuroprotection.

The inhibitors of apoptosis (IAP) molecules are factors known to prevent neuronal cell death under a variety of conditions (Deveraux and Reed, 1999). Among these factors is X-linked IAP (XIAP), which prevents neurodegeneration (Holcik and Korneluk, 2001). In transgenic mice, overexpression of XIAP in neurons resulted in significant neuroprotection from transient cerebral ischemia (Trapp et al., 2003). We have shown that EPO causes XIAP upregulation and facilitates its binding to activated caspase-3, resulting in reduced neuronal death (Digicaylioglu and Lipton, 2001).

## 3 EPO + IGF-I Affords Acute Neuroprotection

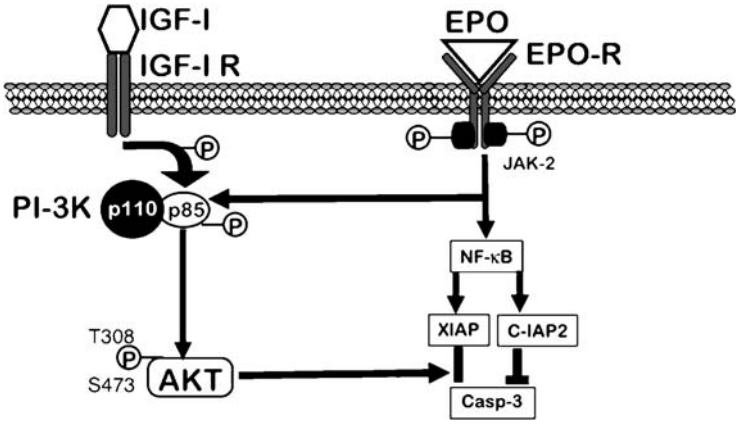
The potential for EPO to be used as an acute neuroprotective agent is limited as preincubation for 3–8 h before the onset of a neurotoxic insult is required (Digicaylioglu and Lipton, 2001; Morishita et al., 1997). Interestingly, we have shown that EPO's efficacy is increased when combined with insulin-like growth factor-I (IGF-I) in an in vitro model (Digicaylioglu et al., 2004a, b). When EPO and IGF-I are given

together, they provide acute and prolonged neuroprotection by synergistically activating antiapoptotic pathways. Using a pharmacological inhibitor, we found that PI-3 kinase is required for the cooperative and acute neuroprotective effects of EPO+IGF-I. Our results suggest that concurrent activation of PI-3 kinase by IGF-I enables EPO to act as an acute neuroprotectant, independently of the more prolonged effects of gene expression mediated by the JAK2/NF-κB pathway that we have previously demonstrated (Digicaylioglu and Lipton, 2001). We also demonstrated that Akt is required for the cooperative and acute neuroprotective effects of EPO+IGF-I by using a dominant interfering mutant of Akt. Moreover, our preliminary results indicate that EPO+IGF-I combination greatly reduces Akt dephosphorylation, thereby enabling a prolonged signaling event. Our results indicate that EPO+IGF-I induces binding of XIAP to active caspase-3 and inhibits the proteolytic activity of the "executioner" caspases, downstream of caspase-3 activation. In addition, EPO+IGF-I increases XIAP and cIAP-2 expression and affords sustained neuroprotection because of the increased association of IAPs with active caspase-3, and subsequent blocking of "executioner" caspases downstream.

NF-κB signaling seems to be uninvolved in the initial phase of EPO+IGF-I neuroprotection. However, Akt is known to phosphorylate and activate IKKα, resulting in enhanced NF-κB function in nonneuronal cells (Ozes et al., 1999; Burow et al., 2000). Akt can also influence NF-κB directly, through activation of MAP3K (Li et al., 1998). The effect of PI-3 kinase on NF-κB activity seems to be mediated primarily by IKKα, and inhibitors of PI-3 kinase block NF-κB DNA binding in IKKα−/− but not IKKβ−/− cells (Gustin et al., 2004). However, in neuronal cells there is only little evidence for the participation of PI3K/Akt pathway in NF-κB activation (Min et al., 2003). In a different experimental model, Bittorf et al confirmed the requirement of NF-κB activation for EPO-mediated neuroprotection, although this signaling pathway was independent of JAK2, but required activation of Src-kinases (Bittorf et al., 2001). Further investigation is required in order to determine NF-κB's role in EPO+IGF-I mediated acute neuroprotection.

The interaction between EPO and IGF-I receptors is depicted in *Figure 8-1*.

**Figure 8-1**
**Signaling pathways of erythropoietin (EPO) and erythropoietin + insulin-like growth factor-I (EPO+IGF-I) in neurons**




## 4 Conclusion and Future Directions

As evidenced by the number of publications and the resulting insights, EPO emerges as a novel neuroprotectant with significant clinical potential. However, detailed knowledge about EPO signaling in neurons is still scarce and more studies about the neuronal signaling pathways involved in EPO-mediated protection are required. EPO is an extremely well studied and tolerated compound in humans, and its use in stroke

patients is promising. Results from a first clinical phase II study showed that intravenous high-dose EPO given to 53 stroke patients was associated with an improved clinical outcome after one month (Ehrenreich et al., 2002). EPO should also be considered for the chronic therapy and the management of neurodegenerative diseases, such as autism, ALS, Alzheimer's disease, Parkinson's disease, and AIDS dementia.

We have described a novel cooperative neuroprotective effect of EPO and IGF-I that is mediated by a signal transduction pathway involving PI-3 kinase and Akt. In addition, our findings suggest that the coadministration of synergistic neuroprotective agents rather than single agents may provide greater benefit to patients suffering from acute brain injury. The combined use of EPO and IGF-I could provide a powerful tool for patients suffering from acute NMDA-receptor-mediated insults such as cerebral ischemia (stroke), head or spinal cord trauma, and epilepsy, stroke and CNS trauma.

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## References

Aleyasin H, Cregan SP, Iyirhiaro G, O'Hare MJ, Callaghan SM, et al. 2004. Nuclear factor-(kappa)B modulates the p53 response in neurons exposed to DNA damage. J Neurosci 24: 2963-2973.

Barkett M, Gilmore TD. 1999. Control of apoptosis by Rel/NF-kappaB transcription factors. Oncogene 18: 6910-6924.

Bhat RV, Shanley J, Correll MP, Fieles WE, Keith RA, et al. 2000. Regulation and localization of tyrosine216 phosphorylation of glycogen synthase kinase-3beta in cellular and animal models of neuronal degeneration. Proc Natl Acad Sci 97: 11074-11079.

Bittorf T, Buchse T, Sasse T, Jaster R, Brock J. 2001. Activation of the transcription factor NF-kappaB by the erythropoietin receptor. Structural requirements and biological significance. Cell Signal 13: 673-681.

Bossy-Wetzel E, Talantova MV, Lee WD, Scholzke MN, Harrop A, et al. 2004. Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K+ channels. Neuron 41: 351-365.

Botchkina GI, Geimonen E, Bilof ML, Villarreal O, Tracey KJ. 1999. Loss of NF-kappaB activity during cerebral ischemia and TNF cytotoxicity. Mol Med 5: 372-381.

Brines ML, Ghezzi P, Keenan S, Agnello D, de Lanerolle NC, et al. 2000. Erythropoietin crosses the blood–brain barrier to protect against experimental brain injury. Proc Natl Acad Sci USA 97: 10526-10531.

Burow ME, Weldon CB, Melnik LI, Duong BN, Collins-Burow BM, et al. 2000. PI3-K/AKT regulation of NF-kappaB signaling events in suppression of TNF-induced apoptosis. Biochem Biophys Res Commun 271: 342-345.

Celik M, Gokmen N, Erbayraktar S, Akhisaroglu M, Konakc S, et al. 2002. Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. Proc Natl Acad Sci USA 99: 2258-2263.

Chin K, Yu X, Beleslin-Cokic B, Liu C, Shen K, et al. 2000. Production and processing of erythropoietin receptor transcripts in brain. Brain Res Mol Brain Res 81: 29-42.

Chong ZZ, Kang JQ, Maiese K. 2002. Hematopoietic factor erythropoietin fosters neuroprotection through novel signal transduction cascades. J Cereb Blood Flow Metab 22: 503-514.

Chong ZZ, Maiese KJ. 2003. Erythropoietin fosters both intrinsic and extrinsic neuronal protection through modulation of microglia, Akt1, Bad, and caspase-mediated pathways. Br J Pharmacol 138: 1107-1118.

Crowder RJ, Freeman RS. 2000. Glycogen synthase kinase-3 beta activity is critical for neuronal death caused by inhibiting phosphatidylinositol 3-kinase or Akt but not for death caused by nerve growth factor withdrawal. J Biol Chem 275: 34266-34271.

D'Andrea AD, Lodish HF, Wong GG. 1989. Expression cloning of the murine erythropoietin receptor. Cell 57: 277-285.

Damen JE, Cutler RL, Jiao H, Yi T, Krystal G. 1995a. Phosphorylation of tyrosine 503 in the erythropoietin receptor (EpR) is essential for binding the P85 subunit of phosphatidylinositol (PI) 3-kinase and for EpR-associated PI 3-kinase activity. J Biol Chem 270: 23402-23408.

Damen JE, Mui AL, Puil L, Pawson T, Krystal G. 1993. Phosphatidylinositol 3-kinase associates, via its Src homology 2 domains, with the activated erythropoietin receptor. Blood 81: 3204-3210.

Damen JE, Wakao H, Miyajima A, Krosl J, Humphries RK, et al. 1995b. Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and Stat5 activation. Embo J 14: 5557-5568.

Datta SR, Dudek H, Tao X, Masters S, Fu H, et al. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231-241.

De-Fraja C, Conti L, Magrassi L, Govoni S, Cattaneo E. 1998. Members of the JAK/STAT proteins are expressed and regulated during development in the mammalian forebrain. J Neurosci Res 54: 320-330.

Deveraux QL, Reed JC. 1999. IAP family proteins—suppressors of apoptosis. Genes Dev 13: 239-252.

Digicaylioglu M, Bichet S, Marti HH, Wenger RH, Rivas LA, et al. 1995. Localization of specific erythropoietin binding sites in defined areas of the mouse brain. Proc Natl Acad Sci USA 92: 3717-3720.

Digicaylioglu M, Garden G, Timberlake S, Fletcher L, Lipton SA. 2004a. Acute neuroprotective synergy of erythropoietin and insulin-like growth factor I. Proc Natl Acad Sci USA 101: 9855-9860.

Digicaylioglu M, Kaul M, Fletcher L, Dowen R, Lipton SA. 2004b. Erythropoietin protects cerebrocortical neurons from HIV-1/gp120-induced damage. Neuroreport 15: 761-763.

Digicaylioglu M, Lipton SA. 2001. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature 412: 641-647.

Ehrenreich H, Hasselblatt M, Dembowski C, Cepek L, Lewczuk P, et al. 2002. Erythropoietin therapy for acute stroke is both safe and beneficial. Mol Med 8: 495-505.

Fry MJ, Waterfield MD. 1993. Structure and function of phosphatidylinositol 3-kinase: a potential second messenger system involved in growth control. Philos Trans R Soc Lond B Biol Sci 340: 337-344.

Fujio Y, Walsh K. 1999. Akt mediates cytoprotection of U S Aendothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J Biol Chem 274: 16349-16354.

Gustin JA, Ozes ON, Akca H, Pincheira R, Mayo LD, Li Q, Guzman JR, Korgaonkar CK, Donner DB. 2004. Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. J Biol Chem 279: 1615-1620.

Gorio A, Gokmen N, Erbayraktar S, Yilmaz O, Madaschi L, et al. 2002. Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. Proc Natl Acad Sci USA 99: 9450-9455.

Grilli M, Pizzi M, Memo M, Spano P. 1996. Neuroprotection by aspirin and sodium salicylate through blockade of NF-kappaB activation. Science 274: 1383-1385.

Grimm C, Wenzel A, Groszer M, Mayser H, Seeliger M, et al. 2002. HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. Nat Med 8: 718-724.

Hetman M, Cavanaugh JE, Kimelman D, Xia Z. 2000. Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal. J Neurosci 20: 2567-2574.

Higuchi M, Onishi K, Masuyama N, Gotoh Y. 2003. The phosphatidylinositol-3 kinase (PI3K)-Akt pathway suppresses neurite branch formation in NGF-treated PC12 cells. Gene Cells 8: 657-669.

Holcik M, Korneluk RG. 2001. XIAP, the guardian angel. Nat Rev Mol Cell Biol 2: 550-556.

Ihle JN, Kerr IM. 1995. Jaks and Stats in signaling by the cytokine receptor superfamily. Trends Genet 11: 69-74.

Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Silvennoinen O. 1995. Signaling through the hematopoietic cytokine receptors. Annu Rev Immunol 13: 369-398.

Jelkmann W. 1992. Erythropoietin: structure, control of production, and function. Physiol Rev 72: 449-489.

Jones DM, Tucker BA, Rahimtula M, Mearow KM. 2003. The synergistic effects of NGF and IGF-1 on neurite growth in adult sensory neurons: convergence on the PI 3-kinase signaling pathway. J Neurochem 86: 11116-11128.

Junk AK, Mammis A, Savitz SI, Singh M, Roth S, et al. 2002. Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. Proc Natl Acad Sci USA 99: 10659-10664.

Kaltschmidt C, Kaltschmidt B, Lannes-Vieira J, Kreutzberg GW, Wekerle H, et al. 1994a. Transcription factor NF-kappa B is activated in microglia during experimental autoimmune encephalomyelitis. J Neuroimmunol 55: 99-106.

Kaltschmidt C, Kaltschmidt B, Neumann H, Wekerle H, Baeuerle PA. 1994b. Constitutive NF-kappa B activity in neurons. Mol Cell Biol 14: 3981-3992.

Karin M, Ben-Neriah Y. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 18: 621-663.

Kirito K, Uchida M, Yamada M, Miura Y, Komatsu N. 1997. A distinct function of STAT proteins in erythropoietin signal transduction. J Biol Chem 272: 16507-16513.

Klingmuller U, Wu H, Hsiao JG, Toker A, Duckworth BC, et al. 1997. Identification of a novel pathway important for proliferation and differentiation of primary erythroid progenitors. Proc Natl Acad Sci USA 94: 3016-3021.

Koury MJ. 1992. Programmed cell death (apoptosis) in hematopoiesis. Exp Hematol 20: 391-394.

Koury MJ, Bondurant MC. 1990. Control of red cell production: the roles of programmed cell death (apoptosis) and erythropoietin. Transfusion 30: 673-674.

Krajewski S, Krajewska M, Ellerby LM, Welsh K, Xie Z, et al. 1999. Release of caspase-9 from mitochondria during

neuronal apoptosis and cerebral ischemia. Proc Natl Acad Sci USA 96: 5752-5757.

Kumral A, Ozer E, Yilmaz O, Akhisaroglu M, Gokmen N, et al. 2003. Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. Biol Neonate 83: 224-228.

LeRoith D, Roberts Jr. CT 1993. Insulin-like growth factors. Ann N Y Acad Sci 692: 1-9.

Li JD, Feng W, Gallup M, Kim JH, Gum J, et al. 1998. Activation of NF-kappaB via a src-dependent ras-MAPK-pp90rsk pathway is required for pseudomonas aeruginosa-induced mucin overproduction in epithelial cells [In Process Citation]. Proc Natl Acad Sci USA 95: 5718-5723.

Lin KI, Di Donato JA, Hoffmann A, Hardwick JM, Ratan RR. 1998. Suppression of steady-state, but not stimulus-induced NF-kappaB activity inhibits alphavirus-induced apoptosis. J Cell Biol 141: 1479-1487.

Liu RY, Snider W. 2001. Different signaling pathways mediate regenerative versus developmental sensory axon growth. J Neurosci 21: RC164.

Lucas JJ, Hernandez F, Gomez-Ramos P, Moran MA, Hen R, et al. 2001. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. Embo J 20: 27-39.

Marti HH, Wenger RH, Rivas LA, Straumann U, Digicaylioglu M, et al. 1996. Erythropoietin gene expression in human, monkey and murine brain. Eur J Neurosci 8: 666-676.

Masuda S, Nagao M, Takahata K, Konishi Y, Gallyas F Jr, et al. 1993. Functional erythropoietin receptors of the cells with neuronal characteristics-comparison with receptor properties from erythroid cells. J Biol Chem 268: 11208-11216.

Mayeux P, Dusanter-Fourt I, Muller O, Mauduit P, Sabbah M, et al. 1993. Erythropoietin induces the association of phosphatidylinositol 3′-kinase with a tyrosine-phosphorylated protein complex containing the erythropoietin receptor. Eur J Biochem 216: 821-828.

Min Y, Park JH, Chong SA, Kim YS, Ahn YS, et al. 2003. Pyrrolidine dithiocarbamate-induced neuronal cell death is mediated by Akt, casein kinase 2, c-Jun N-terminal kinase, and IkappaB kinase in embryonic hippocampal progenitor cells. J Neurosci Res 71: 689-700.

Mora A, Sabio G, Gonzalez-Polo RA, Cuenda A, Alessi DR, et al. 2001. Lithium inhibits caspase 3 activation and dephosphorylation of PKB and GSK3 induced by K+ deprivation in cerebellar granule cells. J Neurochem 78: 199-206.

Morishita E, Masuda S, Nagao M, Sasaki R. 1997. Erythropoietin receptor is expressed in rat hippocampal cerebral cortical neurons, and erythropoietin prevents in vitro glutamate-induced neuronal death. Neuroscience 76: 105-116.

Natarajan K, Manna SK, Chaturvedi MM, Aggarwal BB. 1998. Protein tyrosine kinase inhibitors block tumor necrosis factor-induced activation of nuclear factor-kappaB, degradation of IkappaBalpha, nuclear translocation of p65, and subsequent gene expression. Arch Biochem Biophys 352: 59-70.

Nguyen MH, Ho JM, Beattie BK, Barber DL. 2001. TEL-JAK2 mediates constitutive activation of the phosphatidylinositol 3′-kinase/protein kinase B signaling pathway. J Biol Chem 276: 32704-32713.

Noshita N, Lewen A, Sugawara T, Chan PH. 2002. Akt phosphorylation and neuronal survival after traumatic brain injury in mice. Neurobiol Dis 9: 294-304.

Oda A, Sawada K, Druker BJ, Ozaki K, Takano H, et al. 1998. Erythropoietin induces tyrosine phosphorylation of jak2, STAT5A, and STAT5B in primary cultured human erythroid precursors. Blood 92: 443-451.

Okamoto S, Li Z, Ju C, Scholzke MN, Mathews E, et al. 2002. Dominant-interfering forms of MEF2 generated by caspase cleavage contribute to NMDA-induced neuronal apoptosis. Proc Natl Acad Sci USA 99: 3974-3979.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, et al. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401: 82-85.

Park J, Hill MM, Hess D, Brazil DP, Hofsteenge J, et al. 2001. Identification of tyrosine phosphorylation sites on 3-phosphoinositide-dependent protein kinase-1 and their role in regulating kinase activity. J Biol Chem 276: 37459-37471.

Pleiman CM, Hertz WM, Cambier JC. 1994. Activation of phosphatidylinositol-3′ kinase by Src-family kinase SH3 binding to the p85 subunit. Science 263: 1609-1612.

Prass K, Schraff A, Ruscher K, Lowl D, Muselmann C, et al. 2003. Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. Stroke 34: 1981-1986.

Qin ZH, Chen RW, Wang Y, Nakai M, Chuang DM, et al. 1999. Nuclear factor kappaB nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. J Neurosci 19: 4023-4033.

Quelle FW, Wang D, Nosaka T, Thierfelder WE, Stravopodis D, et al. 1996. Erythropoietin induces activation of Stat5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. Mol Cell Biol 16: 1622-1631.

Rothman SM, Olney JW. 1986. Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann Neurol 19: 105-111.

Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, et al. 2002. Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. J Neurosci 22: 10291-10301.

Sakanaka M, Wen TC, Matsuda S, Masuda S, Morishita E, et al. 1998. In vivo evidence that erythropoietin protects

neurons from ischemic damage. Proc Natl Acad Sci USA 95: 4635-4640.

Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, et al. 1999. NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat Med 5: 554-559.

Schubert M, Gautam D, Surjo D, Ueki K, Baudler S, et al. 2004. Role for neuronal insulin resistance in neurodegenerative diseases. Proc Natl Acad Sci USA 101: 3100-3105.

Schwaiger FW, Hager G, Schmitt AB, Horvat A, Streif R, et al. 2000. Peripheral but not central axotomy induces changes in Janus kinases (JAK) and signal transducers and activators of transcription (STAT). Eur J Neurosci 12: 1165-1176.

Shen W, Zhang C, Zhang G. 2002. Nuclear factor kappaB activation is mediated by NMDA and non-NMDA receptor and L-type voltage-gated Ca(2+) channel following severe global ischemia in rat hippocampus. Brain Res 933: 23-30.

Sinor AD, Greenberg DA. 2000. Erythropoietin protects cultured cortical neurons, but not astroglia, from hypoxia and AMPA toxicity. Neurosci Lett 290: 213-215.

Siren AL, Fratelli M, Brines M, Goemans C, Casagrande S, et al. 2001. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. Proc Natl Acad Sci USA 20: 20.

Socolovsky M, Fallon AE, Wang S, Brugnara C, Lodish HF. 1999. Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. Cell 98: 181-191.

Solaroglu I, Solaroglu A, Kaptanoglu E, Dede S, Haberal A, et al. 2003. Erythropoietin prevents ischemia–reperfusion from inducing oxidative damage in fetal rat brain. Childs Nerv Syst 19: 19-22.

Stoica BA, Movsesyan VA, Lea PM, Faden AI. 2003. Ceramide-induced neuronal apoptosis is associated with dephosphorylation of Akt, BAD, FKHR, GSK-3beta, and induction of the mitochondrial-dependent intrinsic caspase pathway. Mol Cell Neurosci 22: 365-382.

Trapp T, Korhonen L, Besselmann M, Martinez R, Mercer EA, et al. 2003. Transgenic mice overexpressing XIAP in neurons show better outcome after transient cerebral ischemia. Mol Cell Neurosci 23: 302-313.

Uddin S, Kottegoda S, Stigger D, Platanias LC, Wickrema A. 2000. Activation of the Akt/FKHRL1 pathway mediates the antiapoptotic effects of erythropoietin in primary human erythroid progenitors. Biochem Biophys Res Commun 275: 16-19.

Verdier F, Chretien S, Muller O, Varlet P, Yoshimura A, et al. 1998. Proteasomes regulate erythropoietin receptor and signal transducer and activator of transcription 5 (STAT5) activation. Possible involvement of the ubiquitinated Cis protein. J Biol Chem 273: 28185-28190.

Villa P, Bigini P, Mennini T, Agnello D, Laragione T, et al. 2003. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. J Exp Med 198: 971-975.

Weishaupt JH, Rohde G, Polking E, Siren AL, Ehrenreich H, et al. 2004. Effect of erythropoietin axotomy-induced apoptosis in rat retinal ganglion cells. Invest Ophthalmol Vis Sci 45: 1514-1522.

Wilson IA, Jolliffe LK. 1999. The structure, organization, activation and plasticity of the erythropoietin receptor. Curr Opin Struct Biol 9: 696-704.

Yousouffian H, Longmore G, Neumann D, Yoshimura A, Lodish HF. 1993. Structure, Function, and activation of the erythropoietin receptor. Blood 81: 2223-2236.

Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, et al. 2002. Erythropoietin receptor signalling is required for normal brain development. Development 129: 505-516.

Zhou H, Li XM, Meinkoth J, Pittman RN. 2000. Akt regulates cell survival and apoptosis at a postmitochondrial level. J Cell Biol 151: 483-494.

# 9 TNF-α in CNS: Physiologic and Pathologic Roles

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**Abstract:** Tumor necrosis factor-alpha (TNF-α) is a pleuripotent cytokine credited with diverse functions ranging from neuromodulatory roles in a normal brain to immune-responsive Janus-faced involvement in a diseased brain where studies have exposed its neuroprotective as well as its neurodegenerative influences. This chapter aims at illuminating these functions and establishing TNF-α and its receptors (TNF system) as a major proctor of central nervous system (CNS) function.

**List of Abbreviations:** ACTH, adrenocorticotropic hormone; AD, Alzheimer's disease; AP-1, activator protein-1; BBB, blood–brain barrier; c-IAP, cellular inhibitor of apoptosis protein; CNS, central nervous system; COX-2, cyclooxygenase-2; DED, death effector domain; DISC, death-inducing signal complex; FADD, Fas-associated death domain; HAD, HIV-associated dementia; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun N terminal kinase; LPSs, lipopolysaccharides; LTD, long-term depression; LTP, long-term potentiation; MS, multiple sclerosis; mTNF-α, membrane-bound TNF-α; NEMO, NF-κB essential modifier; NF-κB, nuclear factor-kappaB; NGF, nerve growth factor; NIK, NF-κB-inducing kinase; NREM, nonrapid eye movement; PD, Parkinson's disease; PI3K, phosphatidylinositol-3-kinase; RIP1, receptor-interacting protein 1; SDF-1, stromal-derived factor-1; SODD, silencer of death domain; sTNF-α, soluble TNF-α; TNF-α, tumor necrosis factor-alpha; TNF-R, TNF-α receptor; TACE, TNF-α converting enzyme; TRADD, TNF-R-associated death domain; TRAF2, TNF-R-associated factor 2

## 1 Historical Perspective

To the credit of physician William Bradley Coley, the antitumor property of the immune response was identified and clinically utilized more than 100 years ago (Coley, 1891). Coley, the third surgeon-in-chief in New York hospital for special surgeries, reported back in 1893 that cancer patients who developed bacterial infections showed necrosis of tumors. He himself attempted to treat such patients with filtrates of cultured Gram-negative bacteria, which he later marketed under the brand name "Coley Mixed Toxin." Even after Coley's death in 1936, the product was available in USA until the 1960s as a cancer vaccine. However, the vaccine lost its way in history mainly due to extensive side effects generated by its administration. Subsequent to the Coley episode, it took more than three quarters of a century after the surgeon's pioneering report to identify the active principle behind the tumor killing aspect of immune response. In 1975, the active element was identified as a serum constituent of bacillus Calmette–Guérin-treated mice that mimicked lipopolysaccharide (LPS)-induced dramatic hemorrhagic necrosis of solid tumors overnight (Carswell et al., 1975). Owing to this property, the century-old antitumor immune component finally derived the name tumor necrosis factor (TNF). Identified later as a glycoprotein released from host macrophages (Oettgen et al., 1980), TNF-α was found to be the same molecular moiety that was otherwise identified as hormone cachectin secreted from macrophages (Beutler et al., 1985a, b) and had structural and functional relationships with human lymphotoxin-α (later renamed as TNF-β) (Pennica et al., 1984). Subsequently, the cloning of complementary DNA (cDNA) and amino acid sequencing of this cytokine was simultaneously undertaken by several laboratories in the 1980s (Pennica et al., 1985; Haranaka et al., 1986; Aggarwal et al., 1987). The following years saw a rush of information about this protein and by the end of a decade, the therapeutic potential of this acclaimed wonder drug for cancer was also being estimated in other fields as it became clear that, contrary to its name, TNF-α did not induce necrosis or apoptosis in most cell types, including many tumor cells. This paved the pathway for anticytokine therapy, where antibodies generated against TNF-α were shown to prevent bacterial sepsis (Beutler et al., 1985a, b; Tracey et al., 1987), rheumatoid arthritis (RA) (Elliott et al., 1993), and other inflammatory diseases like Crohn's disease (van Dullemen et al., 1995). Involvement of TNF-α in brain function was proposed in the late 1980s and since then a plethora of information has accumulated about the biology and functions of this molecule in the brain. Before discussing its role in the central nervous system (CNS), a brief summary of properties of this cytokine, its receptors, and its signal transduction pathways is presented in the following sections.

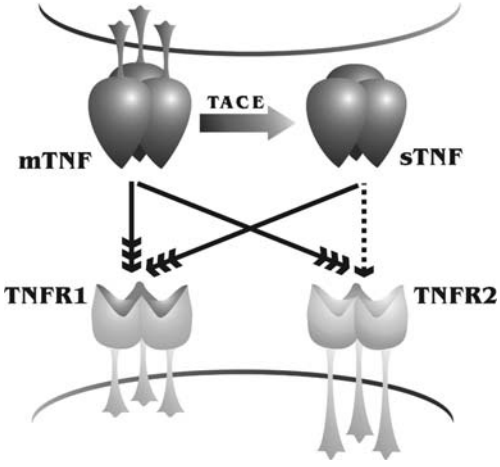
## 2 Biology of TNF-α

### 2.1 The Molecule

TNF-α is the prototypical cytokine member of a family of structurally related biomolecules (TNF ligand superfamily) with conserved bioactivity among vertebrates (Goetz et al., 2004). Human TNF-α is translated as a 233-amino-acid, 26-kDa proprotein that is displayed on the plasma membrane as a type II transmembrane moiety (Kriegler et al., 1988). Membrane-bound form of TNF-α (mTNF-α) is then cleaved by a nonspecific metalloproteinase called TNF-α converting enzyme (TACE) in the extracellular domain to release the 157-amino-acid, 17.3-kDa soluble (sTNF-α) monomer (*Figure 9-1*) (Moss et al.,

**Figure 9-1**

**TNF receptor–ligand interplay. TNF-α is displayed on the membrane as biologically active homotrimers (membrane-bound TNF-α, mTNF) that may be cleaved by metalloproteinase TNF-α converting enzyme (TACE) to release soluble homotrimers (sTNF), which are also active biologically. Two TNF receptors (TNFR1 and TNFR2) have various binding affinity for the ligand. While mTNF has equal affinity for both the receptors, sTNF displays greater affinity for TNFR1**




1997). This 17-kDa monomer is composed of two antiparallel β-pleated sheets with antiparallel β-strands forming a "jelly roll" structure typical of few viral capsid proteins and the TNF ligand family. Both the membrane-bound and secreted forms retain biological activity of the molecule. However, such biological activity is contingent on oligomerization of the molecule into conical homotrimers such that each monomer contacts the remaining two. Adducing the soluble form, this cleaved product exists in solution as a homotrimer of total molecular mass of 52 kDa. Trimers are assembled intracellularly before their membrane display or TACE-mediated cleavage (Tang et al., 1996). Mutational analyses have identified three receptor interaction domains in monomer–monomer interface near the base of the trimer structure (Idriss and Naismith, 2000). We will discuss more about TNF-α ligand–receptor interaction in the next section.

Normal and tumor cells of both hematopoietic and nonhematopoietic origin express TNF-α. This includes immune cells like T cells, B cells, dendritic cells, natural killer (NK) cells, neutrophils, eosinophils, and mast cells. In brain, neurons and glial cells express TNF-α under normal conditions, while in diseased state TNF-α production is greatly enhanced in activated astroglia and microglia.

## 2.2 TNF Receptors

Biological responses of TNF-α are mediated by binding of the cytokine in any of its forms to either of its two structurally distinct receptors: TNF-R1 (CD120a or p55) or TNF-R2 (CD120b or p75) (*Figure 9-1*). Both receptors are transmembrane glycoproteins with multiple cysteine-rich repeats in the extracellular N-terminal domain and are susceptible to metalloproteinase-interceded cleavage. TNF-R2 is cleaved by TACE (Solomon et al., 1999), while the exact proteinase responsible for TNF-R1 has not been identified till date. However, a TNF-R1-interacting protein, called aminopeptidase regulator of TNF-R1 shedding (ARTS-1), has been reported recently whose expression directly correlates with TNF-R1 cleavage (Cui et al., 2002). Significant biological functions of cleaved TNF-R1 are illuminated by cleavage-resistant mutant of TNF-R1 that has been linked to TNF-R1-associated periodic syndrome (TRAPS), a dominant inheritable form of autoimmune disorder (McDermott et al., 1999). However, cell surface proteolytic activity does not eradicate the ligand binding activity of TNF-Rs; thus they may act as decoy receptors and dampen TNF-α bioactivity naturally (Engelmann et al., 1990). However, the affinity of ligand binding is depreciated in soluble receptors form, probably due to lack of cooperativity in ligand binding, which is demonstrated significantly by membrane-bound receptors.

TNF-Rs are present on almost all known cell types with a few exceptions such as erythrocytes and unstimulated T cells. The expression of genes encoding these two receptors is differentially regulated in most cells, thereby generating a cell-specific bias in the TNF-R1:TNF-R2 ratio; this ratio is known to preordain cells to a particular set of responses to the cytokine signal (Vandenabeele et al., 1995). While TNF-R1 gene is controlled by an almost noninducible housekeeping promoter, the TNF-R2 gene is widely regulated in many cell types by external stimuli such as LPS and various mitogens (Erikstein et al., 1991; Tannenbaum et al., 1993).

To understand the interaction of the TNF trimer with its receptor, we will briefly illuminate the structural features of TNF-β and TNF-R1 (Banner et al., 1993). Here, we may mention that both TNF-α and TNF-β mediate their cellular response via TNF-Rs (Wallach et al., 1999). TNF, upon binding to the receptors, aggregates them, leading to formation of TNF-R homotrimers and never heterotrimers (Moosmayer et al., 1994). The receptors remain as elongated structures, with each relating to the others parallel along their long axis. They align themselves along the "groove" present on the monomer–monomer interface of the TNF molecule. The TNF-β and receptor interface involves residues from two adjacent TNF monomers and second and third subdomains of the receptor. Once bound to its ligand, the TNF–TNF-R complex is rapidly internalized by clathrin-coated pits and is degraded in the lysosomes (Mosselmans et al., 1988; Porteu and Hieblot, 1994). Such internalization is specific for TNF-R1, while downregulation of TNF-R2 is mediated mainly by its shedding into the cell medium (Higuchi and Aggarwal, 1994).

The two TNF receptors differ greatly in their binding affinity for TNF-α (*Figure 9-1*). While the binding kinetics for sTNF-α to both the receptors was found to be approximately the same, the dissociation kinetics was far greater in the case of TNF-R2. This renders the sTNF-α:TNF-R1 binding almost irreversible, whereas the ligand:TNF-R2 complex is transient. This leads to an overall greater affinity of sTNF-α for TNF-R1. However, both receptors have similar affinity for mTNF-α, thereby allotting relevant significance to the form of available TNF-α to carry out any particular biological activity. In addition to the form of accessible TNF ligand, as already stated, the ratio of TNF-R1 to TNF-R2 also determines the cellular response.

All biological activities attributed to TNF-R2 can also be exerted by TNF-R1 engagement, usually at a lower density. However, in the presence of both receptors, TNF-R1 monopolizes TNF-induced signaling. Taken together, it is apparent that TNF-R2 plays second fiddle to TNF-R1. Why should cells then carry the burden of expressing TNF-R2 at all? The answer lies in understanding the proposed roles played by TNF-R2. The main among them is augmenting ligand–TNF-R1 interaction by a mechanism called "ligand passing" (Tartaglia and Goeddel, 1992; Tartaglia et al., 1993). Owing to its far greater dissociation rate with TNF ligand, TNF-R1 physically conveys the ligand molecule to the adjoining TNF-R1 cluster, thereby facilitating ligand binding of TNF-R1. This "ligand passing" helping hand model also explains the appositeness of the TNF-R1:TNF-R2 ratio. In this respect it must be understood that ligand passing is more relevant in case of sTNF than in case of mTNF. Moreover, TNF-R2 may have a singular role in conveying signals of mTNF-α. This is suggested by studies in TNF-R2-null murine cells that,

like wild-type cells, are sensitive to a high dose of mTNF-α shock. However, TNF-R1-null cells are resistant to similar insults (Vandenabeele et al., 1995).

It has been proposed recently that TNF-Rs are assembled in preligand binding homomultimer state owing to extracellular pre-ligand-binding assembly domain (PLAD)-mediated homophilic interaction of receptor molecules (Chan et al., 2000). Such receptor arrangements, however, remain silent and attain competence only upon ligand binding. Furthermore, engagement of TNF-R1 and TNF-R2 by lymphotoxin-α (TNF-β) in addition to TNF-α itself (Wallach et al., 1999) further complicates the overall regulatory mechanism of the mediated signaling of the TNF–TNF-R complex.

## 2.3 Signaling via TNF-Rs

While TNF-Rs share structural similarities in their extracellular domains, they differ in their intracellular domain, and consequently in signal transduction. However, both receptors demonstrate trimerization upon binding of noncovalent TNF-α trimers and subsequent recruitment of specific adaptor proteins to their cytoplasmic domain. These adaptor proteins then decide the course of the signal and transduce it further downstream (❯ *Figure 9-2a and b*).

### 2.3.1 Signaling via TNF-R1: Endorsing a Double-Edged Sword

Once activated by ligand binding, TNF-R1 may either summon the apoptotic machinery into play or trigger a diametric survival signal for the cell. Origin of such conflicting signals from the same receptor is indeed superficially paradoxical. The key to understand this paradox lies in the composition of the assembled protein complex near the cytoplasmic tail of the receptor, which essentially houses the characteristic protein–protein interaction domain called death domain (DD). During resting condition, TNF-R1 DD is masked by silencer of death domain (SODD) protein in order to override the intrinsic property of DD to self-trigger a signal, thus keeping the receptor faithful to ligand-mediated activation (Jiang et al., 1999). After binding of a ligand, SODD protein is exchanged for a DD-containing adaptor protein, TNF-R-associated death domain (TRADD) protein, which attaches to TNF-R1 by interaction with its respective DD (Hsu et al., 1995). Thus the TNF-R1–TRADD complex forms the platform on which further adaptor proteins are recruited to generate either a death-inducing or a survival-encouraging conglomerate of signaling proteins.

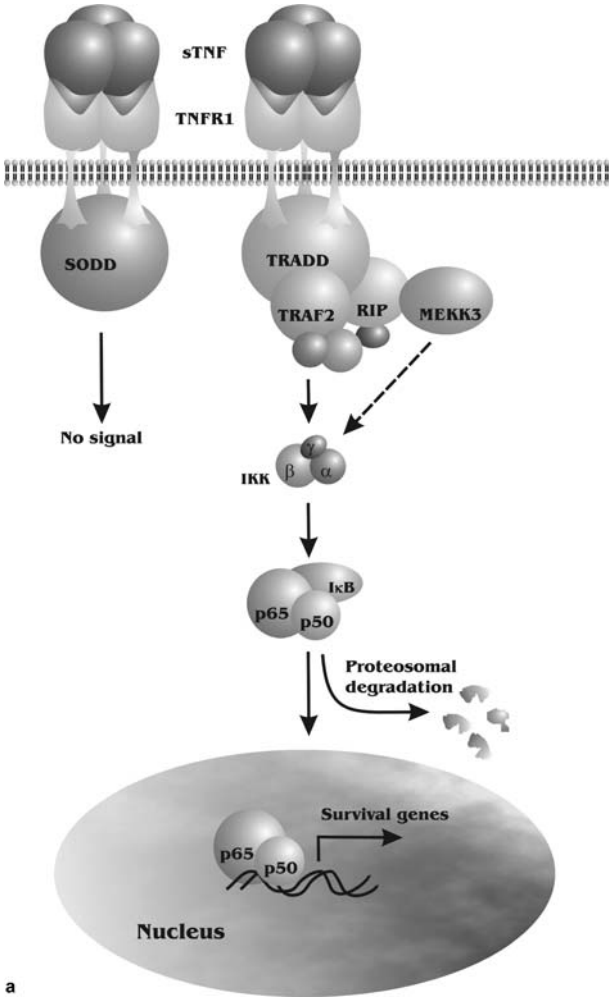
**2.3.1.1 Survival Signals from TNFR1** Origin of survival signals from TNFR1 is contingent on binding of either receptor interacting protein 1 (RIP1) or TNF receptor-associated factor 2 (TRAF2) to TNF-R1. RIP1, a serine/threonine protein kinase, interacts with the TNF-R1–TRADD complex via its C-terminus DD, while TRAF2 interacts with the complex via its C-terminus TRAF domain. Once assembled, the signalosome mediates survival signals via either NF-κB-independent or -dependent pathway (❯ *Figure 9-2a*).

TRAF2, characterized by the presence of N-terminus ring finger and zinc finger motifs, interacts with a variety of survival proteins such as cellular inhibitors of apoptosis proteins (c-IAPs), A-20, and NIK (Aggarwal, 2000). A-20 has been shown to disrupt apoptosis in IκB kinase (IKK) γ-deficient cells from TNF-induced apoptosis by disrupting association of TRADD to TNF-R (He and Ting, 2002). c-IAPs, upon recruitment to TNF-R1, may act to block apoptosis by binding and degrading caspases, as they are otherwise known to do so by interacting with caspases via their N-terminal baculovirus IAP repeat (BIR) domain (Roy et al., 1997). Furthermore, recruitment of NF-κB-inducing kinase (NIK) triggers a kinase cascade, finally activating NF-κB. This transcription factor, as a general rule, mediates transcription and expression of antiapoptotic genes. TRAF2 also engages IKK, member of the kinase cascade leading to NF-κB activation that acts immediately downstream of NIK.

In addition to TRAF2, RIP1 also plays a definite role in NF-κB activation by binding to NF-κB essential modifier (NEMO) (IKKγ), the regulatory subunit of IKK (Zhang et al., 2000). Activation of the NF-κB pathway from TNF-R1 has been proposed to be dependent on the synchronous interplay of TRAF2 and

**Figure 9-2**

**Signaling from TNFR1. (a) Survival signal. TNFR1 remains inactive under normal circumstances by interacting with the silencer of death domain (SODD) protein. However, upon ligand binding, TNFR1 binds to TNF-receptor-associated death domain protein (TRADD) and employs TNF receptor-associated factor-2 (TRAF2) and receptor interacting protein-1 (RIP). These proteins then engage subunits of inhibitory kappaB kinase (IKK) and trigger the NF-κB pathway, which essentially involves nuclear translocation of p65-p50 NF-κB heterodimer after signal-induced proteosomal degradation of inhibitory kappaB (IκB). p65-p50 then mediates transcription of various survival genes (see Table 1). Additionally, RIP may trigger NF-κB via association with MEKK3, a MAP kinase kinase kinase. (b) Death signal. Similar to survival signals, death signal is also induced by exchange of TRADD with SODD at the death domain of TNF-R1 cytoplasmic death domain region. Interaction of TRADD with Fas-associated death domain (FADD) protein facilitates the latter's binding with pro-caspase-8 and its subsequent cleavage. This triggers the caspase cascade, which then leads to apoptosis. On the other hand, apoptotic signals from TNFR1 may also be mediated via c-Jun N terminus kinase (JNK) pathway, where a TRAF-2:MKK-7 interaction recruits SEK, stress-activated protein (SAP) kinase kinase, leading to the activation of JNK. This cascade is conveyed further by phosphorylation of c-Jun by JNK, which then nuclear-translocates and dimerizes with nuclear-resident Fos to form the AP1 transcription factor. AP1 can transactivate inflammatory as well as apoptotic genes**



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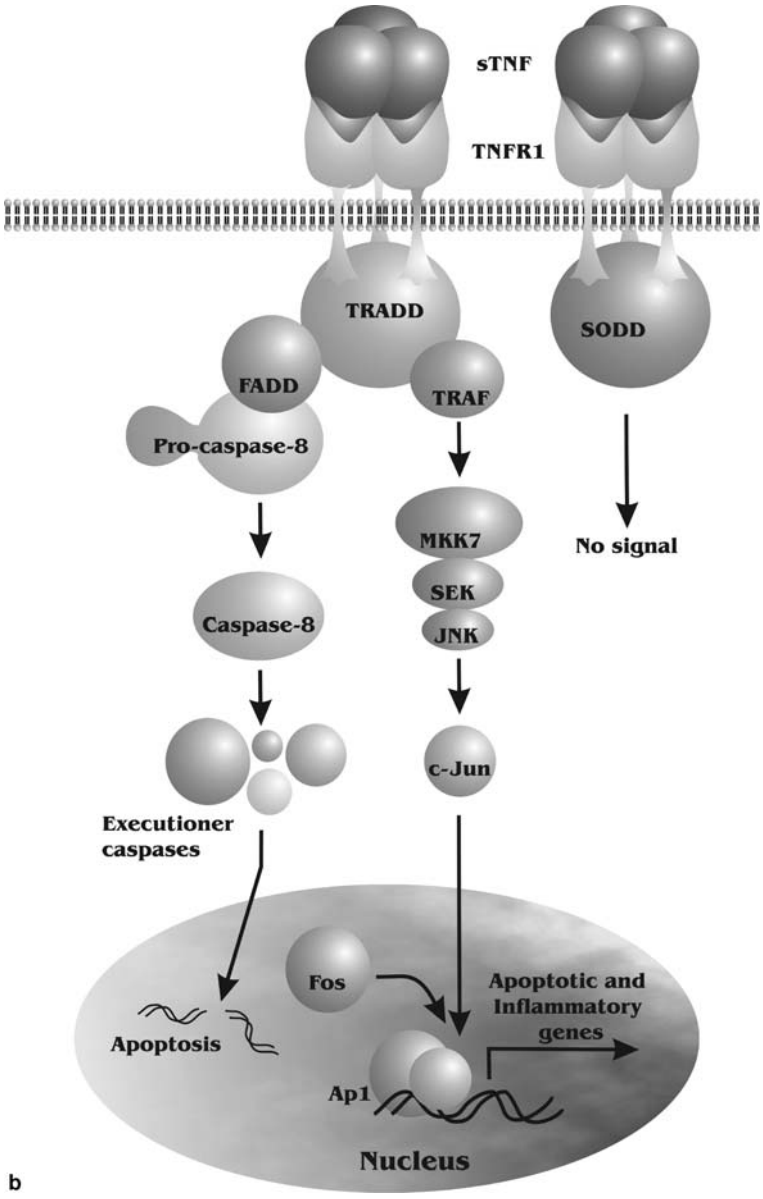



RIP1 (*Figure 9-2a*). While TRAF2 recruits catalytic IKK alpha and beta subunits to TNF-R1, RIP binds to NEMO (Devin et al., 2000, 2001), thereby generating an active IKK trimer complex that ultimately mobilizes active NF-κB to the nucleus via IκB degradation. Although proposed to be the basis of NF-κB activation from TNF-R1, this complex does not involve the kinase activity of RIP. It is the intermediate domain lying between the DD and the kinase domain of the molecule that contributes to NF-κB activation (Hsu et al., 1996). Additionally, RIP may activate IKKs indirectly via mitogen-activated protein kinase

kinase kinase (MEKK3), as MEKK3 interacts with RIP and its deficiency compromises NF-κB activation by TNF-α (Yang et al., 2001) (*Figure 9-2a*).

Synchronized association of these proteins juxtaposing the cytoplasmic tail of TNF-R1 is facilitated by the presence of certain scaffolding proteins. Heat shock protein 90 (Hsp90) and Cdc37 are recruited to TNF-R1 upon ligand binding and are involved in IKK complex formation (Chen et al., 2002). Additionally, a recently described protein called TNF-R-associated ubiquitous scaffolding and signaling (TRUSS) protein interacts with TRADD, TRAF2, and IKK members, thus suggesting its probable role as a scaffolding protein in activating NF-κB activation (Soond et al., 2003).

**2.3.1.2 Apoptotic Signals from TNFR1** Apoptotic signals from TNF-R1 are predominantly generated by recruitment of protein Fas-associated death domain (FADD). As reported for Fas and TNF-related apoptosis inducing ligand (TRAIL) death receptors (members of the TNF receptor superfamily), bound FADD recruits procaspase 8 by death effector domain (DED)-mediated protein–protein interaction. This forms the death-inducing signal complex (DISC) where autolytic cleavage of procaspase 8 renders it active for eliciting the caspase cascade of apoptosis (Fotin-Mleczek et al., 2002). A similar model of caspase activation by TNF-R1 is also proposed (Wajant et al., 2003) (*Figure 9-2b*). However, although FADD directly binds to TNF-R1 DD, the report of successful immunoprecipitation of the DISC complex from TNF-R1 signalosome does not yet exist in current literature. This may hint at absence or instability of such a complex, but it must not be neglected that the default pathway for TNF-R1 is proposed to mediate NF-κB-mediated cell survival in most cell types, where TNF-α is a conditional death ligand whose in vivo death-inducing capability is masked by NF-κB. This is revealed by increased viability of Rel A(−/−) (a NF-κB monomer involved in most of its activities), fibroblasts, and macrophages in response to TNF-α challenge. In this context, it is quite appropriate to suggest that systemic toxicity caused by TNF is related more to its ability to activate the inflammatory response than to induce the apoptotic one.

Such inflammatory responses are mediated in part via activation of NF-κB and c-Jun N terminal kinase (JNK) pathways. In addition to upregulating antiapoptotic genes, NF-κB also upregulates the expression of inflammatory molecules like inducible nitric oxide synthase (iNOS) and TNF-α itself. JNK pathway leads to the activation of the activator protein-1 (AP-1) transcription factor (c-Jun-associated nuclear c-Fos), which has a vivid role in mediating cellular immune response. Synergism between NF-κB and AP-1 factors in upregulating inflammatory responses is well addressed in literature. TRAF2 can elicit both the pathways. Indeed, JNK pathway is known to be triggered by a TRAF2–MKK7-dependent mechanism (Natoli et al., 1997; Reinhard et al., 1997). Is activation of JNK also related to the apoptosis-inducing property of TNF? In mammals, there is evidence for both pro- and antiapoptotic role of JNK (Lin, 2003). Therefore, JNK has been proposed to act as a contextual modulator of TNF-induced apoptosis (Varfolomeev and Ashkenazi, 2004) (*Figure 9-2b*).

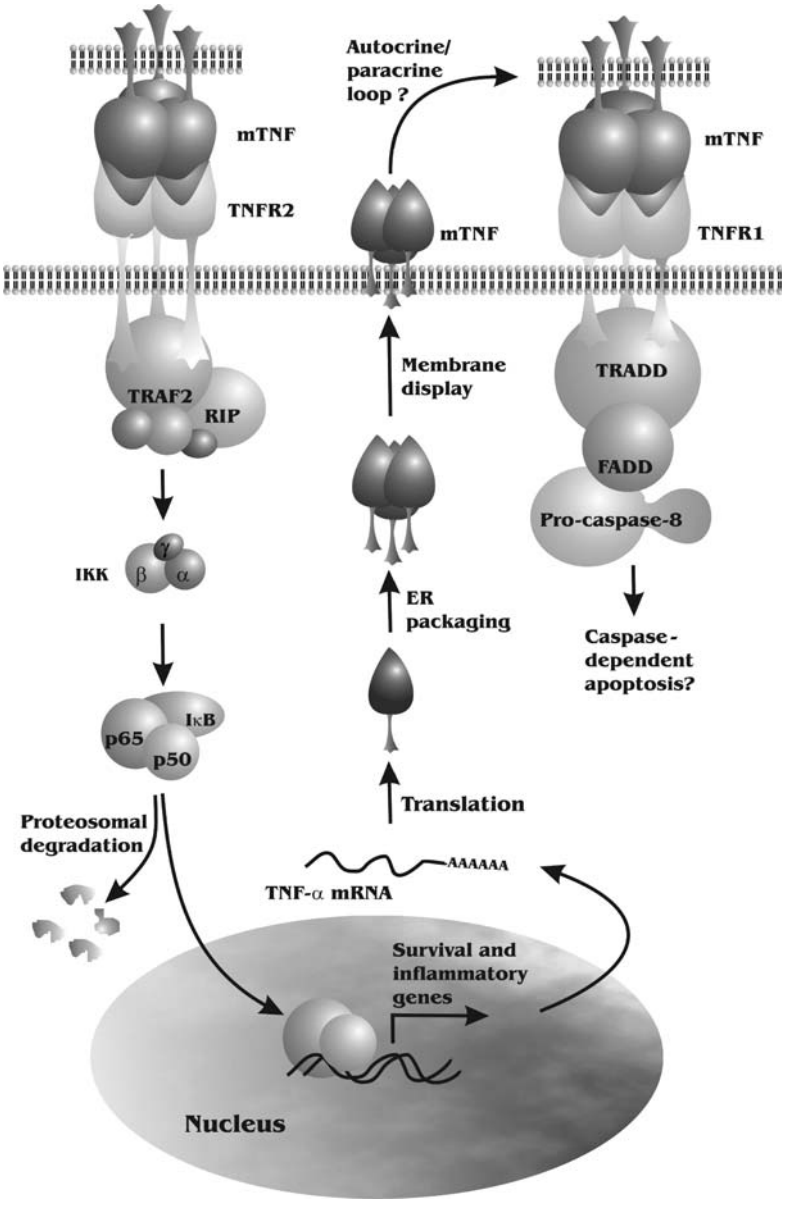
In addition to mediation of death signal by recruiting several proteins, TNF-α is also known to employ cellular lipids to dictate such verdicts. TNF-R1 induces activation of neutral and acidic sphingomyelinases via FADD-dependent but caspase-independent mechanisms, which leads to degradation of sphingomyelin to phosphocholine and ceramide (Fontaine et al., 2002). The latter product, ceramide, an important lipid second messenger, may activate caspases to induce apoptosis or stimulate lysosomes, thus directing the cell toward necrotic cell death (Wajant et al., 2003).

### 2.3.2 Signaling via TNF-R2: Another Double-Edged Sword

TNF-R2 distinctly differs from TNF-R1 in lacking the DD. However, it has a TRAF-binding domain and may, therefore, directly employ TRAF2 without mediation of TRADD. This makes the receptor complex devoid of DD, instantly suggesting a prosurvival role for this receptor. Indeed, TNF-R2 plays a prominent role in mediating prosurvival signals by activating the PI3K–AKT pathway in neuronal cells (Fontaine et al., 2002). Additionally, owing to the presence of TRAF2 that may interact with RIP, it may be presumed that downstream events of TNF-R2 activation resemble that of TNF-R1 in NF-κB activation and subsequent cell survival (*Figure 9-3*).

**Figure 9-3**

**Signaling from TNFR2. Most of the signals from TNFR2 are initiated by membrane-bound TNF-α owing to lesser affinity of the receptor for the soluble form. The cytoplasmic tail of TNFR2, which lacks death domain (DD), interacts with TRAF2, and mediates NF-κB-dependent survival signal like TNFR1 (left side cascade in the diagram). However, NF-κB also transactivates the TNF gene, which is then processed and displayed on the membrane. This may engage TNFR1 and initiate signaling for apoptosis by an autocrine/paracrine loop. This theory appears feasible as the presence of TNFR2 depletes TRAF2 and RIP, thereby facilitating interaction of caspase-activating Fas-associated death domain (FADD) protein with TNFR1**



Is the role of TNF-R2 restricted to eliciting survival signals? By utilizing TNF-R2-specific agonistic antibodies, it has been shown that this receptor by itself may be sufficient to prompt death signals (Grell et al., 1993; Medvedev et al., 1994). How can a DD-lacking receptor perform such a feat? The answer lies in strong induction of NF-κB-dependent TNF-α gene induced by general TNF-R2 signaling. This TNF-α expresses itself as mTNF that subsequently activates TNF-R1 via autocrine/paracrine loops to initiate an apoptotic signal (Grell et al., 1999) (*Figure 9-3*). Additionally, it has also been proposed that TNF-R2 may aid in TNF-R1-induced apoptotic signaling by depleting TRAF2 and c-IAP proteins, thereby accelerating caspase 8 activation by the TNF-R1–TRADD–FADD complex (Fotin-Mleczek et al., 2002).

The above discussion has been presented in a noncomplex linear mode of signal transduction. In reality, TNF signaling is multifarious and almost confusing. As demonstrated by a recent connectivity map of TNF-α-induced NF-κB signal transduction pathways, a total of 221 interactors, including 80 previously unknown molecules, have been reported to undergo activation due to TNF ligand binding (Bouwmeester et al., 2004). Thus, use of the phrase "signal transduction mesh" appears more appropriate for representing this myriad of activated pathways.

## 3 TNF-α in Brain

### 3.1 General Introduction

Despite the dogmatic belief about the brain being an immune-privileged organ, numerous cytokines play diverse functions in the brain. Among them, TNF-α is the most prominent one that is now known to play many roles in the normal and the compromised brain. In addition to being secreted by in-house brain cells (like astroglia and microglia), circulating peripheral TNF-α can diffuse through the blood–brain barrier (BBB) and access brain cells. Most of the customary functions of the cytokine in normal brain are accommodated by TNF-α secreted by brain cells at a low level in certain brain regions. However, as a maxim, the level of brain TNF-α increases dramatically during neuropathological and neurodegenerative conditions, suggesting a greater involvement of the cytokine in these processes (*Figure 9-4*). In subsequent sections, we examine the diverse obligations of TNF-α in conducting various brain functions.

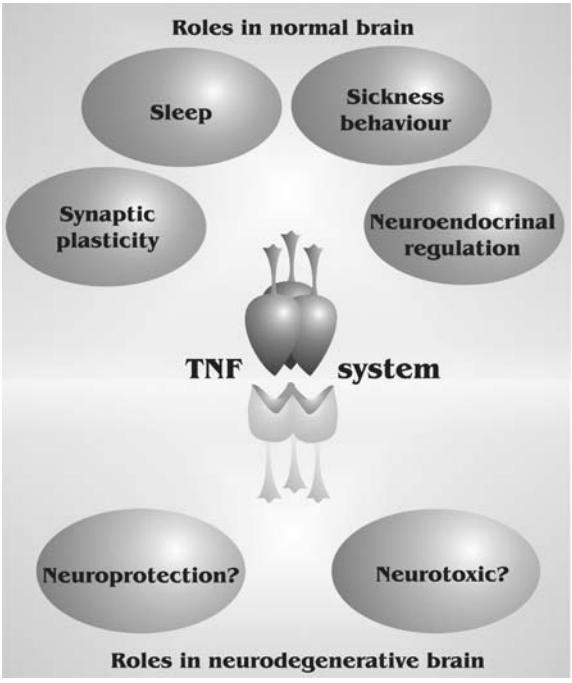
### 3.2 Role of TNF-α in Physiologic Brain Functions

#### 3.2.1 A Thespian Involved in Brain Development

Is TNF-α involved in the development of brain? Studies in sheep neocortex have detected TNF-α at embryonic day 30 as a faint band of immunoreactivity between the ventricular zone and the primordial plexiform layer that decreased in intensity along with progress in ontogeny (Dziegielewska et al., 2000). In developing rodent brain, TNF-α is transiently expressed in immature astrocytes and neurons (Munoz-Fernandez and Fresno, 1998). Is such languid presence significant? The answer is deemed to be affirmative as per the proposal suggesting that at lower concentrations TNF-α may be involved in normal development of the nervous system (Merrill, 1992). This hypothesis was tested in a recent study with TNF-α knockout mice. When exposed to a reference teratogen, cyclophosphamide, excessive apoptosis and suppression of cell proliferation was reported in the brain of TNF-α(−/−) animals compared with wild-type littermates (Torchinsky et al., 2003).

Despite its proposed constructive role in the adult brain system, the cytokine may play the devil during brain development. When overexpressed, TNF-α reduces the weights of whole brain and all brain regions examined during the developmental process (Ye et al., 2003). Additionally, TNF-α has been shown to significantly reduce dendrite development and complexity of developing cortical neurons, consistent with the neuropathology of schizophrenia. Accordingly, it is hypothesized that TNF-α generated from maternal or placental immune machinery in response to infection plays a role in altering brain development, which may subsequently lead to diseases such as schizophrenia (Gilmore et al., 2004).

**Figure 9-4**
**TNF-α in CNS; a brief summary. Involvement of TNF-α in various physiologic and pathophysiologic processes**




### 3.2.2 Regulating Brain Physiology

TNF-α is known to conduct several nonimmunological homeostatic functions in the adult normal brain. This is supported by the constitutive presence of TNF-α and its receptors in discrete brain regions in a fashion consistent with their role in endogenous brain physiology. In normal brain, TNF-α messenger RNA (mRNA) as well as protein demonstrates multifocal expression pattern in areas including the cortex, thalamus, striatum, hypothalamus, caudal raphe nuclei, hippocampus, cerebellum, pons, and brainstem (Breder et al., 1993; see review by Torchinsky et al., 2003 for greater details). However, since the investigators used a soluble fraction of the brain regions, it is difficult to assert whether the expressed TNF-α was in membrane-bound form or was available in brain fluids. In addition to the cytokine, both its receptors are also expressed in all areas displaying the cytokine protein or mRNA (Torchinsky et al., 2003). In contrast to TNF-α expression, which remains confined to neurons under physiologic conditions, both receptors are expressed by all brain cell types, except astrocytes, which predominantly display TNF-R1 (Dopp et al., 1997; Torchinsky et al., 2003). In light of such expression orderliness, it is hardly surprising that TNF-α indeed acts as a neuromodulator performing an array of physiologic assignments in brain, which are described in the following sections.

### 3.2.3 Role in Synaptic Plasticity

Long-term alterations in synaptic strength and transmission (synaptic plasticity) manifested as long-term potentiation (LTP) or long-term depression (LTD) forms the basis of learning and memory. Involvement of TNF-α in synaptic plasticity was reported back in the early 1990s when studies with rat hippocampal slices revealed a slight but prompt increase in basal neurotransmission upon exposure to exogenous TNF-α in a dose-dependent manner (Tancredi et al., 1992). Further examination of LTP in CA1 areas from the slice

suggested an inhibitory role for TNF-α in modulating LTP. Similar observations were also reported in the dentate gyrus (Cunningham et al., 1996). Subsequently, investigations in TNF-R knockout (TNF-R-KO) mice showed that animals lacking TNF-Rs showed impaired LTD response in CA1 neurons (Albensi and Mattson, 2000). As revealed further by the same study, LTP was not affected due to lack of TNF signaling. Thus, despite its suggestive role in LTD, the presence of TNF-α is not essential for LTP, where it probably plays a hindering role. However, lack of TNF signaling does not alter the basal transmission in examined TNF-R-KO mice.

How can TNF-α induce such an effect? Although the definitive answer is still illusive, a few probable avenues to explain this role have been forwarded. LTP and LDP are regulated by potassium influx; thus enhancing action of TNF-α on potassium channel (Houzen et al., 1997) influx may manipulate them. Moreover, regulation of $Ca^{2+}$ homeostasis by TNF-α may mediate its effect on LTP and LTD as calcium influx is a prerequisite for both the processes (Bear and Malenka, 1994). Manipulation of LTP and LTD may also be regulated by altering synaptic strength. TNF-α has been credited with rapid control of synaptic strength at excitatory synapse. Glial-derived constitutive level of TNF-α enhances synaptic efficiency by increasing surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in neurons (Beattie et al., 2002). Preventing activity of endogenous effects of TNF-α retards AMPA trafficking and compromises synaptic efficacy. Thus continual presence of TNF-α is mandatory for preservation of synaptic strength at excitatory synapses.

Regulation of LTP by TNF-α appears to be a biphasic responsibility in which independent signal transduction pathways are recruited in each phase. In a recent report, it has been shown that early-phase LTP is contingent on p38 mitogen-activated protein kinase (MAPK) activity while late-phase LTP is independent of it (Butler et al., 2004). Whether the late phase involves a different TNF signaling pathway or is the result of any autocrine feedback loop of a certain TNF-induced early-phase product has not yet been illuminated.

### 3.2.4 Feeling Sleepy? TNF-α is at Work

The reader is not to be blamed if the boredom of reading a dull review such as this induces sleep symptoms. If so, then the reader is hereby informed that TNF-α is in concert in his or her brain right now. TNF-α, in conjunction with IL-1β, is strongly implicated in regulation of physiologic nonrapid eye movement (NREM) sleep (dreamless sleep) in addition to NREM responses induced by pathological agents (Krueger et al., 2001). Evidences are forwarded by experiments conducted in mice lacking TNF-R1, which are sleepless in comparison with control animals and do not show NREM responses even after exogenous administration of TNF-α (Fang et al., 1997; Deboer et al., 2002). Moreover, inhibition of TNF-α by TNF-soluble receptors inhibits spontaneous sleep and the sleep rebound that occurs after sleep deprivation (Krueger et al., 2001). On the flip side, intracerebroventricular injection of TNF-α promotes sleep (Dickstein et al., 1999).

All these point to a definite role of TNF-α in modulating cycles of sleep and wakefulness. Both peripheral and central sources of TNF-α could contribute to sleep. TNF-α exerts its sleep-inducing effects via TNF-R1 rather than TNF-R2 (Fantino and Wieteska, 1993; Pan et al., 2002). Such somnogenic effects are mediated via well-characterized NREM-regulatory areas of hypothalamic preoptic and basal forebrain area (Obal and Krueger, 2003). How does TNF-α regulate sleep? The answer is yet in its formative stage. However, a generalized proposal includes NF-κB activation, augmented nitric oxide (NO) production by regulating NO synthase, adenosine release, and enhanced prostaglandin ($PGD_2$) synthesis via cyclooxygenase-2 (COX-2) upregulation (Krueger and Majde, 2003).

### 3.2.5 Diurnal Rhythmicity of TNF: Marker of Circadian Rhythm

Sleep is a well-regulated phenomenon showing in-phase rhythmicity among normal subjects. Because TNF is involved in regulating sleep, similar rhythmicity in TNF levels appears to be a valid expectation. Indeed, diurnal light–dark-cycle-oriented variation of TNF-α mRNA concentration in brain is well documented.

Concentration of TNF-α mRNA is highest in hypothalamus, hippocampus, and cerebral cortex of rats at the onset of light phase (rats sleep in the light phase) (Floyd and Krueger, 1997). Moreover, the cytokine level remains high throughout the light rather than the dark phase (Bredow et al., 1997). Conceptually, both peripheral and brain-generated TNF-α may be responsible for such an effect. However, limited circadian variation of circulating TNF-α uptake has been reported (Wichers and Maes, 2002), thus suggesting exclusive involvement of brain-indigenous TNF-α in maintaining the cytokine level variation.

Diurnal rhythm of TNF-α expression has been investigated in great detail and is now the easily understood temporal variation of all neuropeptides in the brain. Such variation may therefore be related to the circadian clock, the 24-h periodic oscillation of biological processes. Any impairment in the rhythmicity of the cytokine oscillation is therefore considered a strong indicator of disease state. The circadian variation of TNF-α has been reported to be significantly impaired in diseases related to sleep disorder, such as obstructive sleep apnea syndrome (Alberti et al., 2003) and chronic insomnia (Vgontzas et al., 2002).

One wonders whether such temporal control of TNF-α gene synthesis is a manifestation of circadian rhythm or does it in some way contribute to sustaining the process. In the light of available evidence, it appears that TNF-α is both the inciter and the consequence of circadian oscillation. As we will see in the next section, on the one hand, TNF-α affects the hypothalamus, the site of circadian clock (suprachiasmatic nucleus) (Besedovsky and del Roy, 1991). On the other hand, TNF-α production is significantly contingent on circadian firing of certain hormones like glucocorticoids (De Rijk et al., 1997).

### 3.2.6 Regulating Neuroendocrinal Circuits

In response to the modulation of the immune system by glucocorticoids, TNF-α and other cytokines activate the hypothalamic–pituitary–adrenal (HPA) axis in a feedback loop in the bidirectional cytokine–HPA axis circuit (Besedovsky and del-Rey, 1991). Where does TNF-α act in this feedback circuit? Administration of subnanomolar concentration of TNF-α in brain induces release of corticotrophin release factor (CRF) from hypothalamus within minutes (Bernardini et al., 1990). Such rapid release of hypothalamic CRF by TNF-α suggests that CNS is the primary site of cytokine action in HPA axis stimulation.

How does TNF-α activate HPA? TNF-α activates HPA axis via CRF release. Pretreatment with the antiserum to CRF completely blocked TNF-α-induced adrenocorticotropic hormone (ACTH) response from pituitary, which is dependent on CRF release from the hypothalamus (Bernardini et al., 1990). The cytokine has been shown to activate neurosecretory CRF-containing neurons in the paraventricular nucleus (PVN) within the hypothalamus (Zhang et al., 2003). However, the effect is not considered to be direct by workers who believe that circulating cytokine molecules are too large to penetrate BBB in a regular fashion. Although more information is needed to understand the cytokine's ability to cross BBB, recent evidence supports the involvement of $PGE_2$ as a likely mediator of the central effects of blood-borne TNF-α (Rivest, 2001).

Is the action of TNF-α limited to its effect on the hypothalamus? TNF-α has been shown to regulate anterior pituitary hormone release in a cell-type-specific way (Harel et al., 1995). Treatment of in vitro pituitary cells by TNF-α rapidly and transiently releases gonadotropins, prolactin, and ACTH (Yamaguchi et al., 1990). This cytokine also influences releases from the adrenal gland, but elaborate discussion on the topic is beyond the scope of this chapter.

Under certain challenging conditions, such as stress, TNF-α expression is significantly enhanced (Holden et al., 1998), which may lead to hyperactivation of HPA. Such dysregulation of HPA leads to depressive behavioral conditions (Wichers and Maes, 2002). In addition to depression, TNF-α influences several other behavioral patterns, which are discussed in the following section.

### 3.2.7 Subjugating Psychophysiology; Brain TNF-α Priming Your Behavior

Administration of exogenous TNF-α in animals prompts sickness behavior (Johnson, 2002), alters nociceptive threshold (Bianchi et al., 1992; Watkins et al., 1995), induces anorectic effects (Bernstein et al., 1991;

Fantino and Wieteska, 1993), perturbs learned taste aversions (Bernstein et al., 1991), incites nausea (Hermann and Rogers, 1995), and manipulates social exploration behavior (Fiore et al., 1998) and spontaneous locomotor action (Bianchi et al., 1992). In humans, a positive relation has been established between anger, verbophysical aggression, and hostility with blood monocyte-generated TNF-α (Suarez et al., 2002).

It is supposed that most of the above actions are mediated by brain TNF-α. For instance, peripheral injections of the cytokine failed to induce anorectic effects generated by injections in the CNS (Fantino and Wieteska, 1993). Despite adequate observations to convincingly corelate TNF-α expression with altered behavior pattern, the responsible mechanisms remain largely elusive. However, it has been proposed that TNF-α elucidates the effects by upregulating certain neuromodulators. For example, altered exploratory and displacement behavior may be mediated by nerve growth factors (NGFs) (Fiore et al., 1998). Similarly, TNF-α sensitizes nociceptors and produces local hyperalgesia via $PGD_2$-mediated pathways (Hori et al., 1998). Regulation of neuroendocrinal parameters by TNF-α may serve as one more avenue of indirect regulation of stress and depression behavior by the cytokine.

However, all behavioral manifestations of TNF-α are not achieved by employing intermediary moieties. This is especially true for regions of brain that lack BBB and are thus doubtlessly accessible to central and peripheral cytokines. For example, medullary dorsal vagus complex (DVC), the loci of gastric motility controlling vagovagal reflex circuits, is essentially devoid of BBB and serves as the site of endogenous TNF-α activity in mediating gastric stasis (nausea) (Hermann et al., 2001). TNF-α is capable of accessing and activating neurons in DVC directly and induces nausea even at subfemtomolar doses (Hermann et al., 2003a, b).

While discussing all the above functions, we have restricted our focus to TNF-α to comply with the title of this chapter. However, it must be emphasized that TNF-α, in most of the above roles, functions in synchrony with other cytokines like IL-1β. Despite such diverse functional utility in the normal brain, the scientific community has all along wondered more about its involvement in the compromised brain of neurodegenerative diseases. In the following sections, we attempt to present an abridged record of observations and opinions about association of TNF-α in the diseased brain.

## 3.3 Role of TNF-α under Pathophysiologic Conditions

### 3.3.1 Involvement of TNF-α in Neurodegenerative Diseases

Enthusiastic research in the past decade has strongly indicated the involvement of TNF-α in inflammatory neurodegenerative disorders like Alzheimer's disease (AD) (Perry et al., 2001), Parkinson's disease (PD) (Nagatsu et al., 2000), human immunodeficiency virus type-1 (HIV)-associated dementia (HAD) (Saha and Pahan, 2003), multiple sclerosis (MS) (Selmaj, 2000), amyotrophic lateral sclerosis (ALS) (Ghezzi and Mennini, 2001) neurotrauma, and stroke (Barone and Parsons, 2000). Let us discuss the evidence pointing strongly at the involvement of TNF-α with these diseases.

TNF-α mRNA level was found to be elevated in autopsy-collected brain tissue of HAD patients (Wesselingh et al., 1993). Subsequently it was found that the increase in severity of dementia in HAD was proportional to the increase in level of TNF-α mRNA (Griffin, 1997). TNF-α protein level, measured by enzyme-linked immunosorbent assay (ELISA) as intrathecal pattern of release in the cerebrospinal fluid (CSF) of AD patients, was found to be 25 times greater in demented patients than in healthy controls (Tarkowski et al., 2003). Similar increase of TNF-α accumulation in CSF of chronic progressive MS patients has also been reported (Sharief and Hentges, 1991). In addition to these studies with soluble forms of TNF-α and its receptors, other studies have revealed the elevated status of membrane-bound form of the cytokine and its receptors. TNF-α immunoreactive glial cells and TNF-R (both) immunoreactive neurons were found in PD brains but not in normal ones (Boka et al., 1994). Similar elevated levels of TNF-R1 in substantia nigra of parkinsonian brains were found by another group (Mogi et al., 2000). However, no difference in levels of TNF-R1 and TNF-R2 in CSF of AD patients was observed, negating any disease-specific tendency of generating sTNF-Rs by cleavage of mTNF-Rs (Tarkowski et al., 2003).

Are elevated TNF components brain cell-derived? Immunohistochemical detection studies suggest that disease-responsive elevated TNF-α and its receptors are products of brain cells and did not originate from systemic compartments. This notion is further supported by the fact that serum TNF-α level was found to be comparatively lower than the CSF level in AD and MS patients (Sharief and Hentges, 1991; Tarkowski et al., 2003).

In addition to variation in protein level, several polymorphisms in genes of TNF-α and its receptors have been related to various neurodegenerative diseases. While looking for any genetic predisposition for development of HAD among HIV-infected patients, it was discovered that TNF-α-308A allele was present in HAD-affected individuals with distinction (Quasney et al., 2001). Similar polymorphism of TNF-α-376A allele marks susceptibility of a segment of Spanish population to MS (Martinez et al., 2004). In addition to polymorphism of the TNF-α gene, several genetic variations have also been reported for TNF-R2. Polymorphism in TNF-R2 exon 6 has been correlated with late-onset AD (Perry et al., 2001). Similarly, polymorphism in exon 10 of the same gene is reported to enhance susceptibility to MS (Ehling et al., 2004).

These demarcating differences in genomic background and altered TNF-α and its receptors' expression level strongly indicate that the TNF system is a major proctor of neurodegenerating diseases. What does the altered level of TNF-α expression signify? The answer lies in knowing the precise role of the cytokine in neurodegenerative diseases. However, opinions about the exact role of TNF-α are a cauldron of contradiction.

### 3.3.2 Apocryphal Role of TNF-α in the Neurodegenerative Brain

Presence of two receptors with their variation in binding affinity for sTNF-α and mTNF-α complicates the TNF system. This complexity is enhanced by the fact that TNF-α ligand binding to its receptors can activate apoptotic as well as survival pathways. Not surprisingly therefore, the cytokine has been implicated both in neurodegeneration and in neuroprotection. On the one hand there is compelling evidence to insinuate a neuroprotective role for the cytokine during disease condition while on the other hand several evidences suggest a neurotoxic role. In the following sections, we discuss evidences that propose mechanisms of TNF action in either role.

**3.3.2.1 Neurocidal Role of TNF-α** Involvement of TNF system in PD neuropathy was illustrated while investigating effects of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), a dopaminergic neurotoxin that mimics some of the key features associated with PD, in mice lacking TNF receptors. Transgenic homozygous mutants for both receptors (but not either one) were protected against MPTP-induced loss of dopaminergic neurons (Sriram et al., 2002). It has also been reported separately that genetic ablation of TNF-α and TNF synthesis inhibitors attenuates MPTP toxicity in mouse striatum (Ferger et al., 2004), suggesting that this cytokine may play a vital role in PD disease development.

It has been postulated that TNF-α kills neurons directly by recruiting apoptotic cascades in them after binding to TNF-R1 (Garden, 2002; Garden et al., 2002), which is similar to its tumor killing property. This theory has been forwarded on the basis of two observations. In the HAD model of neuronal killing, it was observed that blocking TNF-α activity attenuated the neurotoxic effect of the HIV coat protein gp120 (Bezzi et al., 2001). Moreover, inhibition of caspase-8 prevented TNF-α-induced neurotoxicity in mixed cerebellar cultures (Garden et al., 2002). Therefore it is suggested that TNF-α, produced by nonneuronal cells due to gp120 action, activates the caspase cascade by binding to its receptor. Recently, it has been shown that TNF-R1 cytoplasmic DD-associated complex is compulsory for β-amyloid-induced neurotoxicity (Li et al., 2004).

TNF-α may also induce neuronal death by several other indirect methods. Under disease conditions, TNF-α triggers release of several neurotoxins by acting on nonneuronal brain cells. For example, it mediates release of excitotoxic amino acids (EAA) L-cysteine and glutamate from activated microglia and astrocytes, respectively (Yeh et al., 2000; Bezzi et al., 2001). Excessive EAA accumulation in neuronal vicinity exorbitantly activates *N*-methyl-D-aspartate (NMDA)-receptor-operated ion channels leading to unwarranted $Ca^{2+}$ influx (Lipton, 1998) and loss of cellular homeostasis culminating in lysis or apoptosis of the cell

(Foos and Wu, 2002). In addition to release of EAA, TNF-α upregulates several proinflammatory genes in activated glial cells where it stimulates expression and shedding of a plethora of cytokines and cytokine receptors (Rose-John and Heinrich, 1994). Moreover, it also costimulates iNOS production in astrocytes in conjunction with other cytokines (Pahan et al., 2000), leading to NO-mediated enhanced neuronal toxicity.

In addition to its role in the production of many neurotoxins from glial cells, TNF-α has been shown to act synergistically with many toxic elements to augment neuronal dysfunction. TNF-α enhances glutamate-releasing action of stromal-derived factor-1 (SDF-1, an α chemokine) from astrocytes cocultured with microglia (Bezzi et al., 2001). In addition to aiding its release, TNF-α intensifies neurotoxic effect of glutamate in cerebral cortex neurons (Chaparro-Huerta et al., 2002). In HAD, HIV neurotoxins are aided by the cytokine to promote neuronal damage (Saha and Pahan, 2003). Moreover, it has been reported that TNF-α boosts neuronal impairment induced by other cytokines, like IL-1 and IFN-γ, in cell cultures representing the inflammatory milieu of neurodegenerative foci (Chao et al., 1995; Downen et al., 1999).

**3.3.2.2 Neuroprotective Role of TNF-α** The neuroprotective role of the cytokine was initially demonstrated by rescuing hippocampal, septal, and cortical neurons from glucose-deprivation-induced death by pretreatment with TNF-α (Cheng et al., 1994). It was shown that TNF-α attenuated elevation of intracellular $Ca^{2+}$ level due to glucose deprivation by upregulating a calcium-binding protein Calbindin-$D_{28K}$. Furthermore, TNF-α pretreatment of pure neuronal cultures also protected the latter from toxicity of β-amyloid, the etiological reagent of AD (Barger et al., 1995). Additionally, in vivo neuroprotective role of TNF-α is well demonstrated by studies in TNF-R knockout mice, which showed greater susceptibility than normal animals (Bruce et al., 1996; Gary et al., 1998). In yet another study it has been shown that TNF-α had a insignificant effect on hippocampal neurons of TNF-R1(−/−) animals, whereas TNF-R2-negative neurons were vulnerable to TNF-α, even at low doses (Yang et al., 2002).

Most of the neuroprotective roles of TNF-α are mediated via recruitment of NF-κB. As already discussed, both TNF-R1 and TNF-R2 may lead to the activation of NF-κB. In addition to direct IKK-mediated activation, NF-κB can also be activated via pro-survival kinase, Akt (protein kinase B) (Diem et al., 2001). Activated NF-κB dimers enter the nucleus and influence the expression/repression of a complex array of genes constituting the neuronal response to various neurodegenerative insults (Diem et al., 2001). Neuronal NF-κB is proposed to be neuroprotective in nature (Mattson et al., 2000). Several studies have revealed the utility of NF-κB p50 subunit in mediating neuroprotection. Lack of p50 subunit increases vulnerability of hippocampal neurons to excitotoxic injury (Mattson and Camandola, 2001) and striatal neurons in Huntington's disease model (Yu et al., 1999).

How does recruiting NF-κB underwrite TNF-α-induced neuroprotection? A probable mechanism to do so is by upregulating genes encouraging survival. TNF-α upregulates antiapoptotic proteins like Bcl-2 and Bcl-$X_L$ in primary hippocampal neurons (Tamatani et al., 1999). NF-κB-mediated upregulation of these survival proteins has been shown to be at least partially responsible for cell survival in a hypoxia model of neurodegeneration suggesting that TNF-α–NF-κB pathway may trigger more pro-survival genes than these two (❯ *Table 9-1*).

In addition to its direct effect in ensuring survival via recruitment of NF-κB, TNF-α also facilitates survival by manipulating other secondary messengers. It downregulates α-chemokine receptor CXCR4 expression in astrocytes (Han et al., 2001). α-chemokines, like SDF, induce excessive EAA production from activated astrocytes, which makes local neurons more vulnerable to excitotoxicity. In addition to negation of such threats, downregulation of the chemokine receptor in astrocytes may also shield neurons against HIV protein gp120-induced toxicity in HAD (CXCR4 is also a coreceptor for gp120). Additionally, TNF-α induces the production of β-chemokines in activated glial cell (Guo et al., 1998). Greater concentration of β-chemokines helps in preservation of cognitive functions (Letendre et al., 1999). Moreover, TNF-α induces the expression of fractalkine (a δ-chemokine otherwise referred to as CX3CL1) in astrocytes (Yoshida et al., 2001). This δ-chemokine protects neurons from gp120-mediated toxicity and inhibits HIV entry into microglial cells exhibiting its receptor (CX3CR1) (Cotter et al., 2002).

**Table 9-1**
**Upregulation of various NF-κB-dependent genes by TNF-α in brain cells**

| Upregulated gene | Cell type | Effect | Reference |
|---|---|---|---|
| *Bcl-2* | Neuron | Antagonizes apoptosis | Tamatani et al. (1999) |
| *Bcl-X* | Neuron | Antagonizes apoptosis | Tamatani et al. (1999) |
| *MnSOD* | Neuron/ astrocytes | ROS scavenger; promotes survival | Bruce-Keller et al. (1999) |
| *Cu/Zn-SOD* | PC-12 neurons | ROS scavenger; promotes survival | Rojo et al. (2004) |
| *Calbindin* | Neuron | Maintains $Ca^{2+}$ homeostasis; promotes survival | Cheng et al. (1994) |
| *c-IAP-2* | Spinal cord | Relays survival signal from TNFRs; promotes survival | Kim et al. (2001) |
| *Fractalkine* (*CX3CL1*) | Astrocytes | Inhibits gp120-mediated neurotoxicity | Yoshida et al. (2001) |

All these genes have been shown to encourage cell survival in the CNS

### 3.3.3 Current Outlook on the Role of TNF-α in Neurodegenerative Conditions

We have evidence suggesting a dual role (neuroprotective as well as neurodegenerative) of TNF-α. Deriving a conclusive role for the cytokine under these circumstances is a matter of ongoing debate. We believe extreme outlooks (neurotoxic or neurotropic) must be interpreted with caution in the context of experimental limitations. The overall effect of the activation of the TNF system in any cell is decided by a range of several mitigating factors and their interplay. Neuronal type and location, timing and dose of TNF-α expression, soluble or membrane-bound form of available TNF components, and presence or absence of any receptor pretreatment are just a few of them. In the following section we describe a few therapeutically approachable factors that are believed by most neuroimmunologists to be major policymakers of TNF action in brain.

**3.3.3.1 Receptor Specificity Among Cell Types and Their Crosstalk** Different neuronal subtypes may endure TNF-α signal differently depending on the TNF-R1:TNF-R2 ratio. The importance of receptor ratio is well demonstrated in MS patients who show an unbalanced receptor shedding. Spontaneous shedding of TNF-R1 was lower in MS patients than in healthy volunteers, whereas spontaneous generation of sTNF-R2 was significantly higher in MS patients (Selmaj, 2000). Such disparity in receptor ratio at the membrane or soluble form may be responsible for MS etiology. Opposing roles have been allotted to these two receptors in a retinal ischemia study in TNFR-lacking mice. Absence of TNF-R1 significantly attenuated neuronal death whereas augmented neurodegeneration was observed in TNF-R2 knockout mice (Fontaine et al., 2002). Although the role of TNF-R2 in neuroprotection appears well substantiated by other studies as well (Bruce et al., 1996; Yang et al., 2002), the exact role of TNF-R1 remains elusive. In the MPTP model of PD, receptor knockout studies offer contrasting observations. Sriram et al. (2002) (Ferger et al., 2004) showed that mice deficient in TNF-R are protected against dopaminergic neurotoxicity, while Rousselet et al. (2002) reported absence of any influence of TNF-R obliteration in the knockout mice. Such contrasting observations prohibit us from opining conclusively about the exact role of TNF-α system in the MPTP model of PD.

TNF-Rs, apart from utilizing similar downstream messengers, engage in intraneural crosstalk that may also play a pivotal role in framing of the ultimate outcome. We have previously discussed the "ligand passing" role of TNF-R2 and competition between these two receptors for downstream modulators like TRAF2. Such interactions can indeed control differing effects of TNF system. Moreover, such crosstalk could also exist between TNF-Rs and other signaling receptors such as nerve growth factor

receptor (NGF-R) (Haviv and Stein, 1999) and insulin growth factor receptor (IGF-R) (Venters, 2001). Cointeraction of NGF-R and TNF-R has been proposed in mediating β-amyloid-induced neurotoxicity (Perini et al., 2002). Similarly, it has been shown that simultaneous stimulation of IGF-I receptor and TNF-R leads to different outcomes distinct from results of their individual stimulation (Venters, 2001).

In addition to receptor crosstalk, neuronal fate may be decided by ligand crosstalk as well. TNF-Rs are not specific only for TNF-α but are also activated by TNF-β (lymphotoxin-α), which is generally considered to play a lesser role in brain immunology. However, locally upregulated TNF-β is considered to be the principal mediator of murine cerebral malaria (Engwerda et al., 2002). Moreover, in a study with MS patients, serum TNF-β level was found to be more in them than in controls (Kraus et al., 2002). Thus one has to consider the probable interaction of TNF-β with TNF-Rs, which may either compliment or hamper the outcome of receptor interaction with TNF-α.

**3.3.3.2 Timing and Duration of TNF-α Exposure** Most in vitro studies suggesting neuroprotective role of TNF-α involve preconditioning of neurons by TNF-α for 24–48 h. Long-term preconditioning offers neuroprotection by decreasing currents induced by glutamate, NMDA, and AMPA excitotoxic challenges in a NF-κB-dependent fashion (Furukawa and Mattson, 1998). Thus temporal magnitude of TNF-α challenge certainly influences the ultimate cell fate.

The temporal aspect of TNF-α activity is well illuminated by another study with hippocampal organotypic slices. When applied before ischemic stress, TNF-α induces neuroprotection. However, administration of the cytokine after ischemic insult proved to be neurotoxic (Wilde et al., 2000). Other studies involving acute and chronic responses of TNF-α-deficient mice to experimental brain injury suggest that TNF-α could be deleterious during posttraumatic period but facilitates long-term histological repair and behavioral recovery (Scherbel et al., 1999). These studies underline the importance of timing and duration of TNF-α exposure.

**3.3.3.3 Synergistic Modulation** TNF-α does not act alone in vivo. In most of the neurodegenerative immune responses, a plethora of cytokine and chemokines are generated in the diseased milieu. Thus, the extent of neurotoxicity or neuroprotection mediated by this cytokine is often manipulated by other cytokines, chemokines, EAA, or several other toxins like gp120 and NO. This may be illustrated with iNOS gene expression. TNF-α, by itself, is a poor inducer of iNOS in astrocytes. However, it provokes a significant induction when applied in combination with IL-1β or IFN-γ (Pahan et al., 2000). While considering therapeutic approaches to attenuate the toxicity due to enhanced expression of immune components in brain, one must consider the combinatorial mode of activity for the targeted molecules.

## 4 Clinical Perspective of Active TNF System in Brain Diseases

In view of their widespread participation in various diseases, a substantial amount of research has focused on therapies concerning TNF-α and its receptors. Despite the initial hype about its tumoricidal properties, therapies revolving around this cytokine did not prove satisfactory in cancer treatment due to its inclination toward inducing systemic toxicity. The systemic toxicity is generated strictly by sTNF-α as suggested by the cleavage-resistant TNF-α mutant existing only in membrane-bound form that resulted only in cytotoxicity mediated by cell-to-cell contact (Perez et al., 1990). Similarly, some studies involving TNF-R1 and TNF-R2 have suggested a systemic role for TNF-R1 while TNF-R2 has been found to exhibit cytotoxicity only (Van Ostade et al., 1993). This corroborates well with the fact that while TNF-R1 is inducible by sTNF-α, TNF-R2 has greater affinity for mTNF-α and thus depends on cell-to-cell contact for its activation.

Therapeutic strategies involving TNF-α mainly include neutralization of the cytokine via anti-TNF-α antibodies, its soluble receptors, or TNF-R fusion proteins. Glucocorticoids and cyclosporine have also been used as drugs to suppress TNF-α production. One more indirect approach has been to inhibit the action of the cytokine by blocking its secondary signal mediators such as IL-1, IL-6, or NO. In many instances, applications of these therapies have been highly successful. For example, utilization of a chimeric

monoclonal Ig anti-TNF-α antibody (Remicade) to treat intestinal inflammation in Crohn's disease has been clinically successful. Significant success has also been obtained in treatment of RA by utilizing several TNF antagonists (Olsen and Stein, 2004).

Since most of the neurodegenerative diseases have an inflammatory parameter involving TNF-α, anti-TNF-α therapeutic approaches have also been attempted in such diseases. In experimental allergic encephalomyelitis (EAE), animal models of MS, invalidation of TNF-α by soluble TNF-Rs (Lenercept) or with TNF-α neutralizing antibodies showed a positive effect on pathogenesis and demyelination (Klinkert et al., 1997; Korner et al., 1997). However, the administration of Lenercept in 168 patients with relapsing–remitting MS had a detrimental effect, where number of clinical exacerbations was found strikingly higher among patients on Lenercept (the Lenercept Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group, 1999). These patients had longer durations of relapses with earlier exacerbations and more serious neurological deficits compared with the placebo group. Such negative outcomes have led to premature termination of further studies in MS. Furthermore, unsuspected anti-TNF-α therapy for juvenile RA led to the development of MS in a Los Angeles patient (Sicotte and Voskuhl, 2001). Thus, the failed clinical study for anti-TNF-α therapy in MS may serve as a severe warning against exaggeration of positive results in animal studies until significantly extrapolated clinically.

Despite the forced abortion of anti-TNF-α clinical trial in phase II for MS, we have learned important lessons from the enterprise. Therapeutic options are still being forwarded for treatment of various brain diseases. Arresting TNF-α activity by blocking its downstream signal transducer p38 MAPK has been forwarded to treat focal stroke (Barone and Parsons, 2000). Utilization of phosphodiesterase inhibitor ibudilast has been proposed to intercept TNF-α production by activated microglia, which if clinically successful may turn out to be a promising antineurodegenerative drug applicable as primary or adjunct therapy (Mizuno et al., 2004). In addition to curing diseases leading to dementia, excess TNF-α-induced obstruction in gastric motility (nausea) has been relieved by utilizing a TNF-R:Fc construct (Hermann et al., 2003a, b).

Despite its obvious limitations, anti-TNF-α therapy holds promise in curing disorders related to brain malfunction. However, it must be appreciated that TNF-α is by far not the only cytokine associated with disease conditions. For instance, neutralization of TNF-Rs alone does not protect dopaminergic neurons against degeneration in experimental models of PD (Hirsch et al., 2003), suggesting that manipulation of a single signaling pathway may not be sufficient to ensure therapeutic success.

## 5 Conclusion

In addition to several housekeeping functions performed by the cytokine in the normal brain, TNF-α also plays an integral part in the diseased brain. While major research focus is on the latter aspect, the former role of TNF-α must be appreciated as well. The discussion in this chapter is by no means a complete summary of every role performed by the cytokine. We have left out certain proposed roles of TNF-α in the brain to maintain the trimness of the article. For example, TNF-α plays a prominent role in thermoregulation and febrile response where both pyretic and antipyretic roles of the cytokine have been proposed (Leon, 2002). The cytokine is now being widely studied for more than a quarter of a century. However, a paucity of information regarding its role in the brain cannot be denied. Further scope for research includes delineating greater housekeeping roles of TNF-α in brain functioning and pinpointing its precise mode of action during neurodegenerative conditions to open therapeutic avenues.

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## References


Aggarwal BB. 2000. Tumor necrosis factors receptor associated signaling molecules and their role in activation of apoptosis, JNK and NF-kappaB. Ann Rheum Dis 59: i6-i16.

Aggarwal BB, Aiyer RA, Pennica D, Gray PW, Goeddel DV. 1987. Human tumor necrosis factors: structure and receptor interactions. Ciba Found Symp 131: 39-51.

Albensi BC, Mattson MP. 2000. Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. Synapse 35: 151-159.

Alberti A, Sarchielli P, Gallinella E, Floridi A, Mazzotta G, et al. 2003. Plasma cytokine levels in patients with obstructive sleep apnea syndrome: a preliminary study. J Sleep Res 12: 305-311.

Banner DW, D'Arcy A, Janes W, Gentz R, Schoenfeld HJ, et al. 1993. Crystal structure of the soluble human 55 kD TNF receptor–human TNF beta complex: implications for TNF receptor activation. Cell 73: 431-445.

Barger SW, Horster D, Furukawa K, Goodman Y, Krieglstein J, et al. 1995. Tumor necrosis factors alpha and beta protect neurons against amyloid beta-peptide toxicity: evidence for involvement of a kappa B-binding factor and attenuation of peroxide and Ca2+ accumulation. Proc Natl Acad Sci USA 92: 9328-9332.

Barone FC, Parsons AA. 2000. Therapeutic potential of anti-inflammatory drugs in focal stroke. Expert Opin Investig Drugs 9: 2281-2306.

Bear MF, Malenka RC. 1994. Synaptic plasticity: LTP and LTD. Curr Opin Neurobiol 4: 389-399.

Beattie EC, Stellwagen D, Morishita W, Bresnahan JC, Ha BK, et al. 2002. Control of synaptic strength by glial TNFalpha. Science 295: 2282-2285.

Bernardini R, Kamilaris TC, Calogero AE, Johnson EO, Gomez MT, et al. 1990. Interactions between tumor necrosis factor-alpha, hypothalamic corticotropin-releasing hormone, and adrenocorticotropin secretion in the rat. Endocrinology 126: 2876-2881.

Bernstein IL, Taylor EM, Bentson KL. 1991. TNF-induced anorexia and learned food aversions are attenuated by area postrema lesions. Am J Physiol 260: R906-R910.

Besedovsky HO, del-Rey A. 1991. Feed-back interactions between immunological cells and the hypothalamus–pituitary–adrenal axis. Neth J Med 39: 274-280.

Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YC, et al. 1985a. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature 316: 552-554.

Beutler B, Milsark IW, Cerami AC. 1985b. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 229: 869-871.

Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, et al. 2001. CXCR4-activated astrocyte glutamate release via TNFalpha: amplification by microglia triggers neurotoxicity. Nat Neurosci 4: 702-710.

Bianchi M, Sacerdote P, Ricciardi-Castagnoli P, Mantegazza P, Panerai AE. 1992. Central effects of tumor necrosis factor alpha and interleukin-1 alpha on nociceptive thresholds and spontaneous locomotor activity. Neurosci Lett 148: 76-80.

Boka G, Anglade P, Wallach D, Javoy-Agid F, Agid Y, et al. 1994. Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. Neurosci Lett 172: 151-154.

Bouwmeester T, Bauch A, Ruffner H, Angrand PO, Bergamini G, et al. 2004. A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. Nat Cell Biol 6: 97-105.

Breder CD, Tsujimoto M, Terano Y, Scott DW, Saper CB. 1993. Distribution and characterization of tumor necrosis factor-alpha-like immunoreactivity in the murine central nervous system. J Comp Neurol 337: 543-567.

Bredow S, Guha-Thakurta N, Taishi P, Obal F Jr, Krueger JM. 1997. Diurnal variations of tumor necrosis factor alpha mRNA and alpha-tubulin mRNA in rat brain. Neuroimmunomodulation 4: 84-90.

Bruce AJ, Boling W, Kindy MS, Peschon J, Kraemer PJ, et al. 1996. Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. Nat Med 2: 788-794.

Bruce-Keller AJ, Geddes JW, Knapp PE, McFall RW, Keller JN, Holtsberg IW, Parthasarathy S, Steiner SM, Mattson MP. 1999. Anti-death properties of TNF against metabolic poisoning: mitochondrial stabilization by MnSOD. J Neuroimmunol 93: 53-71.

Butler MP, O'Connor JJ, Moynagh PN. 2004. Dissection of tumor-necrosis factor-alpha inhibition of long-term potentiation (LTP) reveals a p38 mitogen-activated protein kinase-dependent mechanism which maps to early- but not late-phase LTP. Neuroscience 124: 319-326.

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, et al. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci USA 72: 3666-3670.

Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, et al. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science 288: 2351-2354.

Chao CC, Hu S, Ehrlich L, Peterson PK. 1995. Interleukin-1 and tumor necrosis factor-alpha synergistically mediate neurotoxicity: involvement of nitric oxide and of *N*-methyl-D-aspartate receptors. Brain Behav Immun 9: 355-365.

Chaparro-Huerta V, Rivera-Cervantes MC, Torres-Mendoza BM, Beas-Zarate C. 2002. Neuronal death and tumor necrosis factor-alpha response to glutamate-induced

excitotoxicity in the cerebral cortex of neonatal rats. Neurosci Lett 333: 95-98.

Chen G, Cao P, Goeddel DV. 2002. TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. Mol Cell 9: 401-410.

Cheng B, Christakos S, Mattson MP. 1994. Tumor necrosis factors protect neurons against metabolic–excitotoxic insults and promote maintenance of calcium homeostasis. Neuron 12: 139-153.

Coley WB. 1891. Contribution to the knowledge of sarcoma. Ann Surg 14: 199-220.

Cotter R, Williams C, Ryan L, Erichsen D, Lopez A, et al. 2002. Fractalkine (CX3CL1) and brain inflammation: implications for HIV-1-associated dementia. J Neurovirol 8: 585-598.

Cui X, Hawari F, Alsaaty S, Lawrence M, Combs CA, et al. 2002. Identification of ARTS-1 as a novel TNFR1-binding protein that promotes TNFR1 ectodomain shedding. J Clin Invest 110: 515-526.

Cunningham AJ, Murray CA, O'Neill LA, Lynch MA, O'Connor JJ. 1996. Interleukin-1 beta (IL-1 beta) and tumour necrosis factor (TNF) inhibit long-term potentiation in the rat dentate gyrus in vitro. Neurosci Lett 203: 17-20.

Deboer T, Fontana A, Tobler I. 2002. Tumor necrosis factor (TNF) ligand and TNF receptor deficiency affects sleep and the sleep EEG. J Neurophysiol 88: 839-846.

De Rijk R, Michelson D, Karp B, Petrides J, Galliven E, et al. 1997. Exercise and circadian rhythm-induced variations in plasma cortisol differentially regulate interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor-alpha (TNF alpha) production in humans: high sensitivity of TNF alpha and resistance of IL-6. J Clin Endocrinol Metab 82: 2182-2191.

Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, et al. 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity 12: 419-429.

Devin A, Lin Y, Yamaoka S, Li Z, Karin M, et al. 2001. The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. Mol Cell Biol 21: 3986-3994.

Dickstein JB, Moldofsky H, Lue FA, Hay JB. 1999. Intracerebroventricular injection of TNF-alpha promotes sleep and is recovered in cervical lymph. Am J Physiol 276: R1018-R1022.

Diem R, Meyer R, Weishaupt JH, Bahr M. 2001. Reduction of potassium currents and phosphatidylinositol 3-kinase-dependent AKT phosphorylation by tumor necrosis factor-(alpha) rescues axotomized retinal ganglion cells from retrograde cell death in vivo. J Neurosci 21: 2058-2066.

Dopp JM, Mackenzie- Graham A, Otero GC, Merrill JE. 1997. Differential expression, cytokine modulation, and specific functions of type-1 and type-2 tumor necrosis factor receptors in rat glia. J Neuroimmunol 75: 104-112.

Downen M, Amaral TD, Hua LL, Zhao ML, Lee SC. 1999. Neuronal death in cytokine-activated primary human brain cell culture: role of tumor necrosis factor-alpha. Glia 28: 114-127.

van Dullemen HM, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, et al. 1995. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 109: 129-135.

Dziegielewska KM, Moller JE, Potter AM, Ek J, Lane MA, et al. 2000. Acute-phase cytokines IL-1beta and TNF-alpha in brain development. Cell Tissue Res 299: 335-345.

Ehling R, Gassner Ch, Lutterotti A, Strasser- Fuchs S, Kollegger H, et al. 2004. Genetic variants in the tumor necrosis factor receptor II gene in patients with multiple sclerosis. Tissue Antigens 63: 28-33.

Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, et al. 1993. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. Arthritis Rheum 36: 1681-1690.

Engelmann H, Novick D, Wallach D. 1990. Two tumor necrosis factor-binding proteins purified from human urine. Evidence for immunological cross-reactivity with cell surface tumor necrosis factor receptors. J Biol Chem 265: 1531-1536.

Engwerda CR, Mynott TL, Sawhney S, De Souza JB, Bickle QD, et al. 2002. Locally up-regulated lymphotoxin alpha, not systemic tumor necrosis factor alpha, is the principle mediator of murine cerebral malaria. J Exp Med 195: 1371-1377.

Erikstein BK, Smeland EB, Blomhoff HK, Funderud S, Prydz K, et al. 1991. Independent regulation of 55-kDa and 75-kDa tumor necrosis factor receptors during activation of human peripheral blood B lymphocytes. Eur J Immunol 21: 1033-1037.

Fang J, Wang Y, Krueger JM. 1997. Mice lacking the TNF 55 kDa receptor fail to sleep more after TNFalpha treatment. J Neurosci 17: 5949-5955.

Fantino M, Wieteska L. 1993. Evidence for a direct central anorectic effect of tumor-necrosis-factor-alpha in the rat. Physiol Behav 53: 477-483.

Ferger B, Leng A, Mura A, Hengerer B, Feldon J. 2004. Genetic ablation of tumor necrosis factor-alpha (TNF-alpha) and pharmacological inhibition of TNF-synthesis attenuates MPTP toxicity in mouse striatum. J Neurochem 89: 822-833.

Fiore M, Alleva E, Probert L, Kollias G, Angelucci F, et al. 1998. Exploratory and displacement behavior in transgenic mice expressing high levels of brain TNF-alpha. Physiol Behav 63: 571-576.

Floyd RA, Krueger JM. 1997. Diurnal variation of TNF alpha in the rat brain. Neuroreport 8: 915-918.

Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, et al. 2002. Neurodegenerative and neuroprotective effects of tumor necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. J Neurosci 22: RC216.

Foos TM, Wu JY. 2002. The role of taurine in the central nervous system and the modulation of intracellular calcium homeostasis. Neurochem Res 27: 21-26.

Fotin-Mleczek M, Henkler F, Samel D, Reichwein M, Hausser A, et al. 2002. Apoptotic crosstalk of TNF receptors: TNF-R2-induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. J Cell Sci 115: 2757-2770.

Furukawa K, Mattson MP. 1998. The transcription factor NF-kappaB mediates increases in calcium currents and decreases in NMDA- and AMPA/kainate-induced currents induced by tumor necrosis factor-alpha in hippocampal neurons. J Neurochem 70: 1876-1886.

Garden GA. 2002. Microglia in human immunodeficiency virus-associated neurodegeneration. Glia 40: 240-251.

Garden GA, Budd SL, Tsai E, Hanson L, Kaul M, et al. 2002. Caspase cascades in human immunodeficiency virus-associated neurodegeneration. J Neurosci 22: 4015-4024.

Gary DS, Bruce-Keller AJ, Kindy MS, Mattson MP. 1998. Ischemic and excitotoxic brain injury is enhanced in mice lacking the p55 tumor necrosis factor receptor. J Cereb Blood Flow Metab 18: 1283-1287.

Ghezzi P, Mennini T. 2001. Tumor necrosis factor and motoneuronal degeneration: an open problem. Neuroimmunomodulation 9: 178-182.

Gilmore CS, Clementz BA, Buckley PF. 2004. Rate of stimulation affects schizophrenia-normal differences on the N1 auditory-evoked potential. Neuroreport 15: 2713-2717.

Goetz FW, Planas JV, Mac Kenzie S. 2004. Tumor necrosis factors. Dev Comp Immunol 28: 487-497.

Grell M, Scheurich P, Meager A, Pfizenmaier K. 1993. TR60 and TR80 tumor necrosis factor (TNF)-receptors can independently mediate cytolysis. Lymphokine Cytokine Res 12: 143-148.

Grell M, Zimmermann G, Gottfried E, Chen CM, Grunwald U, et al. 1999. Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. EMBO J 18: 3034-3043.

Griffin DE. 1997. Cytokines in the brain during viral infection: clues to HIV-associated dementia. J Clin Invest 100: 2948-2951.

Guo H, Jin YX, Ishikawa M, Huang YM, van der Meide PH, et al. 1998. Regulation of beta-chemokine mRNA expression in adult rat astrocytes by lipopolysaccharide, proinflammatory and immunoregulatory cytokines. Scand J Immunol 48: 502-508.

Han Y, Wang J, He T, Ransohoff RM. 2001. TNF-alpha downregulates CXCR4 expression in primary murine astrocytes. Brain Res 888: 1-10.

Haranaka K, Carswell EA, Williamson BD, Prendergast JS, Satomi N, et al. 1986. Purification, characterization, and antitumor activity of nonrecombinant mouse tumor necrosis factor. Proc Natl Acad Sci USA 83: 3949-3953.

Harel G, Shamoun DS, Kane JP, Magner JA, Szabo M. 1995. Prolonged effects of tumor necrosis factor-alpha on anterior pituitary hormone release. Peptides 16: 641-645.

Haviv R, Stein R. 1999. Nerve growth factor inhibits apoptosis induced by tumor necrosis factor in PC12 cells. J Neurosci Res 55: 269-277.

He KL, Ting AT. 2002. A20 inhibits tumor necrosis factor (TNF) alpha-induced apoptosis by disrupting recruitment of TRADD and RIP to the TNF receptor 1 complex in Jurkat T cells. Mol Cell Biol 22: 6034-6045.

Hermann GE, Emch GS, Tovar CA, Rogers RC. 2001. c-Fos generation in the dorsal vagal complex after systemic endotoxin is not dependent on the vagus nerve. Am J Physiol 280: R289-R299.

Hermann GE, Rogers RC. 1995. Tumor necrosis factor-alpha in the dorsal vagal complex suppresses gastric motility. Neuroimmunomodulation 2: 74-81.

Hermann GE, Tovar CA, Rogers RC. 2003a. LPS-induced suppression of gastric motility relieved by TNFR:Fc construct in dorsal vagal complex. Am J Physiol Gastrointest Liver Physiol 284: G634-G639.

Hermann GE, Tovar CA, Rogers RC. 2003b. TNFalpha-stimulation of cFos-activation of neurons in the solitary nucleus is suppressed by TNFR:Fc adsorbant construct in the dorsal vagal complex. Brain Res 976: 69-74.

Higuchi M, Aggarwal BB. 1994. Differential roles of two types of the TNF receptor in TNF-induced cytotoxicity, DNA fragmentation, and differentiation. J Immunol 152: 3550-3558.

Hirsch EC, Breidert T, Rousselet E, Hunot S, Hartmann A, et al. 2003. The role of glial reaction and inflammation in Parkinson's disease. Ann N Y Acad Sci 991: 214-228.

Holden RJ, Pakula IS, Mooney PA. 1998. An immunological model connecting the pathogenesis of stress, depression and carcinoma. Med Hypotheses 51: 309-314.

Hori T, Oka T, Hosoi M, Aou S. 1998. Pain modulatory actions of cytokines and prostaglandin E2 in the brain. Ann N Y Acad Sci 840: 269-281.

Houzen H, Kikuchi S, Kanno M, Shinpo K, Tashiro K. 1997. Tumor necrosis factor enhancement of transient outward potassium currents in cultured rat cortical neurons. J Neurosci Res 50: 990-999.

Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity 4: 387-396.

Hsu H, Xiong J, Goeddel DV. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 81: 495-504.

Idriss HT, Naismith JH. 2000. TNF alpha and the TNF receptor superfamily: structure–function relationship(s). Microsc Res Tech 50: 184-195.

Jiang Y, Woronicz JD, Liu W, Goeddel DV. 1999. Prevention of constitutive TNF receptor 1 signaling by silencer of death domains. Science 283: 543-546.

Johnson RW. 2002. The concept of sickness behavior: a brief chronological account of four key discoveries. Vet Immunol Immunopathol 87: 443-450.

Kim GM, Xu J, Xu J, Song SK, Yan P, Ku G, Xu XM, Hsu CY. 2001. Tumor necrosis factor receptor deletion reduces nuclear factor-kappaB activation, cellular inhibitor of apoptosis protein 2 expression, and functional recovery after traumatic spinal cord injury. J Neurosci 21: 6617-6625.

Klinkert WE, Kojima K, Lesslauer W, Rinner W, Lassmann H, et al. 1997. TNF-alpha receptor fusion protein prevents experimental auto-immune encephalomyelitis and demyelination in Lewis rats: an overview. J Neuroimmunol 72: 163-168.

Korner H, Lemckert FA, Chaudhri G, Etteldorf S, Sedgwick JD. 1997. Tumor necrosis factor blockade in actively induced experimental autoimmune encephalomyelitis prevents clinical disease despite activated T cell infiltration to the central nervous system. Eur J Immunol 27: 1973-1981.

Kraus J, Kuehne BS, Tofighi J, Frielinghaus P, Stolz E, et al. 2002. Serum cytokine levels do not correlate with disease activity and severity assessed by brain MRI in multiple sclerosis. Acta Neurol Scand 105: 300-308.

Kriegler M, Perez C, De Fay K, Albert I, Lu SD. 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell 53: 45-53.

Krueger JM, Majde JA. 2003. Humoral links between sleep and the immune system: research issues. Ann N Y Acad Sci 992: 9-20.

Krueger JM, Obal FJ, Fang J, Kubota T, Taishi P. 2001. The role of cytokines in physiological sleep regulation. Ann N Y Acad Sci 933: 211-221.

Leon LR. 2002. Cytokine regulation of fever: studies using gene knockout mice. J Appl Physiol 92: 2648-2655.

Letendre SL, Lanier ER, McCutchan JA. 1999. Cerebrospinal fluid beta chemokine concentrations in neurocognitively impaired individuals infected with human immunodeficiency virus type 1. J Infect Dis 180: 310-319.

Li R, Yang L, Lindholm K, Konishi Y, Yue X, et al. 2004. Tumor necrosis factor death receptor signaling cascade is required for amyloid-beta protein-induced neuron death. J Neurosci 24: 1760-1771.

Lin A. 2003. Activation of the JNK signaling pathway: breaking the brake on apoptosis. Bioessays 25: 17-24.

Lipton SA. 1998. Neuronal injury associated with HIV-1: approaches to treatment. Annu Rev Pharmacol Toxicol 38: 159-177.

Martinez A, Rubio A, Urcelay E, Fernandez-Arquero M, De Las Heras V, et al. 2004. TNF-376A marks susceptibility to MS in the Spanish population: a replication study. Neurology 62: 809-810.

Mattson MP, Camandola S. 2001. NF-kappaB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 107: 247-254.

Mattson MP, Culmsee C, Yu Z, Camandola S. 2000. Roles of nuclear factor kappaB in neuronal survival and plasticity. J Neurochem 74: 443-456.

McDermott MF, Aksentijevich I, Galon J, McDermott EM, Ogunkolade BW. et al. 1999. Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell 97: 133-144.

Medvedev AE, Sundan A, Espevik T. 1994. Involvement of the tumor necrosis factor receptor p75 in mediating cytotoxicity and gene regulating activities. Eur J Immunol 24: 2842-2849.

Merrill JE. 1992. Tumor necrosis factor alpha, interleukin 1 and related cytokines in brain development: normal and pathological. Dev Neurosci 14: 1-10.

Mizuno T, Kurotani T, Komatsu Y, Kawanokuchi J, Kato H, et al. 2004. Neuroprotective role of phosphodiesterase inhibitor ibudilast on neuronal cell death induced by activated microglia. Neuropharmacology 46: 404-411.

Mogi M, Togari A, Kondo T, Mizuno Y, Komure O, et al. 2000. Caspase activities and tumor necrosis factor receptor R1 (p55) level are elevated in the substantia nigra from parkinsonian brain. J Neural Transm 107: 335-341.

Moosmayer D, Dinkel A, Gerlach E, Hessabi B, Grell M, et al. 1994. Coexpression of the human TNF receptors TR60 and TR80 in insect cells: analysis of receptor complex formation. Lymphokine Cytokine Res 13: 295-301.

Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, et al. 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature 385: 733-736.

Mosselmans R, Hepburn A, Dumont JE, Fiers W, Galand P. 1988. Endocytic pathway of recombinant murine tumor necrosis factor in L-929 cells. J Immunol 141: 3096-3100.

Munoz- Fernandez MA, Fresno M. 1998. The role of tumour necrosis factor, interleukin 6, interferon-gamma and

inducible nitric oxide synthase in the development and pathology of the nervous system. Prog Neurobiol 56: 307-340.

Nagatsu T, Mogi M, Ichinose H, Togari A. 2000. Changes in cytokines and neurotrophins in Parkinson's disease. J Neural Transm 58(Suppl.): 143-151.

Natoli G, Costanzo A, Ianni A, Templeton DJ, Woodgett JR, et al. 1997. Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. Science 275: 200-203.

Obal F Jr, Krueger JM. 2003. Biochemical regulation of non-rapid-eye-movement sleep. Front Biosci 8: 520-550.

Oettgen HF, Carswell EA, Kassel RL, Fiore N, Williamson B, et al. 1980. Endotoxin-induced tumor necrosis factor. Recent Results Cancer Res 75: 207-212.

Olsen NJ, Stein CM. 2004. New drugs for rheumatoid arthritis. N Engl J Med 350: 2167-2179.

Pahan K, Liu X, McKinney MJ, Wood C, Sheikh FG, et al. 2000. Expression of a dominant-negative mutant of p21 (ras) inhibits induction of nitric oxide synthase and activation of nuclear factor-kappaB in primary astrocytes. J Neurochem 74: 2288-2295.

Pan W, Cornelissen G, Halberg F, Kastin AJ. 2002. Selected contribution: circadian rhythm of tumor necrosis factor-alpha uptake into mouse spinal cord. J Appl Physiol 92: 1357-1362.

Pennica D, Hayflick JS, Bringman TS, Palladino MA, Goeddel DV. 1985. Cloning and expression in *Escherichia coli* of the cDNA for murine tumor necrosis factor. Proc Natl Acad Sci USA 82: 6060-6064.

Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, et al. 1984. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 312: 724-729.

Perez C, Albert I, DeFay K, Zachariades N, Gooding L, Kriegler M. 1990. A nonsceretable cell surface mutant of tumor necrosis factor (TNF) kills by cell-to-cell contact. Cell 63: 251-258.

Perini G, Della-Bianca V, Politi V, Della Valle G, Dal- Pra I, et al. 2002. Role of p75 neurotrophin receptor in the neurotoxicity by beta-amyloid peptides and synergistic effect of inflammatory cytokines. J Exp Med 195: 907-918.

Perry RT, Collins JS, Wiener H, Acton R, Go RC. 2001. The role of TNF and its receptors in Alzheimer's disease. Neurobiol Aging 22: 873-883.

Porteu F, Hieblot C. 1994. Tumor necrosis factor induces a selective shedding of its p75 receptor from human neutrophils. J Biol Chem 269: 2834-2840.

Quasney MW, Zhang Q, Sargent S, Mynatt M, Glass J, et al. 2001. Increased frequency of the tumor necrosis factor-alpha-308 A allele in adults with human immunodeficiency virus dementia. Ann Neurol 50: 157-162.

Reinhard C, Shamoon B, Shyamala V, Williams LT. 1997. Tumor necrosis factor alpha-induced activation of c-jun N-terminal kinase is mediated by TRAF2. EMBO J 16: 1080-1092.

Rivest S. 2001. How circulating cytokines trigger the neural circuits that control the hypothalamic–pituitary–adrenal axis. Psychoneuroendocrinology 26: 761-788.

Rojo AI, Salinas M, Martin D, Perona R, Cuadrado A. 2004. Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB. J Neurosci 24: 9324-9334.

Rose- John S, Heinrich PC. 1994. Soluble receptors for cytokines and growth factors: generation and biological function. Biochem J 300: 281-290.

Rousselet E, Callebert J, Parain K, Joubert C, Hunot S, et al. 2002. Role of TNF-alpha receptors in mice intoxicated with the parkinsonian toxin MPTP. Exp Neurol 177: 183-192.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. 1997. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. EMBO J 16: 6914-6925.

Saha RN, Pahan K. 2003. Tumor necrosis factor-alpha at the crossroads of neuronal life and death during HIV-associated dementia. J Neurochem 86: 1057-1071.

Scherbel U, Raghupathi R, Nakamura M, Saatman KE, Trojanowski JQ, et al. 1999. Differential acute and chronic responses of tumor necrosis factor-deficient mice to experimental brain injury. Proc Natl Acad Sci USA 96: 8721-8726.

Selmaj KW. 2000. Tumour necrosis factor and anti-tumour necrosis factor approach to inflammatory demyelinating diseases of the central nervous system. Ann Rheum Dis 59 (Suppl. 1): 94-102.

Sharief MK, Hentges R. 1991. Association between tumor necrosis factor-α and disease progression in patients with multiple sclerosis. N Engl J Med 325: 467-472.

Sicotte NL, Voskuhl RR. 2001. Onset of multiple sclerosis associated with anti-TNF therapy. Neurology 57: 1885-1888.

Solomon KA, Pesti N, Wu G, Newton RC. 1999. A dominant negative form of TNF-alpha converting enzyme inhibits proTNF and TNFRII secretion. J Immunol 163: 4105-4108.

Soond SM, Terry JL, Colbert JD, Riches DW. 2003. TRUSS, a novel tumor necrosis factor receptor 1 scaffolding protein that mediates activation of the transcription factor NF-kappaB. Mol Cell Biol 23: 8334-8344.

Sriram K, Matheson JM, Benkovic SA, Miller DB, Luster MI, et al. 2002. Mice deficient in TNF receptors are protected against dopaminergic neurotoxicity: implications for Parkinson's disease. FASEB J 16: 1474-1476.

Suarez EC, Lewis JG, Kuhn C. 2002. The relation of aggression, hostility, and anger to lipopolysaccharide-stimulated

tumor necrosis factor (TNF)-alpha by blood monocytes from normal men. Brain Behav Immun 16: 675-684.

Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, et al. 1999. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFkappaB activation in primary hippocampal neurons. J Biol Chem 274: 8531-8538.

Tancredi V, D'Arcangelo G, Grassi F, Tarroni P, Palmieri G, et al. 1992. Tumor necrosis factor alters synaptic transmission in rat hippocampal slices. Neurosci Lett 146: 176-178.

Tang P, Hung M-C, Klostergaard J. 1996. Length of the linking domain of human pro-tumor necrosis factor determines the cleavage processing. Biochemistry 35: 8216-8225.

Tannenbaum CS, Major JA, Hamilton TA. 1993. IFN-gamma and lipopolysaccharide differentially modulate expression of tumor necrosis factor receptor mRNA in murine peritoneal macrophages. J Immunol 151: 6833-6839.

Tarkowski E, Liljeroth AM, Minthon L, Tarkowski A, Wallin A, et al. 2003. Cerebral pattern of pro- and anti-inflammatory cytokines in dementias. Brain Res Bull 61: 255-260.

Tartaglia LA, Goeddel DV. 1992. Two TNF receptors. Immunol Today 13: 151-153.

Tartaglia LA, Pennica D, Goeddel DV. 1993. Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa TNF receptor. J Biol Chem 268: 18542-18548.

The Lenercept Multiple Sclerosis Study Group and the University of British Columbia MS/MRI Analysis Group. 1999. TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. Neurology 53: 457–465.

Torchinsky A, Shepshelovich J, Orenstein H, Zaslavsky Z, Savion S, et al. 2003. TNF-alpha protects embryos exposed to developmental toxicants. Am J Reprod Immunol 49: 159-168.

Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, et al. 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 330: 662-664.

Vandenabeele P, Declercq W, Vanhaesebroeck B, Grooten J, Fiers W. 1995. Both TNF receptors are required for TNF-mediated induction of apoptosis in PC60 cells. J Immunol 154: 2904-2913.

Van Ostade X, Vandenabeele P, Everaerdt B, Loetscher H, Gentz R, Brockhaus M, Lesslauer W, Tavernier J, Brouckaert P, Fiers W. 1993. Human TNF mutants with selective activity on the p55 receptor. Nature 361: 266-269.

Varfolomeev EE, Ashkenazi A. 2004. Tumor necrosis factor: an apoptosis JuNKie? Cell 116: 491-497.

Venters HD, Broussard SR, Zhou JH, Bluthe RM, Freund GG, et al. 2001. Tumor necrosis factor(alpha) and insulin-like growth factor-I in the brain: is the whole greater than the sum of its parts? J Neuroimmunol 119: 151-165.

Vgontzas AN, Zoumakis M, Papanicolaou DA, Bixler EO, Prolo P, et al. 2002. Chronic insomnia is associated with a shift of interleukin-6 and tumor necrosis factor secretion from nighttime to daytime. Metabolism 51 (7): 887-892.

Wajant H, Pfizenmaier K, Scheurich P. 2003. Tumor necrosis factor signaling. Cell Death Differ 10: 45-65.

Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, et al. 1999. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol 17: 331-367.

Watkins LR, Maier SF, Goehler LE. 1995. Immune activation: the role of pro-inflammatory cytokines in inflammation, illness responses and pathological pain states. Pain 63: 289-302.

Wesselingh SL, Power C, Glass JD, Tyor WR, McArthur JC, et al. 1993. Intracerebral cytokine messenger RNA expression in acquired immunodeficiency syndrome dementia. Ann Neurol 33: 576-582.

Wichers M, Maes M. 2002. The psychoneuroimmunopathophysiology of cytokine-induced depression in humans. Int J Neuropsychopharmacol 5: 375-388.

Wilde GJ, Pringle AK, Sundstrom LE, Mann DA, Iannotti F. 2000. Attenuation and augmentation of ischaemia-related neuronal death by tumour necrosis factor-alpha in vitro. Eur J Neurosci 12: 3863-3870.

Yamaguchi M, Sakata M, Matsuzaki N, Koike K, Miyake A, et al. 1990. Induction by tumor necrosis factor-alpha of rapid release of immunoreactive and bioactive luteinizing hormone from rat pituitary cells in vitro. Neuroendocrinology 52: 468-472.

Yang J, Lin Y, Guo Z, Cheng J, Huang J, et al. 2001. The essential role of MEKK3 in TNF-induced NF-kappaB activation. Nat Immunol 2: 620-624.

Yang L, Lindholm K, Konishi Y, Li R, Shen Y. 2002. Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways. J Neurosci 22: 3025-3032.

Ye P, Price W, Kassiotis G, Kollias G, D'Ercole AJ. 2003. Tumor necrosis factor-alpha regulation of insulin-like growth factor-I, type 1 IGF receptor, and IGF binding protein expression in cerebellum of transgenic mice. J Neurosci Res 71: 721-731.

Yeh MW, Kaul M, Zheng J, Nottet HS, Thylin M, et al. 2000. Cytokine-stimulated, but not HIV-infected, human monocyte-derived macrophages produce neurotoxic levels of L-cysteine. J Immunol 164: 4265-4270.

Yoshida H, Imaizumi T, Fujimoto K, Matsuo N, Kimura K, et al. 2001. Synergistic stimulation, by tumor necrosis

factor-alpha and interferon-gamma, of fractalkine expression in human astrocytes. Neurosci Lett 303: 132-136.

Yu Z, Zhou D, Bruce- Keller AJ, Kindy MS, Mattson MP. 1999. Lack of the p50 subunit of nuclear factor-kappaB increases the vulnerability of hippocampal neurons to excitotoxic injury. J Neurosci 19: 8856-8865.

Zhang SQ, Kovalenko A, Cantarella G, Wallach D. 2000. Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. Immunity 12: 301-311.

Zhang ZH, Wei SG, Francis J, Felder RB. 2003. Cardiovascular and renal sympathetic activation by blood-borne TNF-alpha in rat: the role of central prostaglandins. Am J Physiol Regul Integr Comp Physiol 284: R916-R927.

# 10 Glia Maturation Factor in Brain Function

*R. Lim · A. Zaheer*

**Abstract:** Glia maturation factor (GMF) is a 141 amino acid, multifunctional, brain-predominant protein. The protein is highly conserved, being identical between humans and cattle, and shows 99% homology between humans and rodents. The gene for GMF has been localized to the long arm of human chromosome 14. GMF is an intracellular protein localized mainly in astrocytes but is also found in some neuronal populations. GMF possesses consensus phosphorylation sites and is rapidly phosphorylated in astrocytes upon phorbol ester stimulation. In in vitro studies, protein kinase A (PKA)-phosphorylated GMF enhances the activity of p38 mitogen-activated protein (MAP) kinase. Overexpression of GMF in C6 glioma cells leads to decreased tumorigenicity and increased differentiation. In C6 cells, as well as in normal astrocytes, GMF overexpression stimulates p38 MAP kinase activity and activates the redox enzyme CuZn superoxide dismutase (CuZnSOD) and the transcription factors nuclear factor-κB (NF-κB) and cAMP response element binding protein (CREB), with downstream induction and secretion of the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Furthermore, overexpression of GMF in astrocytes promotes the production of the cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF), which in turn activates the microglia, the antigen-presenting cells in the nervous system. Overexpression of GMF in PC12 pheochromocytoma cells leads to the sequential activation of p38, MAPKAP kinase-2, and tyrosine hydroxylase (TH), the rate-limiting enzyme for the synthesis of the neurotransmitter norepinephrine. Many of these effects are blocked by the p38 inhibitor, SB203580. Thus, GMF appears to be an intracellular regulator of the stress-related signal transduction, playing a role in neuronal survival and immune activation. GMF-knockout mice are deficient in motor performance and procedural learning. Along with the behavioral deficits, there is neuronal cell loss in the inferior olive, suggesting a role for GMF in the development of the nervous system.

**List of abbreviations:** ATF, activating transcription factor; BDNF, brain-derived neurotrophic factor; CKII, casein kinase II; CMV, cytomegalovirus; CREB, cAMP response element binding protein; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GM-CSF, granulocyte–macrophage colony-stimulating factor; GMF, glia maturation factor; Hsp, heatshock protein; IL, interleukin; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MAPKAK, MAP kinase-activated protein kinase; MHC, major histocompatibility complex; MKK, MAP kinase kinase; NF-κB, nuclear factor-κB; NGF, nerve growth factor; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RPA, ribonuclease protection assay; RSK, p90 ribosomal S6 kinase; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase.

## 1 Introduction

Proteins are universal effectors in the biological world. Because of the complexity of the nervous system, it has been speculated that a greater variety of proteins are expressed in the brain than in any other organ. The brain shares with other organs those proteins that are essential for cell survival, energy utilization, replication, and apoptosis. In addition, the brain can utilize some common proteins to carry out functions unique to the nervous system. Still, a smaller number of proteins are exclusively expressed in neural tissue. These proteins presumably play a role in the specific functions of the nervous system. Over the years, our laboratory has focused on the study of novel brain proteins, using biological clues as a guide to their purification and isolation. The subject of this chapter is a brain-predominant, highly conserved protein called "glia maturation factor" (GMF). Although initially considered to be a growth/differentiation factor, as knowledge on this protein unfolds and a multitude of functions uncovered, the term no longer adequately reflects its biological role. Nonetheless, we retain the original name as a matter of convention, with the understanding that, as is true with many proteins, the name should not be narrowly interpreted.

## 2 Isolation and Sequencing of GMF Protein

The isolation of GMF was the outcome of a protracted attempt to study biologically active proteins in the brain. At a time when DNA technology was not yet available as a tool to identify proteins, we had to rely on

bioassays and brute force conventional fractionation methodology. We also had to start with slaughterhouse materials (bovine brains) in order to compensate for the loss during the tedious purification steps. We were, however, able to utilize two newly available technical aids in our isolation efforts. One was high-pressure liquid chromatography (HPLC). The other was monoclonal antibody technology, for we were able to produce an antibody that appeared to inhibit the cell-stimulating activity of the brain extract. Using these methods, in 1989 our laboratory isolated a unique protein from the bovine brain extract (Lim et al., 1989). The procedure consisted of homogenization, ammonium sulfate precipitation, DEAE–Sephacel chromatography, Sephadex G-75 chromatography, hydroxylapatite chromatography, heparin–Sepharose treatment, and finally reverse-phase HPLC. The protein has an apparent molecular weight of 17 kDa and an isoelectric point at pH 4.9. The primary structure was determined on the digested peptides by microsequencing with automated Edman degradation and by tandem mass spectrometry (Lim et al., 1990). The protein has 141 amino acid residues and possesses no potential N-glycosylation sites. It contains three cysteines, three methionines, and one tryptophan. The amino terminus is an N-acetylated serine, whereas the carboxy terminus is a histidine. The protein sequence, shown in (*Figure 10-1*), reveals that this

**Figure 10-1**

**Amino acid sequence of bovine and human glia maturation factor (GMF). Bovine sequence was determined on the isolated natural protein (Lim et al., 1990). Human sequence was deduced from the cloned complementary DNA (cDNA) (Kaplan et al., 1991). The two sequences are identical. The one-letter abbreviations are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr**

```
         10         20         30         40         50
SESLVVCDVA EDLVEKLRKF RFRKETNNAA IIMKIDKDKR LVVLDEELEG

         60         70         80         90        100
ISPDELKDEL PERQPRFIVY SYKYQHDDGR VSYPLCFIFS SPVGCKPEQQ

        110        120        130        140
MMYAGSKNKL VQTAELTKVF EIRNTEDLTE EWLREKLGFF H
```

protein has never been described before. We initially called it GMF-β, implying the existence of GMF-α. Later experiments, however, did not bear out the presence of GMF-α. Therefore, we subsequently dropped the "β" designation. (Note: In this chapter, the term "GMF" is used interchangeably with "GMF-β," unless specified.)

## 3 cDNA and Deduced Amino Acid Sequence

Recombinant GMF was cloned soon after the protein was isolated (Kaplan et al., 1991). Using oligonucleotide probes based on the sequences of three tryptic peptides derived from the bovine GMF, we screened a human brain stem complementary DNA (cDNA) library in λgt11. A 0.7-kb clone was isolated and sequenced in its entirety. The nucleotide sequence encodes a polypeptide of 142 amino acids (including the methionine initiation codon). The deduced amino acid sequence from the human cDNA is identical to the empirically determined sequence from the bovine protein. Both contain no potential N-linked glycosylation site. Further, there was no secretory leader sequence observed in the GMF cDNA. Recombinant human GMF (rhGMF) was expressed in *Escherichia coli*, using the plasmid pET-3b translation vector. Recombinant human GMF is identical to natural bovine GMF in all respects, including amino acid composition, partial N-terminal and C-terminal sequences, and behavior in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western immunoblotting, enzyme-linked immunosorbent assay (ELISA), and HPLC chromatography of the tryptic peptides. We subsequently cloned recombinant

GMF from the rat brain (Zaheer et al., 1993). The rat GMF cDNA was obtained by reverse transcription of rat brain total RNA and amplified by polymerase chain reaction (PCR) using 5′ and 3′ primers flanking the entire GMF coding region, based on the known sequence of the human cDNA. The sequence of the rat cDNA, in comparison with that of human cDNA, is shown in *Figure 10-2*. The coding region of the rat cDNA differs from that of the human by a total of 44 nucleotides. However,

**Figure 10-2**

**Nucleotide and deduced amino acid sequence of rat glia maturation factor (GMF). The rat complementary DNA (cDNA) was obtained by RT-PCR of rat total RNA, using primers (*underlined*) from human sequence. For comparison, human nucleotides and amino acid residues that are different from the rat are indicated below the rat sequence. Note only three amino acids are different. (From Zaheer et al., 1993)**

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | 1 | | | | | | | | | | 10 |
| | | | | | | | | | CCGCTGACGGCCGGAAGGAAA | ATG | AGT | GAG | TCC | TTG | GTG | GTT | TGT | GAT | GTT | GCT |
| | | | | | | | | | | | SER | GLU | SER | LEU | VAL | VAL | CYS | ASP | VAL | ALA |
| | | | | | | | | | | | | | T | | T | | | | | C |
| | | | | | | | | | | 20 | | | | | | | | | | | 30 |
| GAA | GAT | TTA | GTG | GAA | AAG | CTG | AGA | AAG | TTT | CGT | TTT | CGA | AAA | GAA | ACC | CAC | AAT | GCT | GCT | | |
| GLU | ASP | LEU | VAL | GLU | LYS | LEU | ARG | LYS | PHE | ARG | PHE | ARG | LYS | GLU | THR | HIS | ASN | ALA | ALA | | |
| | | | | | | | | | | | | C | | | G | A | C | | | | |
| | | | | | | | | | | | | | | | | ASN | | | | | |
| | | | | | | | | | | 40 | | | | | | | | | | | 50 |
| ATT | ATT | ATG | AAG | ATT | GAC | AAG | GAT | AAA | CGC | TTG | GTG | GTG | CTG | GAT | GAG | GAG | CTC | GAG | GGT | | |
| ILE | ILE | MET | LYS | ILE | ASP | LYS | ASP | LYS | ARG | LEU | VAL | VAL | LEU | ASP | GLU | GLU | LEU | GLU | GLY | | |
| | A | | | | | | | | | C | | A | | | | | T | | C C | | |
| | | | | | | | | | | 60 | | | | | | | | | | | 70 |
| GTC | TCT | CCA | GAT | GAA | CTT | AAA | GAT | GAA | CTA | CCT | GAA | CGG | CAA | CCT | CGC | TTC | ATT | GTG | TAT | | |
| VAL | SER | PRO | ASP | GLU | LEU | LYS | ASP | GLU | LEU | PRO | GLU | ARG | GLN | PRO | ARG | PHE | ILE | VAL | TYR | | |
| A T | A | | | | | | | | | | | A | | | | | | | | | |
| ILE | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 80 | | | | | | | | | | | 90 |
| AGT | TAT | AAA | TAC | CAG | CAC | GAC | GAT | GGC | CGG | GTC | TCC | TAC | CCT | CTG | TGC | TTT | ATC | TTC | TCC | | |
| SER | TYR | LYS | TYR | GLN | HIS | ASP | ASP | GLY | ARG | VAL | SER | TYR | PRO | LEU | CYS | PHE | ILE | PHE | SER | | |
| | | | T | A | T | T | | A | A A | T | A | T | | | | | T | | | | |
| | | | | | | | | | | 100 | | | | | | | | | | | 110 |
| AGT | CCT | CTG | GGG | TGC | AAA | CCT | GAG | CAG | CAG | ATG | ATG | TAC | GCT | GGG | AGT | AAG | AAC | AAG | CTG | | |
| SER | PRO | LEU | GLY | CYS | LYS | PRO | GLU | GLN | GLN | MET | MET | TYR | ALA | GLY | SER | LYS | ASN | LYS | LEU | | |
| | | G T | A | T | G | | A | A | | | | T | | A | | | T | | A | | |
| | | VAL | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | 120 | | | | | | | | | | | 130 |
| GTC | CAG | ACC | GCC | GAA | CTA | ACC | AAG | GTA | TTT | GAA | ATA | AGA | AAC | ACC | GAA | GAC | CTG | ACT | GAA | | |
| VAL | GLN | THR | ALA | GLU | LEU | THR | LYS | VAL | PHE | GLU | ILE | ARG | ASN | THR | GLU | ASP | LEU | THR | GLU | | |
| | | A | T | | | | | | | | | | T | | | | A | | | | |
| | | | | | | | | | | 141 | | | | | | | | | | | |
| GAA | TGG | TTA | CGT | GAG | AAA | CTT | GGA | TTT | TTC | CAC | TAA | TGTGAACTTCTGTGTTTCTTAAGTATTTATGTA | | | | | | | | | |
| GLU | TRP | LEU | ARG | GLU | LYS | LEU | GLY | PHE | PHE | HIS | *** | | | | | | | | | | |
| | | | | | | | | | T | | | | | | | | A | | | | |

TTAACCTGACCATACTGGATCCAGACATAA

when translated into amino acid sequence, there are only three altered residues. The deduced rat sequence contains $His^{27}$ instead of Asn, $Val^{51}$ instead of Ile, and $Leu^{93}$ instead of Val. The high degree of conservation of GMF over the course of evolution is impressive.

## 4 GMF Isoforms and Homologs

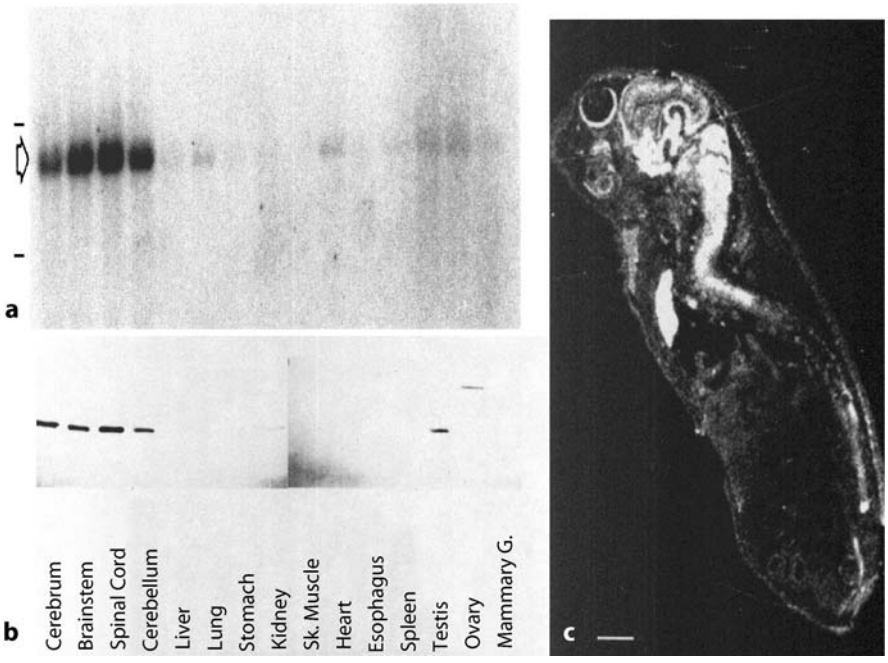
An isoform of GMF-β, called GMF-γ, has been identified in humans (Asai et al., 1998). It is highly homologous to human GMF-β, with 82% amino acid identity. Unlike the GMF-β gene, which is present in human chromosome 14 (International Human Genome Sequence Consortium, 2001), the GMF-γ gene is mapped to human chromosome 19 (Kawai et al., 2003). The human GMF-γ gene and its promoter activity have been characterized (Kawai et al., 2003). The 5′-flanking region of the first exon contains putative elements for binding transcription factors Sp-1, GATA-1, AML-1a, Lyf-1, and Ets-1, but there are no TATA or CAAT boxes within a 226-bp sequence upstream from the initiation codon. There are multiple transcription initiation sites within the region −84 to −70 nucleotides from the first ATG codon. A 226-bp region exhibits promoter activity. GMF-γ cDNA has also been isolated from rat, showing 79% homology with rat GMF-β (Tsuiki et al., 2000). Down the evolution scale, GMF homologs have been found in nonvertebrates. It was reported that the filarial nematode *Brugia malayi* possesses a GMF-like gene, which encodes a predicted protein of 138 amino acids with 52% identity and 75% similarity to the mammalian GMF-β (Liu et al., 1997). The gene has six predicted exons, with the first encoding only the initiation methionine, as in GMF-β gene. The filarial GMF is *trans*-spliced with the nematode spliced leader sequence SL1 and is expressed in microfilariae but not in the adult worms. A homolog of GMF is also present in the genome of *Drosophila melanogaster* (Ashburner et al., 1999). Thus, the GMF superfamily spans a wide range of species and implies an essential role in cellular function. On the other hand, GMF is found to be remotely related to a group of proteins called actin-depolymerizing factors (ADF). But unlike the ADFs, GMF does not bind actin or calcium, nor does it contain the consensus actin-depolymerizing motif (Liu et al., 1997).

## 5 Distribution and Localization

The expression of GMF mRNA in the rat was studied using Northern blotting with a rat complementary RNA (cRNA) probe corresponding to the entire coding region (Zaheer et al., 1993). GMF mRNA is predominantly expressed in the brain and spinal cord, although trace levels are found in other organs, including testis and ovary (*Figure 10-3*). Western blot shows similar organ distribution for GMF protein. In the brain, GMF mRNA is detectable at as early as embryonic day 10 and persists through postnatal month 14, the oldest age tested, with minor variations in between. On the other hand, GMF protein exhibits more obvious developmental changes in the brain, with its level increasing slowly prenatally and plateauing at 1 week after birth. Thereafter, GMF protein remains high throughout life. In cultured cells, GMF is high in cells of neural origin, including astrocytes, astrocytomas, oligodendrogliomas, and neuroblastomas, and is lower in cells of other origin, such as fibroblasts, fibrosarcomas, hepatomas, and intestinal epithelial cells. It is low in normal Schwann cells but high in schwannomas. Using immunohistochemistry, GMF is localized to astrocytes and Bergmann glia, but not oligodendroglia (Wang et al., 1992). However, it is present in some neurons, including Purkinje cells, deep cerebellar nuclei, midbrain nuclei (oculomotor nucleus, red nucleus, and substantia nigra pars compacta), spinal cord motor neurons, lateral vestibular (Deiters) nucleus, basal ganglia (mainly globus pallidus), and spinal ganglion cells. In the sciatic nerve, GMF is normally absent in myelin, but is intensely but transiently expressed by Schwann cells distal to the site of axotomy following transection or crush injury, suggesting a role in nerve regeneration (Bosch et al., 1989). The intracellular location of GMF has been determined with immunofluorescence on cultured astrocytes (Lim et al., 1987) and cultured Schwann cells (Bosch et al., 1989). In both instances GMF exists mainly as a cytoplasmic protein with no specific correlation with the organelles. *Figure 10-4* shows some of the localizations described above.

**Figure 10-3**

**Distribution of glia maturation factor (GMF) in rat organs. (a) Northern blot for mRNA; (b) Western immunoblot for protein; (c) in situ hybridization for mRNA. Figure 10-3(a) and (b) are of adult rats (Zaheer et al., 1993); (c) is of newborn rat (adopted from Nishiwaki et al., 2001). Note "A" and "B" strongly localize GMF in cerebrum, brain stem, spinal cord and cerebellum; "C" identifies GMF in the central nervous system, retina, and thymus**

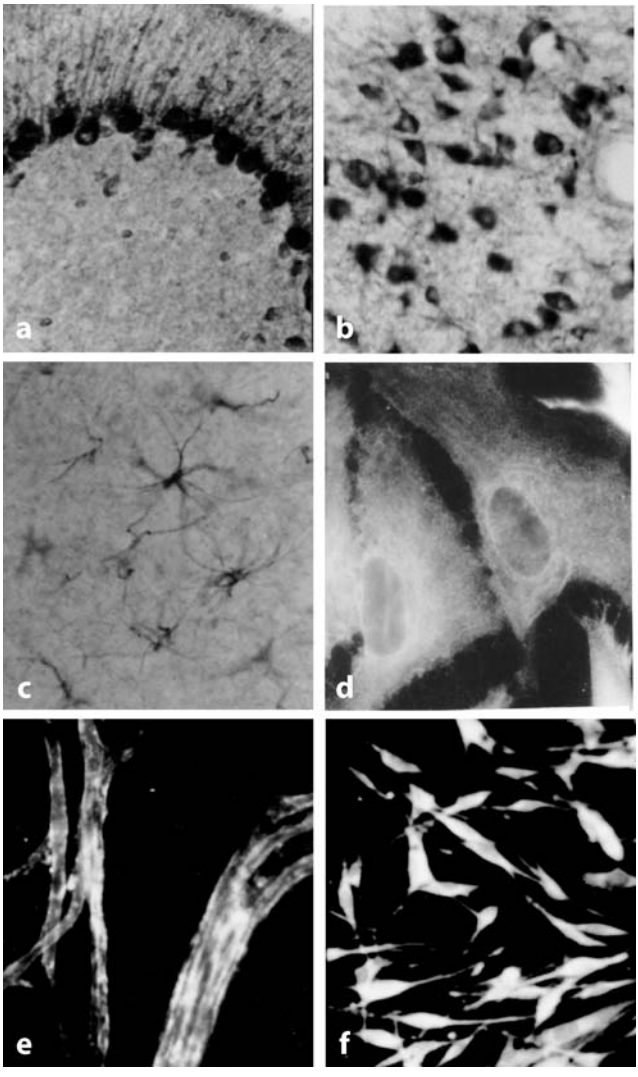



The distribution of GMF was also studied by Asai et al. (1998), Tsuiki et al. (2000), Nishiwaki et al. (2001), and Inagaki et al. (2004). These authors confirmed the brain predominance of GMF localization, although minor differences were detected. The Northern blot study of Asai et al. (1998) showed a very strong expression of GMF in the human brain, with a weaker expression in the heart, placenta, kidney, and pancreas. Likewise, Tsuiki et al. (2000) detected using Northern blotting the major expression of GMF in the rat brain, with lower levels in thymus, heart, and lung. Using in situ hybridization, Nishiwaki et al. (2001) found GMF to be intensely expressed in the brain, spinal cord, thymus, and retina in the newborn rat (*Figure 10-3*). In the adult rat retina, GMF is localized to the Muller cells. Using a sensitive two-site enzyme immunoassay (EIA), Inagaki et al. (2004) found, among the rat organs, high levels of GMF in the cerebral cortex, cerebellum, and midbrain, but a lower level in the spinal cord. The level in the colon is comparable with that in the brain, and the thymus also expresses a lower but significant amount of the protein. GMF is also detectable in human serum (Inagaki et al., 2004).

In contrast to GMF-β, GMF-γ is not a brain-predominant protein. Using Northern blot analysis on human organs, Asai et al. (1998) found that GMF-γ mRNA is primarily expressed in the lung, with a lower expression in the placenta and heart. The expression in the brain is nil. Unlike GMF-β, GMF-γ is not detectable in the nervous system of newborn rats using in situ hybridization, although it is detectable in the thymus (Nishiwaki et al., 2001). Enzyme immunoassay study showed low levels of GMF-γ in the brain, lung, and liver, and high levels in the spleen, colon, and thymus, among human organs (Inagaki et al., 2004). In rats, Northern blot analysis revealed the expression of GMF-γ in thymus and testis (Tsuiki et al., 2000). In newborn rats, in situ hybridization detected the presence of GMF-γ in the thymus but not in the nervous system (Nishiwaki et al., 2001).

**Figure 10-4**

**Morphological localization of glia maturation factor (GMF) in rat. (a–c) Immunostaining showing positive Purkinje cell bodies and Bergmann glial processes in (a), positive deep cerebellar neurons in (b), and positive astrocytes in (c). (d–f) Immunofluorescence showing positive cultured astrocytes in (d), positive teased peripheral myelin sheaths following crush injury in (e), and positive cultured Schwann cells in (f). Note that GMF disappears from myelin after regeneration (not shown). (Collated from Lim et al., 1987; Bosch et al., 1989; Wang et al., 1992) (Photos not to scale)**



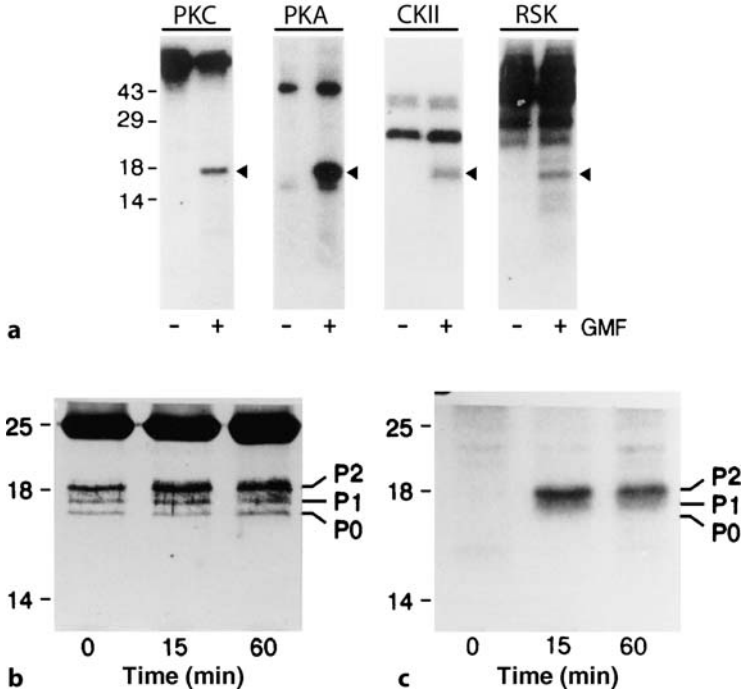

A developmental study was carried out by Bourgeois et al. (2001). Using a differential display paradigm to select genes expressed in proliferating neuroblasts, they cloned a GMF-β cDNA, which is preferentially expressed in mouse telencephalon and peaks at E15 embryonic age. That GMF may be developmentally regulated is further strengthened by the presence of both GMF-β and GMF-γ in mouse embryonic stem (ES) cells. Mao et al. (1998) and Zhang et al. (2000) also reported the presence of GMF-γ in umbilical cord blood CD34+ cells.

## 6 In Vitro Studies

The GMF protein contains several consensus phosphorylation sites (Pearson and Kemp, 1991). Serine 71 (SYK) is a putative substrate for protein kinase C (PKC), which fits the most canonical sequence of S/T-X-K/R; serine 82 (RVS) is a putative substrate for protein kinase A (PKA), which fits the most canonical sequence of RXS; serine 52 (SPDEL) is a putative substrate for casein kinase II (CKII), which fits the most canonical sequence of S/T-X-X-E-X; threonine 26 (RFRKET) is a putative substrate for p90 ribosomal S6 kinase (RSK), which is in agreement with the consensus sequence of R-X-R-X-X-S/T. To test if GMF can be phosphorylated in the test tube, we incubated rhGMF with various protein kinases (Lim and Zaheer, 1995) and found that GMF is indeed phosphorylated by PKC, PKA, CKII, and RSK (*Figure 10-5*), but not by

**Figure 10-5**

**Phosphorylation of glia maturation factor (GMF). (a) In vitro phosphorylation of GMF by protein kinase C (PKC), protein kinase A (PKA), casein kinase II (CKII), and p90 ribosomal S6 kinase (RSK). *Arrow* indicates phosphorylated GMF on autoradiogram of Western blot. (b and c) Phosphorylation of cultured astrocytes after phorbol ester stimulation. (b), Western blot; (c), autoradiogram. Timescale refers to the length of exposure to phorbol ester. Po, nonphosphorylated GMF; P1, lightly phosphorylated GMF; P2, heavily phosphorylated GMF. Note strong phosphorylation of GMF after 15 min of phorbol ester stimulation. (From Lim and Zaheer, 1995)**




other kinases such as extracellular signal-regulated kinase (ERK), mitogen-activated protein (MAP) kinase, MEK, or cdc-2-like kinase. Phosphoamino acid analysis confirmed that GMF is phosphorylated by PKC, PKA, and CKII at the serine residue, and by RSK at the threonine residue. We subsequently synthesized peptide fragments of GMF containing the above putative phosphorylation sites and confirmed the phosphorylation of serine 71 by PKC, serine 82 by PKA, serine 52 by CKII, and threonine 26 by RSK. In addition, PKA also phosphorylates threonine 26 when the synthetic peptide was used as the substrate (Zaheer and Lim, 1997). *Table 10-1* shows the results of peptide phosphorylation.

**Table 10-1**
**Phosphorylation of GMF peptides containing putative targets of protein kinases**

| | Phosphorylation detected (cpm)[b] | | | |
|---|---|---|---|---|
| GMF peptide tested[a] | PKA | PKC | RSK | CKII |
| I. $^{21}$RFRKE(T)$^{26}$NNAA$^{30}$ | 34,140±671 | 0 | 5,470±705 | 0 |
| II. $^{47}$ELEGV(S)$^{52}$PDEL$^{56}$ | 0 | 0 | 0 | 51,420±145 |
| III. $^{66}$RFIVY(S)$^{71}$YKYQ$^{75}$ | 0 | 25,380±929 | 0 | 0 |
| IV. $^{77}$DDGRV(S)$^{82}$YPLC$^{86}$ | 7,630[c]±374 | 0 | 0 | 0 |

Four peptides were synthesized according to the human sequence of GMF. Each peptide contained a single serine or threonine residue (in parentheses) that by consensus was a probable phosphorylation site of one or more of the kinases known to phosphorylate GMF. Each peptide was tested as a substrate for the kinases and the results of phosphorylation are expressed in terms of $^{32}$P radioactivity. (From Zaheer and Lim, 1997)

[a]Synthetic peptides were *N*-acetyl blocked and *C*-amido blocked. Numbers correspond to locations in the intact GMF protein

[b]Values are mean±SD of triplicate reaction tubes from one set of experiment. A second experiment yielded similar results

[c]A 15-mer version of peptide IV stretching from amino acid position 75(Gln) to 89(Phe) gave the same phosphorylation value.

To verify that endogenous, intracellular GMF undergoes phosphorylation, we stimulated cultured astrocytes with the phorbol ester, phorbol 12-myristate 13-acetate (PMA) (Lim and Zaheer, 1995). PMA causes a rapid and transient phosphorylation of GMF, detectable within 15 min, and gradually subsides over the next 24 h (*Figure 10-5*). The phosphorylated residues are primarily serine and secondarily threonine. The process is not blocked by protein synthesis inhibitors, and there is no change in the mRNA level of GMF, suggesting that it is strictly a posttranslational phenomenon and not one of protein or mRNA turnover.

The function of phosphorylated GMF was studied in test tube and in intact cells (Lim and Zaheer, 1996). It was demonstrated with immune complex kinase assay that when PKA-phosphorylated GMF is incubated with the three major isoforms of the MAP kinase superfamily, it enhances the activity of p38 but inhibits that of ERK, with little effect on c-Jun N-terminal kinase (JNK) (*Figure 10-6*). Nonphosphorylated GMF has no effect, nor has GMF phosphorylated by PKC, RSK, or CKII. In the intact cells, forskolin, an activator of PKA, enhances phosphorylation of endogenous GMF. The interaction between GMF and p38 in the cell can be demonstrated by their co-immunoprecipitation. Thus, one of the major functions of GMF could be the regulation of signal transduction at the level of p38 MAP kinase.

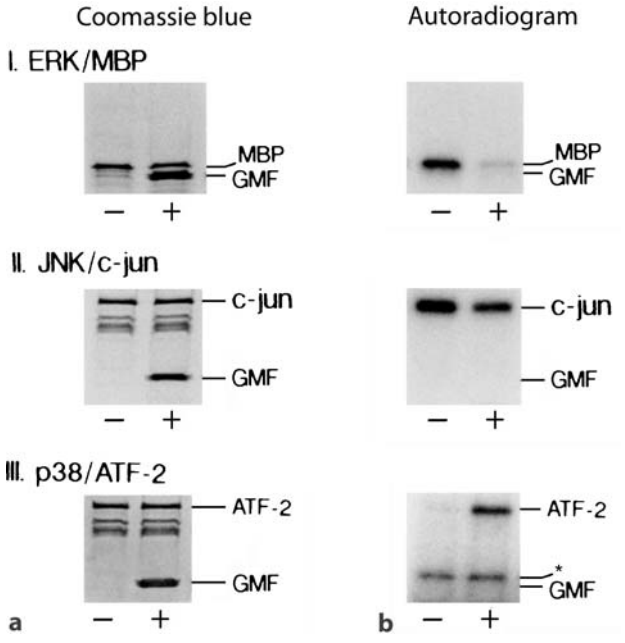
It is of interest to note that both PKA-phosphorylated GMF and PKC-phosphorylated GMF stimulate the catalytic activity of PKA. This activity is not shared by CKII-phosphorylated GMF or RSK-phosphorylated GMF (Zaheer and Lim, 1997). The mutual augmentation of GMF and PKA implies a positive feedback loop between the two, which could be important in the regulation of signal transduction.

## 7 Overexpression Studies

A powerful tool to study protein function is to express the protein in question in excessive amounts and to observe the outcome of the perturbation in the cell. To this end we prepared a transfection agent by attaching the full-length coding sequence of rat GMF cDNA to the replication-defective human adenovirus vector (serotype 5) using the cytomegalovirus (CMV) promoter. The construct, called Ad5CMVGMF, was

**Figure 10-6**

**Effects of protein kinase A (PKA)-phosphorylated glia maturation factor (GMF) on the three pathways of mitogen-activated protein (MAP) kinase (extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), and p38) using their respective substrates of phosphorylation, MBP, c-jun, and activating transcription factor (ATF-2). *Minus* and *plus* signs refer to the absence and presence of GMF in the immune complex kinase assay. Note that GMF enhances kinase activity of p38, inhibits that of ERK, and has no effect on JNK. Autoradiogram for p38 was purposely underexposed in order to bring out the difference. (From Lim and Zaheer, 1996)**




used to infect cultured cells, both normal and tumor-derived. We also prepared stable transfectants from the C6 cell line using the pcDNA3 plasmid. GMF-transfected cells overexpress GMF but do not secrete the protein into the conditioned medium (*Figure 10-7*).

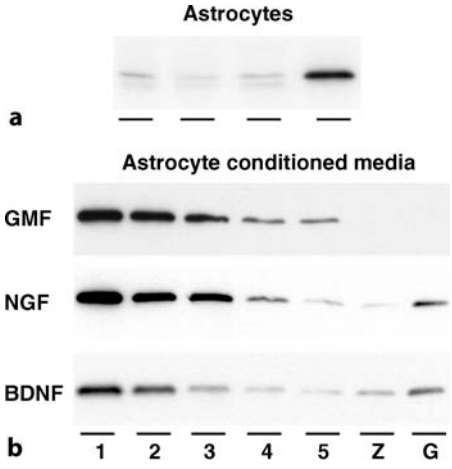
## 7.1 C6 Rat Glioma Cells

Stable C6 transfectants overexpressing GMF grow with a lower saturation density and show a more differentiated morphology (process outgrowth), compared to the original C6 cells (Lim et al., 1998). There is a 3.5-fold increase in the activity of the redox enzyme CuZn-dependent superoxide dismutase (CuZnSOD), but not that of the Mn-dependent isoform (MnSOD). The increase in CuZnSOD activity is accompanied by an increase in the enzyme protein, suggesting enzyme induction. When inoculated into the nude mice, these stable transfectants show a lower tumorigenicity and express the mature astrocytic marker glial fibrillary acidic protein (Lim et al., 1998).

C6 cells infected with the GMF–adenovirus construct show a robust expression of GMF and a slower growth curve compared with the control (Lim et al., 1998). GMF/virus infection of C6 leads to the activation of the transcription factor nuclear factor-κB (NF-κB), as evident in electrophoretic mobility shift assay (EMSA) of the nuclear extract, using a double-stranded oligonucleotide probe containing the consensus binding sequence for NF-κB (Lim et al., 2000). The specificity of binding is demonstrated by competition with an unlabeled probe and by the nonbinding of the mutant probe. Binding is detectable as

**Figure 10-7**

**Overexpression of glia maturation factor (GMF) in cultured astrocytes showing absence of GMF secretion but enhanced secretion of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). (a) Western blot of astrocyte cell lysate following transfection of GMF using adenovirus/GMF construct with cytomegalovirus (CMV) promoter. M, mock transfection; C, transfected with empty adenovirus vector containing CMV promoter; Z, transfected with adenovirus/*lacZ* construct; G, transfected with adenovirus/GMF construct. (b) Western blot of standard proteins (1–5) and conditioned medium concentrates (Z and G) of GMF-transfected astrocytes. The amounts of standard proteins applied are (1–5): 50, 25, 12.5, 6.25, and 3.1 ng per lane. The conditioned media were concentrated 200-fold and applied at 20 μl per lane. Note enhanced secretion of NGF and BDNF and absence of secretion of GMF by the transfected cells. The amount of GMF in the conditioned medium was calculated to be less than 1 ng/ml. Similar results were obtained with C6 cells (not shown). (From Zaheer et al., 2001, 2002)**

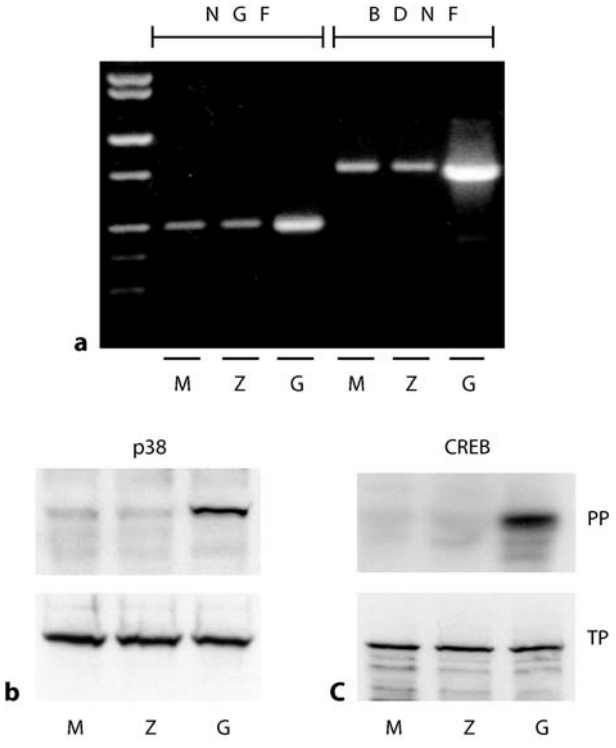



early as 3 h after transfection, peaks at 6 and 12 h, and gradually declines thereafter. The observed NF-κB activation is reduced by cotransfection with catalase and by the presence of high concentrations of pyruvate in the medium, suggesting the involvement of $H_2O_2$. The p38 MAP kinase inhibitor SB-203580 also suppresses the GMF-activated NF-κB, suggesting the involvement of the p38 signal transduction cascade. Along with NF-κB activation is an enhanced expression of CuZnSOD, which is suppressed if NF-κB nuclear translocation is blocked by its specific decoy DNA, implicating NF-κB as an upstream mediator of this redox enzyme. The p38 inhibitor SB203580 also blocks the GMF-activated SOD.

GMF/virus -infected C6 cells show an increased expression of the neurotropic factors, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), detected by reverse-transcription polymerase chain reaction (RT-PCR) (*Figure 10-8*) (Zaheer et al., 1999; Pantazis et al., 2000). There is also an increase in the NGF and BDNF proteins in the conditioned medium (*Figure 10-7*) (Pantazis et al., 2000). That the neurotrophins in the conditioned medium are biologically active is demonstrable by the ability of the medium to promote neurite outgrowth and neurite resprouting in PC12 rat pheochromocytoma cells (Zaheer et al., 1999) and to exert trophic effect on primary cultures of cerebellar granule neurons while protecting these cells against ethanol toxicity (Pantazis et al., 2000). A soluble TrkB–IgG fusion protein, which selectively binds BDNF and prevents its binding to the neuronal TrkB receptor, eliminates the trophic effect of the conditioned medium on granule cells, whereas anti-NGF antibody is ineffective in preventing this effect, suggesting that the neurotrophic effect on cerebellar granule cells is due to BDNF. On the other hand, both the TrkB–IgG fusion protein and anti-NGF reduce protection against ethanol, suggesting that both BDNF and NGF contribute to the neuroprotection of the granule cells provided by the conditioned medium (Pantazis et al., 2000). Thus, GMF upregulates the expression of BDNF and NGF in C6 cells, and these factors exert neurotrophic and neuroprotective functions on primary neurons.

**Figure 10-8**

**Overexpression of glia maturation factor (GMF) leads to activation of p38 and cAMP response element binding protein (CREB) and increase in nerve growth factor (NGF) and brain-derived neurotrophic factors (BDNF) mRNAs. (a) RT-PCR of GMF-overexpressed C6 cells showing increase in NGF and BDNF mRNAs (from Pantazis et al., 2000). PCR was carried out to 35 cycles for NGF and to 32 cycles for BDNF. Astrocytes gave similar results (not shown). (b and c) Western blot for phosphorylated p38 and cAMP response element binding protein (CREB) on GMF-overexpressed astrocytes using phosphospecific antibodies. PP, phosphorylated protein; TP, total protein (nonphosphorylated plus phosphorylated). Note increased phosphorylaion of p38 and CREB in GMF-transfected cells. In contrast, there is no increase in the phosphorylation of MAP kinase kinase (MKK), the kinase upstream of p38 (not shown). For meanings of M, Z, and G, see Figure 10-7. (From Zaheer et al., 2001)**




## 7.2 PC12 Rat Pheochromocytoma Cells

In order to study the intracellular regulatory function of GMF in neuronal cells, we achieved a tenfold overexpression of GMF in PC12 cells by infection with the GMF/virus construct (Zaheer and Lim, 1998). These cells showed a 3.6-fold increase in the activity of p38 MAP kinase, a 3.8-fold increase in the activity of MAP kinase-activated protein kinase 2 (MAPKAP-K2), and a 4.2-fold increase in the activity of tyrosine hydroxylase (TH). There is also an increase in the phosphorylation of TH and the 25-kDa heatshock protein (Hsp25) without a concomitant increase in the corresponding protein levels, suggesting a posttranslational phenomenon. It was previously established that in PC12 cells, MAPKAP-K2 is an immediate downstream target of p38, and both TH and Hsp25 are immediate downstream targets of MAPKAP-K2. The current in vivo results are in concordance with our earlier in vitro finding that GMF promotes the activity of p38, and implicate the participation of GMF in stress-induced catecholamine synthesis through the p38 signal pathway. The fact that PC12 possesses endogenous GMF suggests that the observed effects of GMF transfection reflect an enhanced physiologic function.

## 7.3 Primary Astrocytes

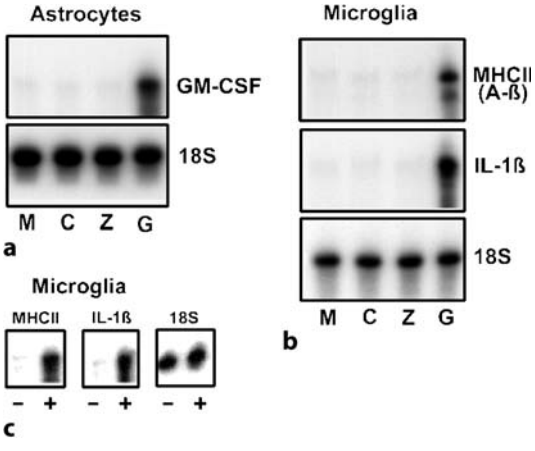
### 7.3.1 Signal Transduction, Transcription Activation, and Neurotrophin Secretion

In primary astrocyte cultures derived from near-term embryonic rat brains, GMF overexpression from GMF/virus infection results in a number of changes (◉ *Figure 10-8*) (Zaheer et al., 2001). Among the three isoforms of MAP kinase, p38 shows a big increase in phosphorylation, as detected by Western blotting using a phosphospecific antibody. Likewise, there is a substantial increase in the phosphorylation of the transcription factor cAMP response element binding protein (CREB). Using EMSA, we found stimulation of the transcription factor NF-κB. The activation of CREB and NF-κB is blocked by the inhibitor of p38 MAP kinase (SB-203580). There is an increased secretion of BDNF and NGF into the conditioned medium (◉ *Figure 10-7*), along with an increase in their messenger RNA (◉ *Figure 10-8*). The induction of BDNF and NGF is also blocked by inhibiting p38 with SB-203580, and by inhibiting NF-κB with a decoy DNA sequence. Taken together, the results suggest that GMF functions intracellularly in astrocytes as a modulator of MAP kinase signal transduction, leading to a series of downstream events including CREB and NF-κB activation, resulting in the induction and secretion of the neurotrophins. That the probable site of GMF action is at the p38 level and not anywhere upstream is supported by the lack of increased MAP kinase kinase (MKK3/6) phosphorylation, the kinase immediately above p38 (Zaheer et al., 2001).

### 7.3.2 Cytokine Secretion and Immune Activation

Overexpression of GMF in astrocytes also leads to the enhanced production and secretion of the cytokine granulocyte–macrophage colony-stimulating factor (GM-CSF) (◉ *Figure 10-9*) (Zaheer et al., 2002). Other

**◘ Figure 10-9**
**Ribonuclease protection assay showing induction of granulocyte–macrophage colony-stimulating factor (GM-CSF) in glia maturation factor (GMF)-overexpressed astrocytes (a) and response of microglia to the astrocyte-conditioned medium by production of MHC class II protein and IL-1β (b). For comparison, (c) shows a similar response of microglia to recombinant GM-CSF. The 18S ribosomal RNA was used as sample control. For meanings of M, C, Z, and G, see ◉ Figure 10-7. *Minus* and *plus* signs indicate absence and presence of recombinant GM-CSF. (From Zaheer et al., 2002)**




cytokines such as interleukin-3 (IL-3) and interleukin-5 (IL-5) are not affected. Although the GMF/virus does not directly transfect microglia, coculture of GMF-transfected astrocytes with microglia leads to the activation of the latter as evidenced by the microglial expression of interleukin-1β (IL-1β) and the major

histocompatibility complex class II (MHC II) protein, detected by the Affymetrix DNA microarray system. That the astrocyte-secreted agent responsible for microglial activation is indeed GM-CSF is verified by the following: increased GM-CSF mRNA in astrocytes detectable by RT-PCR and ribonuclease protection assay (RPA); ability of the astrocyte-conditioned medium to activate microglia as shown by the latter's production of MHC II and IL-1β (detected by RT-PCR and RPA); mimicry of the conditioned medium effects on microglia by recombinant GM-CSF; and lastly, the direct demonstration of a 40-fold increase in GM-CSF in the conditioned medium of GMF-transfected astrocytes by ELISA (Zaheer et al., 2002). MHC class II proteins are unique to antigen-presenting cells such as microglia and monocytes. Antigens are presented to T lymphocytes during the initial phase of the immune cascade. Thus, our finding suggests that GMF in astrocytes can initiate a series of events leading to immune activation in the nervous system. The enhancement of GM-CSF production in astrocytes following the overexpression of GMF is blocked by MAP kinase inhibitors.

The downstream effects of GMF are summarized in *Table 10-2*.

**Table 10-2**
**Cellular effects following overexpression of GMF**

| Activation/increase | Inhibition/decrease |
|---|---|
| p38 MAP kinase | ERK MAP kinase |
| CREB | Catalase |
| NGF | Glutathione peroxidase |
| BDNF | |
| GM-CSF | |
| CuZnSOD | |
| NF-κB | |
| MAPKAP-kinase 2 | |
| Hsp 25 | |
| Tyrosine hydroxylase | |

## 8 Gene Knockout Studies

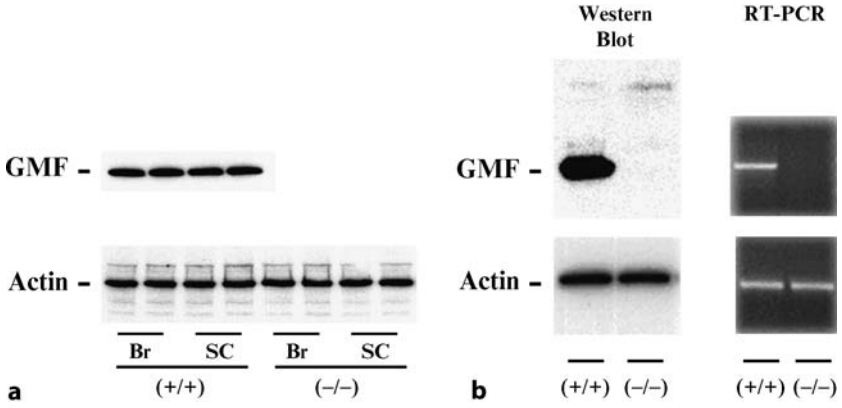
### 8.1 Production of GMF-Null Mice

Powerful as it is, overexpression conveys only one aspect of a protein's function. The alternative approach of total deletion can provide complementary information not otherwise obtainable. To this end, we have produced GMF-knockout (KO) mice by homologous recombination (Zaheer et al., 2004).

A 14-kb DNA fragment containing the entire mouse GMF gene was identified and cloned from the mouse 129/SVJ BAC library. All seven exons of GMF were identified. A targeting construct was prepared by using a 2.1-kb region containing exon 1 as the upstream homology and a 3.1-kb region containing exon 7 as the downstream homology. After homologous recombination, a region of 7 kb containing exon 2 to 6 was deleted and replaced with the bacterial neomycin gene. With this deletion, more than 80% of the amino acid residues are removed from the GMF protein. After electroporation of the targeting construct into ES cells, the drug-resistant colonies were screened by PCR, using primers designed to amplify across the upstream homology region of the targeting construct. Eight colonies were selected that contained both wild type and knockout alleles, with a targeting frequency of 4.3%. Three ES cell lines were used to generate chimeras. Germline transmission was obtained from a line designated BQ50. The knockout mice showed no trace of GMF protein and mRNA (*Figure 10-10*) (Zaheer et al., 2004). Knockout mice were maintained by backcross breeding to C57BL/6 mice.

Absence of GMF gene is nonlethal to the mice, which develop to maturity showing no difference in weight and no gross morphological abnormality. Routine observation, handling, and checking failed to

**Figure 10-10**
**Analysis of glia maturation factor (GMF)-knockout mice. (a) Western blot showing absence of GMF protein in brain (Br) and spinal cord (SC). (b) Absence of GMF protein and mRNA in cultured astrocytes derived from knockout mice. (+/+), wild type; (–/–), homozygous knockout. (From Zaheer et al., 2004)**




detect gross behavioral differences like free locomotion and exploration, tail movement, body posture, paw grip, muscle strength, muscle tone, spontaneous urination, and defecation between the knockout and the wild-type animals.

For housekeeping proteins that are necessary for maintenance of life, the deletion results in fetal death or shortened life span. Regulatory proteins, on the other hand, are not lethal when deleted, but the animal may react abnormally when challenged. The fact that GMF is a highly conserved protein yet nonlethal when knocked out is consistent with the concept of a regulatory protein that performs important adaptive functions.

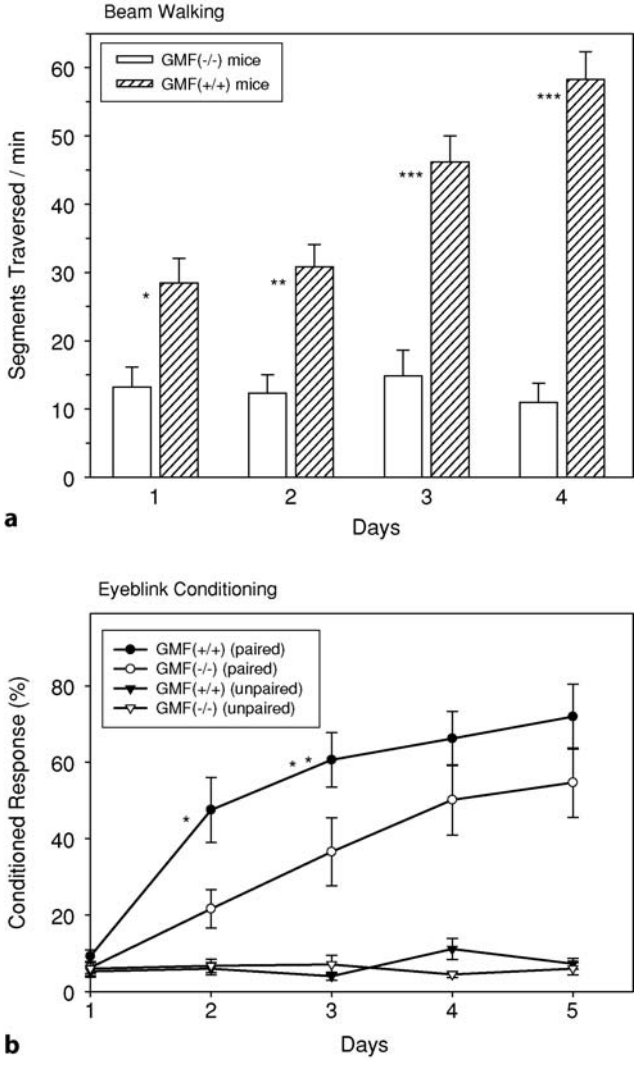
## 8.2 Defective Motor Performance and Learning

When tested with an elevated "plus" maze, both GMF-knockout and wild-type mice crossed the intersection the same number of times and spent the same fraction of time in the enclosed area, indicating no difference in activity level and anxiety. Again, there was no difference when tested for simple position discrimination with a T-maze, using positive reinforcement. Still, no difference was noted when tested for spatial memory in a Morris water maze, suggesting no difference in hippocampus-dependent spatial learning. The last point was corroborated by our failure to detect a difference in long-term potentiation (LTP) elicited in hippocampal slices (Lim et al., 2004).

However, when the mice were forced to walk on a narrow beam, the knockout mice performed poorly and failed to improve upon repeated training (*Figure 10-11a*). Since poor motor performance can mask motor learning, we subjected the animals to eyeblink conditioning, a test designed for motor learning involving specific cerebellar circuitry, with very little, if any, performance component. Again, GMF-knockout mice were deficient in eyeblink-conditioned response (*Figure 10-11b*). Thus, the absence of GMF affects both motor performance and motor learning, but not the hippocampus-dependent declarative learning (Lim et al., 2004).

Histological examination of the GMF-null brains revealed neuronal loss in the inferior olive (*Figure 10-12*), a defect that explains both poor beam-walking (inability to balance) and poor eyeblink conditioning (inability to learn), since inferior olive is part of the circuitry for both responses (Freeman and Nicholson, 2000; Nicholson and Freeman, 2000).

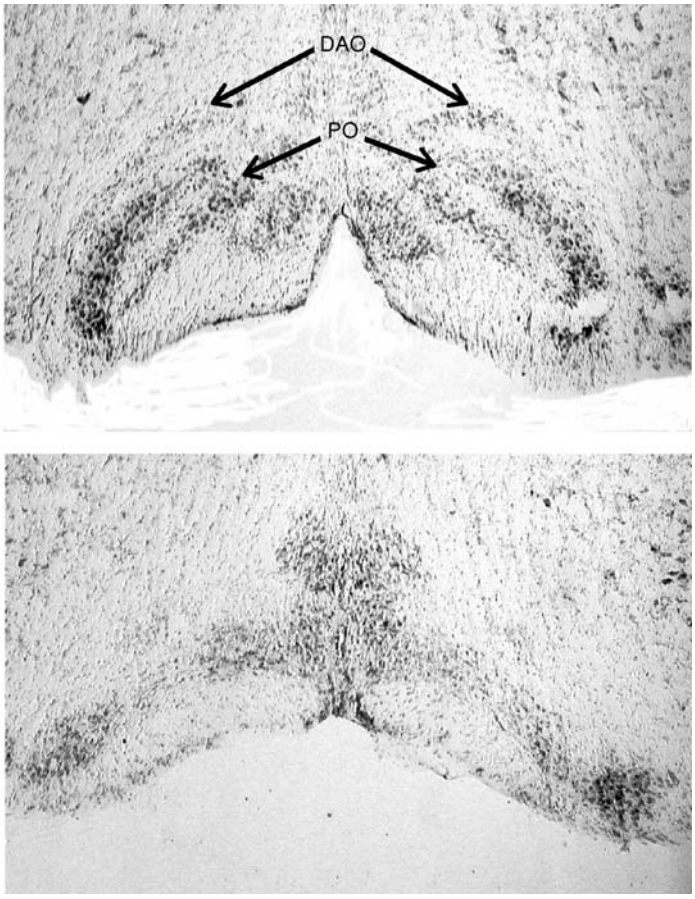
**Figure 10-11**
**Behavioral deficit in glia maturation factor (GMF)-null mice. (a) Beam walking. (b) Eyeblink conditioning using sound as conditioned stimulus and electric shock as unconditioned stimulus. Note deficit of knockout mice in both tasks. (From Lim et al., 2004)**




## 8.3 Alterations of Redox Enzymes

Astrocyte cultures derived from GMF-knockout mice become more resistant to $H_2O_2$. This is accompanied by a decrease in the activity of the redox enzyme CuZnSOD and an increase in the activities of catalase and glutathione peroxidase (Zaheer et al., 2004). CuZnSOD converts the toxic mitochondrial by-product superoxide to $H_2O_2$, also a potentially harmful agent, which in turn is reduced to water by catalase and glutathione peroxidase. CuZnSOD therefore is a double-edged sword, capable of imparting benefit or harm to the cell, depending on whether or not the accumulated $H_2O_2$ is cleared by the two downstream enzymes. An increase in the activity of these enzymes results in a lower intracellular level of $H_2O_2$ and explains why

**Figure 10-12**
**Anatomical deficit in glia maturation factor (GMF)-null mice. (a) Wild-type mice showing intact inferior olives; (b) knockout mice showing neuronal loss in the same structure. DAO, dorsal accessory nucleus of inferior olive; PO, primary nucleus of inferior olive. (From Lim et al., 2004)**




the GMF-null cells are more resistant to exogenous $H_2O_2$. The decrease in CuZnSOD is in agreement with our earlier finding that GMF overexpression induces CuZnSOD (Lim et al., 1998, 2000).

Unlike the life-sustaining proteins, adaptive proteins are more flexible, and could be involved in both physiologic and pathologic processes. A case in point is glutamate, which is essential in neural function and memory but whose aberration contributes to neuronal death. Or one can recall cytochrome *c*, which is both a life-supporting and a terminating agent. GMF may be one of these multifunctional proteins whose ultimate effect depends on the environmental context, both inside and outside the cell. Although $H_2O_2$ is traditionally considered a toxic by-product of biological oxidation, recent data suggest that it is also essential for certain signal transduction steps (Keyes and Emslie 1992; Abe et al., 1996; Lo et al., 1996) and transcription activation (Sun and Oberley, 1996). There is evidence that $H_2O_2$ is produced as a normal response to some growth factors and cytokines (Thannickal and Fanburg, 2000), and it has been suggested that oxidation of cysteine and histidine residues by $H_2O_2$ can activate a protein by conformational changes in a manner akin to protein phosphorylation (Finkel, 1998). Therefore, by affecting the steady-state level of $H_2O_2$ through SOD and other enzymes, GMF could be one of the redox regulators that determine cell survival, proliferation, differentiation, or death.

## 9 GMF in Extraneural Functions

### 9.1 Oxidative Stress in Renal Cells

Although GMF is normally low in kidney, Kaimori et al. (2003) reported that GMF is induced in renal proximal tubular (PT) cells in experimental proteinuria produced by albumin overloading in mice. When PT cell lines are permanently transfected to overexpress GMF, the cells are more susceptible to cell death upon stimulation with tumor necrosis factor-α (TNF-α) and angiotensin II, both of which have been found to produce oxidative stress. Moreover, when GMF-overexpressing PT cells are exposed to $H_2O_2$, the cells are more likely to undergo apoptosis. There is a sustained increase in intracellular $H_2O_2$ when these cells are stimulated by tumor necrosis factor, angiotensin II, or $H_2O_2$. The increase in intracellular $H_2O_2$ is due to an increase in the activity of CuZnSOD, which produces $H_2O_2$, and a decrease in the activities of catalase and glutathione peroxidase, which destroy $H_2O_2$. The downstream effects of GMF are blocked by the p38 MAP kinase inhibitor SB203580. These results are consistent with our data from brain cells with respect to the GMF stimulation of SOD and accumulation of $H_2O_2$.

### 9.2 T Cell Differentiation

The thymus, apart from the nervous system, expresses GMF in large amounts (Nishiwaki, 2001). Utsuyama et al. (2003) identified GMF as a protein in the thymic epithelial cells essential for the differentiation of T cells. They arrived at this conclusion by producing a monoclonal antibody that inhibits T cell development in an in vitro culture system of the embryonic thymus. cDNA of the protein recognized by the antibody carries a sequence identical to that of GMF. Both Northen blot and immunoblot analyses confirm the presence of GMF in the thymus. Finally, recombinant GMF influences T cell development in favor of an increase in CD4+ T cells.

## 10 Concluding Remarks and Prospects

It appears that GMF is an intracellular regulatory protein. Although not necessary for basic survival, it plays a role in many adaptive functions, including reaction to stress, immune regulation, and maintenance of redox homeostasis. In the brain, the downstream effects of GMF include the production of neurotrophins and cytokines. Evidence has accumulated that GMF probably acts on the signal transduction cascade at the level of p38 MAP kinase. Future research will focus on the detailed mechanism of this action, along with the regulation of GMF gene expression. The immune modulation role of GMF suggests new avenues of investigation that may be relevant to autoimmune disorders in the central nervous system, such as multiple sclerosis.

## Acknowledgments

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## References

Abe J, Kusuhara M, Ulevitch RJ, Berk BC, Lee JD. 1996. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. J Biol Chem 271: 16586-16590.

Asai K, Fujita K, Yamamoto M, Hotta T, Morikawa M, et al. 1998. Isolation of novel human cDNA (hGMF-γ) homologous to glia maturation factor-β gene. Biochem Biophys Acta 1396: 242-244.

Ashburner M, Misra S, Roote J, et al. 1999. An exploration of the sequence of a 2.9-Mb region of the genome of *Drosophila melanogaster*: the Adh region. Genetics 153: 179-219.

Bosch EP, Zhong W, Lim R. 1989. Axonal signals regulate expression of glia maturation factor in Schwann cells: an immunohistochemical study of injured sciatic nerves and cultured Schwann cells. J Neurosci 9: 3690-3698.

Bourgeois F, Guimiot F, Mas C, Bulfone A, Levacher B, et al. 2001. Identification and isolation of a full-length clone of mouse GMFB (Gmfb), a putative intracellular kinase regulator, differentially expressed in telencephalon. Cytogenet Cell Genet 92: 304-309.

Finkel T. 1998. Oxygen radicals and signaling. Curr Opin Cell Biol 10: 248-253.

Freeman JH Jr, Nicholson DA. 2000. Developmental changes in eye-blink conditioning and neuronal activity in the cerebellar interpositus nucleus. J Neurosci 20: 813-819.

Inagaki M, Aoyama M, Sobue K, Yamamoto N, Morishima T, et al. 2004. Sensitive immunoassay for human and rat GMFB and GMFG, tissue distribution and age-related changes. Biochem Biophys Acta 1670: 208-216.

International Human Genome Sequence Consortium. 2001. Nature 409: 860-921.

Kaimori JY, Takenaka M, Nakajima H, Hamano T, Horio M, et al. 2003. Induction of glia maturation factor-β in proximal tubular cells leads to vulnerability to oxidative injury through p38 pathway and changes in antioxidant enzyme activities. J Biol Chem 278: 33519-33527.

Kaplan R, Zaheer A, Jaye M, Lim R. 1991. Molecular cloning and expression of biologically active human glia maturation factor-beta. J Neurochem 57: 483-490.

Kawai Y, Asai K, Miura Y, Inoue Y, Yamamoto M, et al. 2003. Structure and promoter activity of the human glia maturation factor-gamma gene: a TATA-less, GC-rich and bidirectional promoter. Biochem Biophys Acta 1625: 246-252.

Keyse SM, Emslie EA. 1992. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. Nature 359: 644-647.

Lim R, Hicklin DJ, Ryken TC, Miller JF. 1987. Endogenous immunoreactive glia maturation factor-like molecule in astrocytes and glioma cells. Dev Brain Res 33: 49-57.

Lim R, Miller JF, Zaheer A. 1989. Purification and characterization of glia maturation factor beta: a growth regulator for neurons and glia. Proc Natl Acad Sci USA 86: 3901-3905.

Lim R, Zaheer A. 1995. Phorbol ester stimulates rapid intracellular phosphorylation of glia maturation factor. Biochem Biophys Res Commun 211: 928-934.

Lim R, Zaheer A. 1996. In vitro enhancement of p38 mitogen-activated protein kinase activity by phosphorylated glia maturation factor. J Biol Chem 271: 22953-22956.

Lim R, Zaheer A, Khosravi H, Freeman JH Jr, Halverson HE, et al. 2004. Impaired motor performance and learning in glia maturation factor-knockout mice. Brain Res 1024: 225-232.

Lim R, Zaheer A, Kraakevik JA, Darby CJ, Oberley LW. 1998. Overexpression of glia maturation factor in C6 cells promotes differentiation and activates superoxide dismutase. Neurochem Res 23: 1445-1451.

Lim R, Zaheer A, Lane WS. 1990. Complete amino acid sequence of bovine glia maturation factor beta. Proc Natl Acad Sci USA 87: 5233-5237.

Lim R, Zaheer A, Yorek MA, Darby CJ, Oberley LW. 2000. Activation of nuclear factor-kappa B in C6 rat glioma cells after transfection with glia maturation factor. J Neurochem 74: 596-602.

Liu LX, Xu H, Weller PF, Shi A, Debnath I. 1997. Structure and expression of a novel filarial gene for glia maturation factor. Gene 186: 1-5.

Lo YY, Wong JM, Cruz TF. 1996. Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. J Biol Chem 271: 15703-15707.

Mao M, Fu G, Wu JS, et al. 1998. Identification of genes expressed in human CD34+ hematopoietic stem/progenitor cells by expressed sequence tags and efficient full-length cDNA cloning. Proc Natl Acad Sci USA 95: 8175-8180.

Nicholson DA, Freeman JH Jr. 2000. Developmental changes in eye-blink conditioning and neuronal activity in the inferior olive. J Neurosci 20: 8218-8226.

Nishiwaki A, Asai K, Tada T, Ueda T, Shimada S, et al. 2001. Expression of glia maturation factor during retinal development in the rat. Mol Brain Res 95: 103-109.

Pantazis NJ, Zaheer A, Dai D, Zaheer S, Green SH, et al. 2000. Transfection of C6 glioma cells with glia maturation factor upregulates BDNF and NGF: trophic effects and protection against ethanol toxicity in cerebellar granule cells. Brain Res 865: 59-76.

Pearson RB, Kemp BE. 1991. Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol 200: 62-81.

Sun Y, Oberley LW. 1996. Redox regulation of transcriptional activators. Free Radic Biol Med 21: 335-348.

Thannickal VJ, Fanburg BL. 2000. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279: L1005-L1028.

Tsuiki H, Asai K, Yamamoto M, Fujita K, Inoue Y, et al. 2000. Cloning of a rat glia maturation factor-gamma (rGMFG) cDNA and expression of its mRNA and protein in rat organs. J Biochem 127: 517-523.

Utsuyama M, Shiraishi J, Takahashi H, Kasai M, Hirokawa K. 2003. Glia maturation factor produced by thymic epithelial cells plays a role in T cell differentiation in the thymic microenvironment. Int Immunol 15: 557-564.

Wang BR, Zaheer A, Lim R. 1992. Polyclonal antibody localizes glia maturation factor beta-like immunoreactivity in neurons and glia. Brain Res 591: 1-7.

Zaheer A, Fink BD, Lim R. 1993. Expression of glia maturation factor beta mRNA and protein in rat organs and cells. J Neurochem 60: 914-920.

Zaheer A, Lim R. 1997. Protein kinase A (PKA)- and protein kinase C-phosphorylated glia maturation factor promotes the catalytic activity of PKA. J Biol Chem 272: 5183-5186.

Zaheer A, Lim R. 1998. Overexpression of glia maturation factor (GMF) in PC12 pheochromocytoma cells activates p38 MAP kinase, MAPKAP kinase-2, and tyrosine hydroxylase. Biochem Biophys Res Commun 250: 278-282.

Zaheer A, Mathur SN, Lim R. 2002. Overexpression of glia maturation factor in astrocytes leads to immune activation of microglia through secretion of granulocyte-macrophage-colony stimulating factor. Biochem Biophys Res Commun 294: 238-244.

Zaheer A, Weiss JL, Goyal P, Lim R. 1999. Enhanced expression of neurotrophic factors by C6 rat glioma cells after transfection with glia maturation factor. Neurosci Lett 265: 203-206.

Zaheer A, Yang B, Cao X, Lim R. 2004. Decreased copper-zinc superoxide dismutase activity and increased resistance to oxidative stress in glia maturation factor-null astrocytes. Neurochem Res 29: 1473-1480.

Zhang QH, Ye M, Wu XY, et al. 2000. Cloning and functional analysis of cDNAs with open reading frames for 300 previously unidentified genes expressed in CD34+ hematopoietic stem/progenitor cells. Genome Res 10: 1546-1560.

Zaheer A, Yorek MA, Lim R. 2001. Effects of glia maturation factor overexpression in primary astrocytes on MAP kinase activation, transcription factor activation, and neurotrophin secretion. Neurochem Res 26: 1293-1299.

# 11 Nitric Oxide Synthases in Brain Function

*S. Murphy · T. Coughlan*

**Abstract:** The nitric oxide synthase (NOS) enzymes, three gene products that are highly homologous, are variously expressed in the nervous system. Gene regulation is complex, and they are the only flavoheme enzymes that require tetrahydrobiopterin (BH4) as a redox cofactor. The nNOS and eNOS isoforms are constitutive enzymes found typically in some neurons and in the endothelium, respectively, while iNOS is transcriptionally activated in response to injury and infection. Distinct N- and C-terminal motifs in the NOS proteins target these enzymes to discrete cellular compartments where they associate with specific scaffold and cytoskeletal proteins. Further oxidation of nitric oxide (NO) generates a wide variety of products that can react with DNA and proteins, resulting in a short-term change and also long-lived effects on gene expression, cell cycle, and differentiation. On the basis of the phenotype of gene-deficient mice, and the use of enzyme inhibitors with only partial selectivity, roles for reactive nitrogen species (RNS) have been invoked in almost every aspect of nervous system function and in the pathology that accompanies acute injury and degeneration. This chapter focuses on the NOS enzymes, generation of RNS and their molecular targets, and involvement in neurodegeneration, acute injury, and the host response to infection.

**List of Abbreviations:** AD, Alzheimer's disease; ALS, amyotropic lateral sclerosis; BBB, blood–brain barrier; BH4, tetrahydrobiopterin; CSF, colony stimulating factor; COX, cyclooxygenase; EAE, experimental allergic encephalomyelitis; IFN, interferon; IL, interleukin; LTP, long-term potentiation; MS, multiple sclerosis; NO, nitric oxide; NOS, nitric oxide synthase; NT, nitrotyrosine; $ONOO^-$, peroxynitrite; PD, Parkinson's disease; PDZ, PSD-95/discs large/zona occludens-1; PSD, postsynaptic density; RNS, reactive nitrogen species; sGC, soluble guanylyl cyclase; SNO, S-nitrosothiol; SOD, superoxide dismutase; TBI, traumatic brain injury; TNF, tumor necrosis factor

## 1 Introduction

The excretion of nitrate, an oxidation product of nitric oxide (NO), was rediscovered in human subjects (Green et al., 1981). Production was observed to be independent of bacterial involvement, and was seen to increase during inflammation. Subsequently, mouse macrophages were shown to produce nitrite and nitrate as part of the host response to bacterial challenge (Stuehr and Marletta, 1985). There followed a flurry of activity resulting in some classic publications, and by the end of that decade it had been established that the smooth muscle relaxing factor released by vascular endothelium (Furchgott and Zawadski, 1980) was NO (Ignarro et al., 1987; Palmer et al., 1987), that neurons also produced NO (Garthwaite et al., 1988), and that in both cases the attendant rise in cGMP was due to NO binding to the heme moiety of soluble guanylyl cyclase (sGC). With almost unseemly haste *Science* magazine named NO as molecule of the year in 1992, and three of those intimately involved in the early work (Robert Furchgott, Louis Ignarro, and Ferid Murad) shared the Nobel Prize for Physiology and Medicine in 1998; from discovery to universal acclaim in a mere 20 years!

The source of NO is arginine. The first crude separation and purification of the nitric oxide synthase (NOS) enzyme involved in NO synthesis was reported simultaneously by a number of groups (Schmidt et al., 1989; Bredt and Snyder, 1990). The brain enzyme was found to be dependent upon calcium/calmodulin, active as a dimer with monomeric mass 150 kDa, and to require a number of cofactors. The gene was cloned from rat brain (Bredt et al., 1991) and named nNOS (later type I or NOS-1). A second gene was cloned from activated macrophages by a number of groups within the year (Lowenstein et al., 1992; Lyons et al., 1992; Xie et al., 1992) and named macNOS or inducible NOS (type II, NOS-2). That same year a third gene was cloned from human and bovine endothelium (Janssens et al., 1992; Lamas et al., 1992; Marsden et al., 1992; Nishida et al., 1992; Sessa et al., 1992) and named eNOS (type III, NOS-3).

These three gene products are highly homologous and display splice variants to a greater or lesser degree. They are active as homodimers, with each monomer displaying a catalytic oxygenase domain and a reductase domain. Bacterial genomes also code for a NOS oxygenase domain precursor, similar in sequence and biochemistry to the mammalian NOS oxygenase domain. To function, the bacterial domain requires an electron-donating partner. Study of the snail *Helix pomatia* implied the existence of a new type of NOS without obvious homology to the three previously described. More recently, the *Aribidopsis* AtNOS1 protein

was identified as having homology similar to that of the snail, and this protein also has NOS activity. Using database search strategies Zemojtel et al. (2004) have recently described close homologs in the genomes of all metazoans. These proteins have a GTPase and an NOS domain, an integration point of two fundamental signaling mechanisms. We are waiting to see what functional roles these proteins have in mammals.

Following the discovery that various NOS isoforms are expressed in the nervous system, interest has been on identifying the products and molecular targets, and their roles in development, normal central nervous system (CNS) function, and pathology. First, we describe the NOS proteins and how their expression is regulated in specific cells and cellular compartments within the CNS.

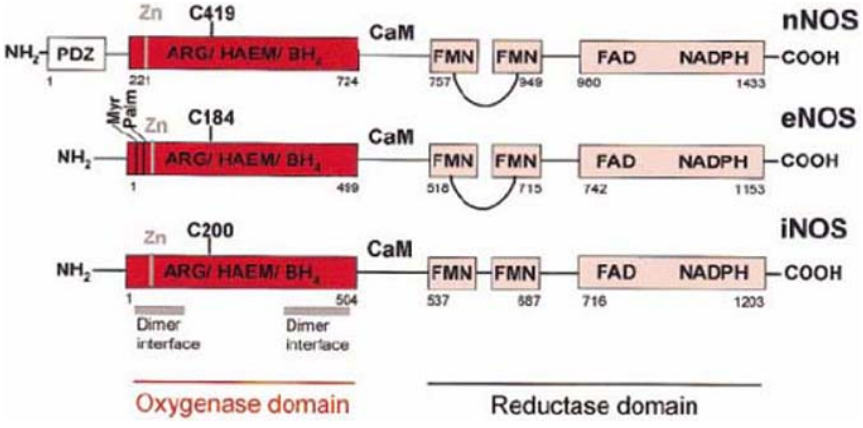
## 2 Structure and Activity

### 2.1 Protein Structure

There are three isoforms of the classical mammalian NOS, namely NOS-1 (nNOS, neuronal NOS); NOS-2 (iNOS, inducible, immunologic NOS); and NOS-3 (eNOS, endothelial NOS). All three NOS isoforms are characterized by an N-terminal oxygenase domain and a C-terminal reductase domain, which generate NO via the combination of L-arginine and molecular oxygen, and reduction of NADPH, to form citrulline and NADP as by-products (Alderton et al., 2001; Stuehr et al., 2004). The three classical NOS are products of different single-copy genes and share roughly 50% amino acid homology (*Figure 11-1*). Common

**Figure 11-1**

**Structure of human nNOS, eNOS, and iNOS. The amino acid residue numbers that define the major domains are shown. The cysteine residues that ligate the heme and CaM-binding site, and zinc, are indicated. Reproduced with permission from Alderton et al. 2001. Biochem J, 357: 593–615. Copyright of the Biochemical Society**




features include binding domains for the substrates arginine and NADPH, and for the cofactors heme, tetrahydrobiopterin (BH4), CaM, FMN, FAD, and $Zn^{2+}$. There is an N-terminal PDZ domain in nNOS, a C-terminal PDZ-binding ligand motif in iNOS, and myristoylation and palmitoylation sites on eNOS, all of which are involved in protein targeting. The flavin cofactors bound to the reductase domain act as electron transporters, accepting electrons from NADPH and donating them to the heme domain. The electron flow is intermonomeric rather than within the same molecule, which may account for the inactive state of the monomer. The essential requirement of CaM for NOS function may be due to the reduced speed of the electron transfer in the absence of CaM-$Ca^{2+}$. The diverse proposed roles for BH4 in NO activity include potentiation of NOS active state, promotion of dimer formation, and promotion of coupling of NADPH oxidation to NO synthesis.

A comparison of human eNOS and iNOS oxygenase structure shows they are similar in overall shape and cofactor, stereochemical relationships (Fischmann et al., 1999). Human iNOS residues 82–508 of the oxygenase domain form an elongated curved shape, with a novel nonmodular, single-domain α–β fold. A 30 å deep, funnel-shaped channel is formed by dimerization, creating an active center whose entrance is wide enough to permit diffusion of both arginine and citrulline. Arginine binds with its side-chain terminus in the narrow part of the cavity and with the guanidino group coplanar to the heme. In mouse iNOS, the guanidinium group of arginine forms two hydrogen bonds with the two carboxy oxygens of Glu371, and this localizes the substrate over the heme. The BH4 cavity is located near the dimer interface, buried within the protein and inaccessible to the solvent. Indole aromatic rings of human iNOS Trp463 and the BH4 pterin are stacked in parallel, with hydrogen bonding between heteroatoms of the pterin and Arg365, His461, and Ala446. The identity of these residues is, however, not critical for BH4 binding and activity (Ghosh et al., 1999). A Zn tetrathiolate center is located at the bottom of the dimer interface, which is tetrahedrally coordinated with two cysteines (Cys 110 and 115) from each subunit. The Cys115 has been shown to be essential for dimer stability (Chen et al., 1995). The Zn is located between the two hemes where Cys115 is in proximity to Ser119, which hydrogen-bonds to BH4. This suggests that Zn aids structural integrity of the BH4-binding site. Previous studies show Zn is important in dimer stability of nNOS, but not in activity (Hemmens et al., 2000).

## 2.2 Dimerization

Studies utilizing protein dissociation and limited proteolysis have revealed that dimerization is essential for NOS activity, facilitating correct structure of the active site for heme and arginine binding (Ghosh and Stuehr, 1995). Dimers of iNOS consist of subunits aligned head to head, with the oxygenase domains forming the dimer, while the reductase domains are monomeric extensions. This indicates that it is the heme, BH4, and the arginine-binding domain alone that maintain the dimeric structure. In fact, these cofactors not only participate in catalytic activity but help form and maintain dimeric structure in all three NOS isoforms (Baek et al., 1993). In the case of nNOS and eNOS, however, there are additional interactions, both between the two reductase domains and between the oxygenase and reductase domains of each monomer. For these two molecules, BH4 is not essential for dimerization (as it is for iNOS), though, together with arginine, it stabilizes the heme-containing dimers. The nNOS has an extra 250-amino-acid N-terminal leader sequence, which is not required for dimerization. The two dissociated fragments of iNOS, consisting of dimerized oxygenase domains complete with bound heme and isolated reductase domains, function independently but cannot generate NO. However, the subunits can reassociate and regain substantial NOS activity upon incubation with arginine and BH4. The reductase domain of the enzyme first binds FAD, FMN, and CaM in the case of iNOS. The reductase-active monomers then dimerize, requiring sufficient heme and involving arginine and BH4. A heme-containing monomer may be an intermediate. The presence of stably incorporated BH4, along with arginine, alters the heme environment and enables electron transfer in the active dimer.

## 2.3 Catalytic Function

Catalytic activity of the NOS enzymes is novel and involves a unique role for BH4. Although the precise mechanisms and products of NOS catalytic activity remain controversial, one simple explanation invokes a two-step process whereby arginine is initially hydroxylated to form $N^G$-hydroxy-L-arginine, followed by oxidation of this intermediate using an electron from NADPH to form citrulline and NO (Stuehr et al., 2004). The enzyme can also catalyze the production of other products including superoxide ($O_2^-$), depending on the reaction environment. Electron flow is from the NADPH to FAD, then FMN, and finally to heme iron. Flow from FMN to heme is gated by bound CaM (site of the $Ca^{2+}$ dependency of nNOS and eNOS) while, in iNOS, the electron flow occurs constantly. The oxygenase and reductase domains of NOS have been shown to be independently catalytically active (Sheta et al., 1994), such that the isolated reductase

domain is capable of transferring electrons from NADPH via FAD and FMN to cytochrome *c*, and the oxygenase domain can catalyze the second step of NO synthesis (Ghosh and Stuehr, 1995). However, NOS function cannot occur for the two domains without their correct association as a homodimer. Like all enzymes, the availability of substrate and cofactors affects the function of NOS. For example, regulated cellular arginine uptake and availability is essential for iNOS activity and, just like heme and BH4 cofactors, availability is affected by involvement in other cellular processes (reviewed in Aktan, 2004). The proposed alternative reaction pathways and products of NOS activity are discussed in Section 4.1.

## 2.4 Role of Calcium

The two constitutively expressed NOS isoforms, eNOS and nNOS, are activated by transient changes in intracellular $Ca^{2+}$ concentrations, which lead to noncovalent (reversible) $Ca^{2+}$/CaM binding to the enzyme. However, iNOS is insensitive to changes in intracellular [$Ca^{2+}$], because calcium is tightly bound to CaM following protein synthesis. NO and cGMP might also attenuate $Ca^{2+}$ influx and initiate a removal mechanism, decreasing $Ca^{2+}$ in a negative feedback fashion (Yao and Huang, 2003).

## 2.5 NO Feedback Inhibition

As a key signaling molecule, NO can alter redox-sensitive signaling processes, as well as regulate multiple gene families, either directly or indirectly via protein modification or upregulation of cGMP. Alderton et al. (2001) discuss the possibility that NO can inhibit the activity of enzymes, including NOS, that form reduced ferrous heme intermediates by reacting with, and forming, a stable $Fe^{2+}$–NO complex. It is now well established that iNOS itself can be autoregulated by NO. Inhibition of NO formation causes an increase in iNOS transcription and, conversely, inhibition of iNOS transcription occurs upon prolonged exposure of cells to NO (Luss et al., 1994; Park et al., 1994). Perez-Sala et al. (2001) demonstrated that NO can modulate iNOS expression by a posttranscriptional mechanism involving a cGMP-mediated decrease in messenger RNA (mRNA) stability. Several studies have revealed that NO can reduce transcription from NF-κB-induced promoters, which include other genes as well as iNOS (Peng et al., 1995; Park et al., 1997).

Some studies report NO feedback inhibition of eNOS function. This can be via dissociation of caveolin-1 platforms and subsequent reversible silencing of signal transduction in caveolae (Li et al., 2001), or by means of a superoxide-mediated decrease in enzyme activity (Sheehy et al., 1998). The NO generated from nNOS can likewise inhibit nNOS activity via suppression of *N*-methyl-D-aspartate (NMDA) receptor signaling (Kim et al., 1999).

## 2.6 Phosphorylation

All three isoforms of NOS can be phosphorylated. García-Cardeña et al. (1996) demonstrated that eNOS becomes tyrosine-phosphorylated in endothelial cells, causing a reduction in enzyme activity and an associated increase in interaction with caveolin-1. Phosphorylation also occurs at multiple sites, including Ser 116, Thr 497, and Ser 1179 (Shaul, 2002). Phosphorylation can increase as well as decrease eNOS activity, and phosphorylation at the different sites has been shown to be differentially regulated by membrane targeting (Gonzalez et al., 2002), and may alter subcellular localization of eNOS (Rizzo et al., 1998). Matsubara et al. (2003) have further shown that phosphorylation of eNOS Thr 497 (in the CaM-binding site) results in reduced affinity of eNOS for CaM. Phosphorylation of eNOS can be increased upon stimulation by agonists such as flow-induced shear stress, estradiol, and VEGF in a $Ca^{2+}$/CaM-independent manner. Several protein kinases are implicated in the process. Phosphorylation of nNOS Ser847 leads to a decrease in enzyme activity and is mediated by CaM-K IIα (Hayashi et al., 1999). This process has been shown to be directly promoted by PSD-95 (Watanabe et al., 2003). The phosphorylation of nNOS has also

been shown to be mediated by protein kinase A (PKA) and protein kinase C (PKC) (Brune and Lapetina, 1991). The relevance of iNOS phosphorylation by tyrosine kinases immediately following translation (Pan et al., 1996), resulting in an increase in enzyme activity, is less clear. This may be important in cellular localization and functioning of iNOS.

## 3 Regulation and Expression of the NOS Enzymes

### 3.1 Transcription

The major transcriptional start site in human nNOS is 28 nucleotides downstream of a TATA box, with two inverted CAAT boxes also being present. Several potential control element recognition sites, including NF-1, AP-2, CREB/ATF, Ets, NRF-1, and NF-κB, exist in the 5′ flanking region. Human nNOS has $(CA/TG)_n$ polymorphic alleles in the promoter, which may affect activity (Förstermann et al., 1998). There is a similar microsatellite repeat in the 3′ UTR of nNOS, which has polymorphic alleles and may do the same. The transcription of nNOS is regulated in neurons in response to stress induced physically, chemically, or electrically, which can occur in the normal brain and is associated with neuropathology.

The human eNOS promoter lacks a TATA box, but a CCAAT box is present at −286. The Sp1 and GATA sites are consistent with the constitutive, endothelial cell type expression pattern of eNOS. Putative sequence motifs for AP-1, AP-2, NF-1, IL-6, NF-κB, and PEA3, heavy metal, and shear-stress response elements, among others, are also present. Posttranscriptional regulation of eNOS can occur via TNF-α-mediated increase in mRNA degradation (Yoshizumi et al., 1993). Transcription can be affected by shear stress, hypoxia, cell proliferation, steroids, and cytokines (Cirino et al., 2003).

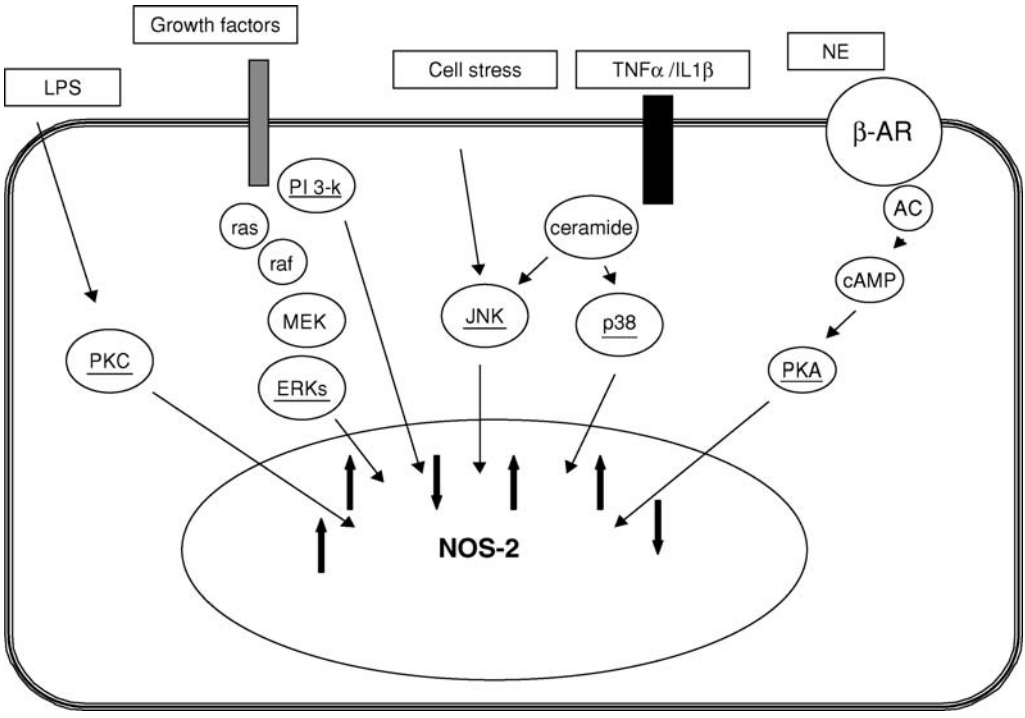
The human iNOS promoter contains binding sites for NF-κB, Jun/Fos heterodimers, C/EBT, CREB, STAT, AP-1, AP-2, Sp-1, as well as TNF-RE, NF-IL6, and a number of γ-IRE response elements (Aktan, 2004). The 3′ UTR of iNOS mRNA contains AU-rich motifs, which have been demonstrated to destabilize the transcript (Rodriguez-Pascual et al., 2000). Transcription of iNOS in vitro can be regulated by an array of factors (❯ *Figure 11-2*).

### 3.2 Splice Variants

Alternative splicing affects the processing, localization, translational efficiency, and protein half-life, and is a method of regulation of the NOS enzymes, both spatially and temporally. nNOS is the only isoform to exist as catalytically active truncated forms that can differ subtly in function (Panda et al., 2003). A variety of splice variants of nNOS have been identified to date, with this gene being among the most complex described in humans. A combination of three mechanisms of alternative splicing account for the increasing number of observed variants, namely alternative promoters, exon deletions, or insertions, and the use of alternative polyadenylation signals (Förstermann et al., 1998).

Four conserved peptide isoforms of nNOS (α, β, μ, and γ) have been identified. Splice variants from which peptides could be translated exist but there is no current evidence for their products. nNOSα originates from the full-length transcript, and this 150-kDa protein accounts for most nNOS activity in the mouse brain. nNOSβ and γ lack exon 2, and possess alternative first exons (1a and 1b). The protein products have no membrane-localizing PDZ domain, which results in their localization to the cytoplasmic rather than membrane fraction. nNOSβ is a 136-kDa protein with six unique N-terminal amino acids, while nNOSγ is translated from a start site in exon 5, resulting in a truncated 125-kDa protein. nNOSμ is expressed mainly in skeletal muscle and is absent from brain. It is fully functional and possesses an in-frame insertion of 34 amino acids between the oxygenase and reductase domains. In vitro studies have revealed that nNOSβ activity compares with that of nNOSα, while nNOSγ displays no significant catalytic ability (Brenman et al., 1996). Low level of expression of a human nNOS transcript with an in-frame deletion of exons 9 and 10 was observed in brain (Wang et al., 1995), which may correspond to the inactive NOS1-(144) peptide found to be expressed during synaptogenesis (Ogilvie et al., 1995). Mitochondrial NOS is in

**Figure 11-2**
**Transcriptional activation of the iNOS gene. Reproduced from Murphy and Pearce (2005)**




fact nNOSα, but with novel posttranslational modifications including acylation with myristic acid and phosphorylation at the C-terminus (Elfering et al., 2002).

There are at least four alternatively spliced iNOS transcripts, resulting from deletions in either exon 5, exons 8 and 9, exons 9–11, or exons 15 and 16 (Eissa et al., 1996, 1998). These exhibit tissue-specific regulation, although only the exon 5-deleted form is significantly expressed in brain. This form predicts a truncated protein resulting from a frame-shift and premature stop codon, and is not predicted to generate NO (Eissa et al., 1996). The authors speculate that such transcripts may posttranscriptionally regulate NO production simply via the generation of excess prematurely terminated transcripts, and there is similar evidence in eNOS (Lee et al., 1995). Reduction in iNOS may be important in terminally differentiated brain tissue. The only iNOS splice variant known to be translated into protein is that lacking exons 8 and 9, which results in a protein with only a reductase domain. This protein may differ in function from full-length iNOS, and may use an alternative electron acceptor (Eissa et al., 1998). There are two transcription start sites in eNOS, though both generate the same 133-kDa protein.

## 3.3 Translation

There are many co- and posttranslational modifications of the NOS enzymes, some of which having already been mentioned. Heat shock protein hsp90 has emerged as an important modulator of the NOS enzymes, being a ubiquitous cellular molecular chaperone. It functions to ensure correct folding of proteins but is also associated with newly synthesized proteins, including eNOS (Venema et al., 1996). Stimulation of NO production in endothelial cells results in an enhancement of hsp90-eNOS binding, concomitant with NO production. Dynamin-2 is a large GTPase family member involved in trafficking, and has

been shown to colocalize and bind eNOS in the Golgi complex, resulting in the upregulation of eNOS activity. Protein inhibitor of NOS (PIN) is an 89-amino-acid protein that has been found to bind to the N terminus of nNOS, and may inhibit activity or transport by mechanisms unknown (Fan et al., 1998).

There is evidence that iNOS can be regulated at the posttranslational level, and corticosteroids and sodium salicylate are examples (Rao, 2000; Bogdan, 2001). Two novel iNOS-binding proteins, kalirin and NAP110 (NOS-associated protein 110kDa), have been shown to bind iNOS monomers. Thus, they could inhibit iNOS protein function by forming inactive heterodimers. In addition, members of the Rho-like GTPase family, Rac1 and 2, can interact with iNOS to potentiate activity and alter subcellular distribution (Kuncewicz et al., 2001).

## 3.4 Targeting and Trafficking

The regulation of nNOS and eNOS is primarily via intracellular $Ca^{2+}$. The NOS enzymes are localized to particular subcellular domains, and this facilitates selective responses to $Ca^{2+}$ mobilization from specific sources (Bredt, 2003). Discrete localization also positions NOS close to specific downstream targets. All three NOS isoforms possess motifs that enable their targeting to appropriate subcellular sites.

The catalytic function of nNOS in neurons can be activated by $Ca^{2+}$ influx through the NMDA receptor, and these proteins are physically associated via PSD protein scaffolds (Brenman et al., 1996). PSD-95 possesses three PDZ domains that mediate binding of the C-terminal tail of the NMDA receptor subunits and a region in nNOS just C-terminal to its own PDZ domain. The ternary complex so formed is essential for the coupling of $Ca^{2+}$ influx to the generation of NO. The complex also tethers nNOS to the postsynaptic density, thus determining downstream signaling specificity. The nNOS can also bind CAPON, an adaptor protein, by means of the PDZ domain, and this targets nNOS to the nerve terminal where nNOS-bound CAPON interacts with synapsin I (Jaffrey et al., 2002). Saitoh et al. (2004) have recently identified a novel nNOS-interacting DHHC domain-containing protein (NIDD) that targets nNOS to the synaptic membrane. The interaction is via C-terminal binding to the nNOS PDZ domain. The phosphoprotein CtBP (carboxyl-terminal binding protein) has also been demonstrated to bind the nNOS PDZ domain, resulting in a more cytoplasmic localization of nNOS (Riefler and Firestein, 2001).

In a similar manner the human iNOS protein has been shown to interact with the PDZ domains of EBP50 (ezrin–radixin–moesin (ERM)-binding phosphoprotein 50) via a C-terminal S-A-L motif (Glynne et al., 2002). This association promotes NOS-2 targeting to the apical domain of epithelial cells, within a submembranous protein complex tightly bound to cortical actin. This localization of iNOS is believed to direct the production of NO in an appropriate "vectorial" fashion. In addition, iNOS is palmitoylated at Cys-3, and this is required both for activity and for appropriate intracellular localization (Navarro-Lerida et al., 2004).

The N-terminal myristoylation and palmitoylation of eNOS subcellularly targets the protein to membranes. Myristoylation is essential for membrane targeting and maintaining membrane association, whereas palmitoylation appears to direct trafficking of eNOS from the Golgi complex to the plasma membrane of endothelial cells, where it is localized within specialized caveolae (Shaul, 2002). In these lipid-rich domains, Caveolin-1, the scaffolding protein of caveolae (cav-1 in brain), holds eNOS in a tonically inhibited state via binding to an eNOS consensus site (García-Cardeña et al., 1996). Following agonist stimulation of receptors, eNOS dissociates and translocates to the cytosol in a $Ca^{2+}$-dependent process, which may be mediated via enzymatic depalmitoylation of eNOS. Prolonged incubation with agonists causes subsequent relocation of eNOS to the cell membrane, invoking the involvement of some eNOS recycling mechanism. Two novel proteins that interact with eNOS and affect subcellular localization have been identified: NOSIP (eNOS interacting protein) binds to the eNOS oxygenase domain and lowers activity, possibly by relocating eNOS away from caveolae; NOSTRIN (eNOS traffic inducer) binds eNOS via a C-terminal SH3 domain, translocates eNOS away from the plasma membrane, and reduces activity (Dedio et al., 2001).

### 3.5 Ubiquitination

Selective proteolytic degradation is a complex regulatory mechanism by which proteins are targeted for destruction within the cell. This can involve covalent modification of the target protein, via conjugation of one or more ubiquitin molecules, to be degraded by the large multi-subunit cellular protease, 26 S proteasome. The degradation of NOS is regulated by many factors, including corticosteroids and neurotoxins, which may involve the proteasomal pathway, or alternatively an upregulation of calpain can cause proteolytic cleavage (Osawa et al., 2003). The proteasomal degradation of nNOS can be induced either by inactivation via the active process of ubiquitination or by inhibition of the hsp90 chaperone system that is necessary for correct cellular localization. It appears to be the heme-deficient nNOS monomer that is preferentially ubiquitinated. Dunbar et al. (2004) further demonstrated that metabolism-based inactivation of nNOS can lead to targeting for ubiquitination and proteasomal degradation. Inactivating substances can cause covalent alteration of nNOS leading to accelerated degradation, and one example is the loss of the heme group leading to the formation of the "apo-nNOS" monomer. The key prerequisite for the ubiquitination proteasome pathway is in fact destabilization of the nNOS dimers, rather than inactivation per se. BH4 modification, as well as heme alteration, has been implicated as a possible mechanism for proteasomal targeting of NOS. It is envisaged that any event resulting in inhibition of dimerization, such as the depletion of cofactors, could lead to enhanced proteasomal degradation (Osawa et al., 2003).

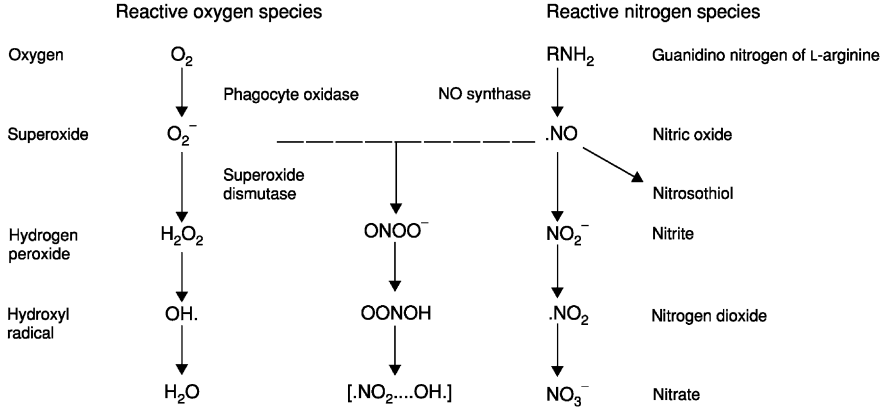
The iNOS protein can also be proteasomally degraded (Musial and Eissa, 2001), and in fact the proteasome can additionally upregulate iNOS at the transcriptional level via degradation of the constitutive inhibitor of NF-κB, IκB (Griscavage et al., 1996). Ubiquitination does not only serve the function of marking a protein for degradation, and nor are all proteasomally degraded proteins first ubiquitinated. However, Kolodziejski et al. (2002) demonstrated that iNOS is indeed ubiquitinated, and that ubiquitination is necessary for degradation.

## 4 Products of NOS Catalysis and Their Molecular Targets

### 4.1 Reactive Nitrogen Species

Reactive nitrogen species (RNS) are oxidation states and adducts of the products of NOS that form in physiological environments. These include NO, nitrogen dioxide ($NO_2$), nitrite ($NO_2^-$), trioxide and tetroxide ($N_2O_3$, $N_2O_4$), S-nitrothiols, peroxynitrite anion ($ONOO^-$), and dinitrosyl-iron complexes (*Figure 11-3*). At low pH, accumulating $NO_2^-$ can be protonated to nitrous acid, which can dismutate

**Figure 11-3**
**Production of reactive oxygen and nitrogen species. Reproduced from Murphy (2005)**



to NO. The NO can react with thiols to yield storage forms that can return an electron-deficient nitrosonium ($NO^+$). Reactive oxygen species are intermediate reduction products of molecular $O_2$ en route to water, namely, $O_2^-$ (superoxide), $H_2O_2$ (hydrogen peroxide), and $OH^-$ (hydroxyl anion). There are three major classes of pro-oxidant enzymes: NOS, cyclooxygenase (COX) and NADPH oxidase, and myeloperoxidase (MPO). It is estimated that between 2% and 5% of electron flow in brain mitochondria produces $O_2^-$ and this is scavenged by superoxide dismutases (SODs), glutathione peroxidase, and catalase. Cellular antioxidants include glutathione, ascorbate, and α-tocopherol. As a radical, NO reacts rapidly with other species that contain unpaired electrons. In reaction with $O_2$, NO can form an intermediate species ($N_2O_3$) that efficiently nitrosates thiols and amines. Outcompeting SOD, NO reacts with $O_2^-$ to produce $ONOO^-$, which if protonated decomposes almost instantly to a hydroxyl radical. This $ONOO^-$ reacts rapidly with $CO_2$ (acting as a catalyst) in a complex manner that produces several short-lived reaction intermediates, such as $NO_2$ and $CO_3^-$. These intermediates are probably responsible for many of the reported toxic effects of NO.

## 4.2 The Arginine Supply

The intracellular availability of arginine is rate-limiting for the production of NO, and nitrite formation is only detected in neurons in vitro if astrocytes are also present, suggesting that these cells form the source of substrate. Immunocytochemical evidence in vivo suggests that arginine is stored in glia and that the NOS coproduct (citrulline) is in neurons (Pow, 1994). This observation has led to the idea that arginine is transferred to the nerve terminal via the y+ cationic amino acid carrier system to replenish the neuronal precursor pool. The localization of aspartate in glia indicates that citrulline is transferred back to these cells and recycled, and citrulline is found only in neurons that express NOS (Arnt-Ramos et al., 1992). Therefore, the supply of substrate appears to regulate NO production. The important question is what prompts arginine transfer from glia (for a review, see Wiesinger, 2001).

The concentration of arginine within cells far exceeds the $K_m$ for NOS, and yet adding arginine to cells can drive enzyme activity (the so-called arginine paradox). There are endogenous NOS inhibitors, such as Nω, Nω-dimethyl-L-arginine, and Nω-methyl-L-arginine, and it could be that there is an exchange of intracellular inhibitors with circulating arginine. Alternatively, compartmentation of NOS and the arginine transporter may be responsible. For example, the enzymes involved in the regeneration of arginine cofractionate with caveolae. There may be two pools of arginine, with one being independent of changes in extracellular arginine. A resolution to this paradox has been proposed by Lee et al. (2003), who found that when activated iNOS is transcribed the uptake of arginine leads to derepression of a translational control mechanism and enhanced levels of iNOS protein. Deprivation of extracellular arginine, or overexpression of intracellular arginase, results in lower intracellular arginine and a decrease in iNOS translation (*Figure 11-4*).

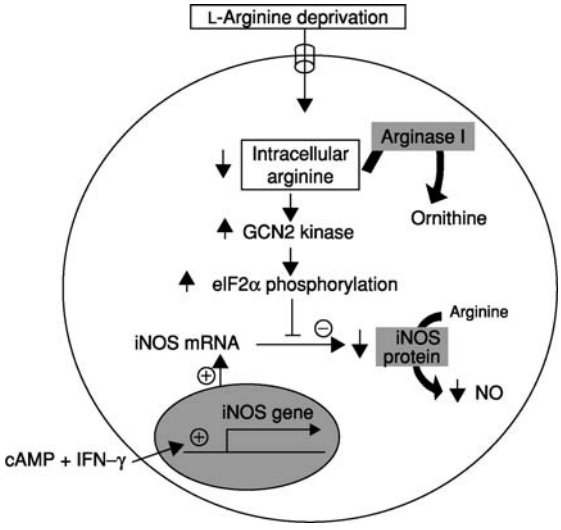
## 4.3 Nitration and S-Nitrosylation

The fastest reaction in biology is that between NO and $O_2^-$ (to form $ONOO^-$), such that it cannot be measured directly. In the presence of $CO_2$, this $ONOO^-$ readily modifies proteins to form nitrotyrosine (NT). In addition, NT can be formed by peroxidation of nitrite and $H_2O_2$ (Hurst, 2002). This posttranslational modification can alter protein function (Ischiropoulos and Beckman, 2003; Radi, 2004). Peroxynitrite does not react directly with tyrosine but oxidizes and nitrates through its radical products. Any significant biological participation of $ONOO^-$ in nitration has been questioned, as the yield of NT is very low in vitro (1–5 residues per 1000), and the reaction is only efficient when fluxes of the precursor radicals NO and $O_2^-$ are equal, which is unlikely.

S-nitrosothiols (SNOs) are produced posttranslationally by the S-nitrosylation of cysteine thiol by NO (Foster et al., 2003). There is a high degree of specificity for a single thiol within each protein target, the identity of which is revealed by consensus motifs ("acid/base" or "hydrophobic"). These SNOs are not

**Figure 11-4**

**A model for translational regulation of iNOS by arginine. Translation is governed by transport of arginine into the cell that charges tRNAs. Reproduced with permission from Lee et al. 2003. Proc Natl Acad Sci USA, 100: 4843–4848. Copyright (2003) National Academy of Sciences, USA**




produced by the direct reaction between NO and thiol but require dioxygen, transition metals, or other relevant electron acceptors. The SNO can also be formed by the reaction of a thiol group with alternative physiological nitrosants. The formation of SNO requires higher concentrations of NO than does the activation of sGC, and has slower kinetics. A variety of readily S-nitrosylated proteins have been identified in brain lysates (Ahern et al., 2002), including ion channels such as the calcium-activated K channel (activity increased), the ryanodine receptor (increased), and the sodium channel (decreased).

## 4.4 Molecular Targets for RNS

NO signaling is crucial for effecting long-lasting changes in cells, including gene expression, cell cycle arrest, apoptosis, and differentiation.

### 4.4.1 DNA

The chemical alteration of DNA underlies a variety of pathological states. NO can potentially damage DNA either through RNS ($ONOO^-$, $N_2O_3$), the inhibition of DNA repair processes, or by increased production of genotoxic (alkylating) agents. A species such as $N_2O_3$ causes mutations in cells, chemically altering DNA. Deamination of cytosine, adenine, and guanine results in conversion to uracil, hypoxanthine, and xanthine, respectively. As well as modification, NO can form or modulate the activity of carcinogens. Nitrosamines are metabolized to alkylating species which lesion DNA.

### 4.4.2 Transcription

The ability to nitrosate and to nitrate key amino acids in proteins (transcriptional regulators, enzymes, receptors) explains many of the actions of RNS, in terms of gene regulation and alterations in cell signaling

pathways. Transcription factors, the regulatory proteins that bind to gene promoters and recruit RNA polymerase, display sequences specific for DNA binding and also a transactivation domain. A number of transcriptional activators have been shown to be regulated by NO, either posttranslationally through direct modification of the protein, or indirect regulation via alteration in the rate of their own transcription. In so doing, NO can turn off constitutively expressed genes, activate transcriptionally regulated genes, or prevent their activation. The best examples to date are the transcription factors NF-κB and AP-1. Their activation can be blocked by NO and the proteins can also be directly nitrosated, with a reduction in promoter association. These two transcription factors are involved in the regulation of expression of a very large number of genes. Initially NO mediates potentiation of NF-κB activity and then, as the concentration of NO increases, it inhibits activity (Connelly et al., 2001). Serine substitution of a cysteine residue at the N-terminal region of the p50 NF-κB subunit reduces DNA binding. Specific binding of NF-κB is also inhibited reversibly by NO, and these effects appear to be mediated by nitrosation of the same cysteine residue. There is also good evidence that NO stabilizes the NF-κB inhibitory protein IκBb and activates transcription, thus inhibiting NF-κB translocation from the cytoplasm to the nucleus. AP-1 is a dimeric complex of Jun-Jun or Jun-Fos proteins. The key to dimer formation resides in two cysteine residues in the leucine zipper and basic regions of Fos and Jun, and nitrosation of cys 252 in the DNA-binding domain decreases AP-1 binding to DNA. HIF 1α, ERK, and p53 are all regulated by distinct threshold concentrations of NO (Thomas et al., 2004). Hemish et al. (2003) have determined the temporal order of gene activation induced by NO in mammalian cells using microarray technology, and describe three distinct waves. The first group of genes are induced within 30 min and represent the primary targets of NO, including the immediate early genes and those for transcription factors. The second wave includes direct targets of such transcription factors. The third wave starts 12 h later and represents the targets of the second group of genes and also reflects changes inherent in the cell cycle arrest status that are induced by NO.

### 4.4.3 Enzymes

Critical signaling proteins can be influenced by NO, functioning at the transcriptional and/or posttranscriptional level either as an activator (poly ADP-ribose synthetase, p21ras, sGC) or as an inhibitor (adenylyl cyclase type I, PKC, cytochrome P450, NOS, lipoxygenase). The mitochondrial respiratory chain is susceptible. For example, NO inhibits cytochrome *c* oxidase (complex IV) in a reaction that is reversible and competitive with oxygen, while $ONOO^-$ irreversibly inhibits respiratory complex I–III as glutathione levels decrease. NO can have diverse effects on cell death, initiating or protecting against apoptosis depending on the cell type, NO concentration, and redox environment. The family of protein-cleaving enzymes known as caspases are targets for NO, which inhibits their activity in a reversible manner. The antiapoptotic effects of NO are also mediated by reversibly inhibiting the permeability transition pore in mitochondria (Beltran et al., 2000; Brookes et al., 2000). NO activates sGC, which contains a prosthetic iron heme group, accelerating the production of cGMP many 100-fold and stimulating PKG activity. Virtually all ion channels have PKG consensus phosphorylation sites. Low levels of NO enhance and high levels block MPO activity through the formation of a nitrosyl complex (Abu-Soud and Hazen, 2000).

The COX enzymes catalyze the conversion of arachidonic acid to biologically active prostanoids. Like NOS, COX exists in both constitutive (COX-1) and inducible (COX-2) isoforms, and their products play roles in both physiological and pathological conditions (O'Banion, 1999). The stimuli and intracellular signaling pathways responsible for promoting COX-2 expression are remarkably similar to those known to upregulate NOS-2 expression, and coinduction has been demonstrated in a number of peripheral cell types and animal models of inflammation (Salvemini, 1997). These studies have pointed to a functional relationship between these enzymes in the initiation, progression, and resolution of the inflammatory response and have prompted investigators to determine whether NO and the prostanoids are involved in the reciprocal regulation of enzyme expression and/or activity. To date, the weight of evidence favors the idea that NO produced by NOS-2 activates COX-2 and thus amplifies the inflammatory response (Salvemini, 1997). The precise mechanism by which NO potentiates COX-2 activity remains to be elucidated. Alone, NO is incapable of increasing the catalytic capacity of COX-2, and certain interactions

should result in enzyme inhibition rather than activation (Goodwin et al., 1999). The most convincing evidence for COX-2 activation by NO involves the generation of $ONOO^-$. Goodwin et al. (1999) suggest that the increased $O_2^-$ levels are provided by enhanced NADPH oxidase activity in inflammatory cells, and that $ONOO^-$ then acts as a peroxidase substrate to oxidize the iron heme moiety in COX-2, thus releasing the tyrosyl radical required for the oxygenation of arachidonic acid.

### 4.4.4 Cytokines

Exposing cells to NO, coupled with the use of NOS inhibitors, has revealed complex effects on the production of cytokines. Some members of the caspase family participate in the maturation of cytokines. The precursors of IL-1β and IL-18 are cleaved by caspase-1, and NO can block this process reversibly, thereby acting as an antiinflammatory agent. However, NO can be proinflammatory, increasing release of other cytokines such as TNFα and IL-6. Chemokines, small chemoattractant cytokines, can be quite specific in determining the immigration patterns of lymphocytes and macrophages to certain inflammatory regions. There appears to be an interesting crosstalk between NO and chemokines in that the expression of IL-8 and MCP-1 are inhibited by NO, whereas MCP-1 can prevent the expression of NOS-2.

### 4.4.5 Membrane Receptors

Excessive activation by glutamate of the NMDA receptor in mature neurons mediates cell death through calcium entry and downstream events, which may or may not involve the activation of NOS-1. In addition, NO in its various redox states is reported to downregulate NMDA receptor activity at modulatory sites consisting of critical cysteine sulfhydryl or thiol groups. The induction of MHC class II by IFNγ is blocked by NO donors, and NO inhibits the induction of adhesion molecules such as ICAM and VCAM-1 via effects on NF-κB.

### 4.4.6 Structural Proteins

The nitration of tyrosine residues in proteins is a convenient marker of reactive nitrogen-centered oxidants being produced. Whether it is $ONOO^-$ or another oxidation product that is responsible for this nitration in vivo is the subject of debate. If these tyrosine residues are sites for regulation via PTK-mediated phosphorylation, then functional activity of the target protein can be affected. Important structural proteins such as neurofilament L show evidence of nitration in diseases of the nervous system. Nitration converts a negatively charged hydrophilic residue and so disrupts assembly into polymers, so important for axonal integrity.

## 5 Role of NOS in CNS Development

While NO is strongly linked to neurodegenerative and protective actions (see below) it has an important role in cell survival and differentiation (Contestabile and Ciani, 2004). The nNOS protein has also been implicated in postnatal development of cerebellar granule cells (Ogilvie et al., 1995), and in regulating the growth and differentiation effects of nerve growth factor and brain-derived neurotrophic factor (Peunova and Enikolopov, 1995). During migration, granule cells display periodic fluctuations of cytoplasmic calcium. At comparable stages of cerebellar development, a similar permissive role toward migration from the external to the internal granular layer is played by NO. Using in vitro slice cultures, it was demonstrated that a NOS inhibitor significantly decreased the migratory index of granule cells (Tanaka et al., 1994). NO can stimulate the growth and branching of dendrites and neurite extension in PC12 cells

(Poluha et al., 1997). Through an ability to nitrosylate, and thus modify proteins such as synaptotagmin and SNAP-25 involved in exocytosis, NO can induce the release of transmitters from vesicular stores (Meffert et al., 1994). Inhibition of NOS-1 activity during postnatal development also results in disturbance of layer formation in the cerebellum (Wang et al., 1998). NO is a negative regulator of neuronal precursor cell proliferation. For example, in mature dorsal root ganglia only 5% of the cells express nNOS, but this increases to 100% after peripheral nerve lesion. Neurons die following axotomy if nNOS is inhibited, or in nNOS(−/−) mice. Inhibition of NO production in cerebellar granule cells or spinal cord motor neurons for 3–4 days results in progressive apoptotic death, which can be rescued by cGMP analogs or NO donors. NO is important in terminal synaptogenesis and neural map formation, and in the earlier stages of neurogenesis. Packer et al. (2003) have observed nNOS-positive cells in brain areas that retain proliferative activity, such as the subventricular zone. If nNOS is blocked then there is an increase of 60–70%. However, NO donors and arginine can also increase precursor populations. Zhang et al. (2001) found that administration of a NO donor to rats increased neurogenesis after cerebral ischemia, and recipients exhibited significant improvements in neurological outcome. Zhu et al. (2003) found that NOS-2(−/−) mice do not display the predicted neurogenic response following ischemia. There is a dominant role for eNOS in both angiogenesis and vasculogenesis (Duda et al., 2004).

## 6 NOS and Homeostatic Functions

### 6.1 Cerebral Circulation

NO is a potent vasodilator of cerebral blood vessels both in vitro and in vivo, either via sGC in vascular muscle or by the activation of potassium channels (Faraci and Heistad, 1998). Although peroxynitrite can also cause relaxation of blood vessels, the concentrations needed are 50- to 1000-fold higher than with NO. Constitutive levels of expression of NOS in endothelium are sufficient to influence tone in cerebral blood vessels under basal conditions. Inhibitors of NOS decrease basal levels of cGMP, cause contraction of cerebral arteries, and decrease cerebral blood flow. These effects are absent in mice deficient in the expression of eNOS, suggesting that endothelium is the primary source of NO that influences vascular tone. Basal activity of eNOS can be further stimulated by acetylcholine to produce endothelium-dependent relaxation, as do a variety of substances (bradykinin, arginine vasopressin, oxytocin, substance P, histamine, endothelin, ADP, ATP, UTP, and prostaglandin $F_{2\alpha}$). Basic fibroblast growth factor and some opioids also produce NO-dependent dilatation of cerebral vessels in vivo that is presumably endothelium-dependent. The promoter of the iNOS gene contains shear-stress and hypoxia response elements, suggesting that expression is modulated by blood flow and oxygen tension. Although expression of iNOS is generally associated with inflammatory or pathophysiological conditions, it appears that the gene may be active during development. For example, mRNA and protein for inducible NOS have been detected in parenchymal microvessels in brain during normal embryonic development and in the newborn. The significance is not clear, but may relate to vascular remodeling.

### 6.2 Neuronal Excitability

Through cGMP and S-nitrosylation, NO can expand and enrich neuronal excitability. Modulation of voltage-gated channels allows NO and cGMP to alter the firing behavior of a neuron. Modulation of ligand-gated channels can alter postsynaptic responses, and modulation of voltage-gated channels alters neurosecretion (Ahern et al., 2002). NO acts as a neurotransmitter for nonadrenergic, noncholinergic (NANC) synapses found in peripheral tissues. Activity of the NANC innervation of the respiratory tract is a component of ventilation/perfusion matching. The NO generated by nitrergic innervation and the respiratory epithelium mediates the relaxation of the bronchiolar and vascular smooth muscle. In humans, while stimulation of NOS-containing neurons of the pelvic plexus results in the vasodilation of cavemosum vessels and penile erection, modulation by NANC neurons results in the relaxation of uterine smooth

muscle during pregnancy. Gastrointestinal peristalsis is regulated in part by NO release from neurons located in the submucosa and myenteric plexus that stimulates smooth muscle relaxation. Intestinal absorption and secretion of fluid and electrolytes may be mediated in a similar NANC–NO-dependent fashion.

The neurotransmitter activity of NO may be involved in long-term potentiation (LTP), a model of learning and memory in the hippocampus. The process of LTP is initiated by the activation of postsynaptic NMDA receptors with the subsequent production of NO.NO produced by the postsynaptic neuron acts as a retrograde messenger activating sGC in the presynaptic neuron and effecting changes in the amount, or ease of release of, neurotransmitter during a future depolarization. This NO signaling from post- to presynaptic neuron results in an increase in the synaptic strength or efficiency. Glutamate, AMPA receptor activation, and NO are components of long-term depression (LTD), a similar model of learning and memory in the cerebellum. In the model of LTD, however, NO acts as an anterograde messenger and synaptic strength and efficiency are reduced.

## 7 NOS in CNS Pathology/Recovery of Function

Roles for the NOS enzymes have been invoked in a variety of neuropathologies. Here we focus on a few as examples of degeneration, acute injury, and infection.

### 7.1 Degeneration

#### 7.1.1 Multiple Sclerosis

Inflammation is associated with demyelinating disease, and the generation of RNS is increased dramatically in conditions involving inflammation. Oligodendrocytes are particularly sensitive to nitrative stress and reactive species can damage the myelin sheath directly, promoting the attention of macrophages. In active multiple sclerosis (MS) lesions, numerous cell types express NOS-2, and elevated levels of CSF $NO_3^-$ correlate with clinical relapse. Calabrese et al. (2002) report iNOS and NOS activity in the CSF from patients with MS, and detect NT immunostaining of CSF proteins. There is also evidence for nitrosylation (Boullerne et al., 2002).

Associated with increased proinflammatory gene expression in an experimental model of MS (experimental autoimmune encephalomyelitis, EAE) is expression of iNOS mRNA, protein, and evidence for the generation of RNS. There is also evidence for the formation of NT and nitroso-S-cysteine (Boullerne et al., 2002), indicating interaction of RNS with proteins. Antiinflammatory steroids block cytokine-mediated NO production and reduce EAE symptoms, NOS inhibitors reduce passively transferred EAE, encephalitogenic T cells cause macrophages to produce NO, and NOS-2 expression correlates with disease severity. However, a study of the therapeutic effects of different NOS inhibitors in both adoptive transfer and actively induced EAE revealed no protection, and some exacerbation of disease (Zielasek et al., 1995). With one of the first iNOS-selective inhibitors (L-NIL), Gold et al. (1997) found protection in adoptive EAE and exacerbation of actively induced disease. However, the emergence of iNOS knockout mice and the observation that NO is important in recovery and refractoriness to active reinduction point to the potential protective role of NO in actively induced EAE. There is now abundant evidence that NO is beneficial, or that NOS-2 is not necessarily harmful (for a review, see Murphy, 2005).

It is well known that NO has a number of effects on immune responses that would prevent T cell expansion. This appears to be a specific impairment of Th1, while sparing Th2 cells. Since EAE is a function of Th1 cells, the increase in NO may selectively limit proliferation of the encephalitogenic effector population. In addition, expression of VCAM and ICAM-1 are downregulated by NO, significantly altering lymphocyte migration. In target tissue, NO can induce both apoptosis and necrosis in T effector cells and protect oligodendrocytes against destruction by lipid peroxidation. There is a destructive role for $ONOO^-$ in MS and EAE, based on the observation that a natural antioxidant scavenger, uric acid (UA), ameliorates

disease. The normal level of UA in humans confers enhanced protection against free radical-mediated cell injury, and low UA may predispose toward the development of CNS diseases involving $ONOO^-$, which include amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD). Some of the novel $ONOO^-$ scavengers, mercaptoethylguanidines, readily cross the blood–brain barrier (BBB) (Scott et al., 2001) and delay the onset and reduce the incidence of MBP-induced EAE. Surprisingly, these scavengers do not alter the severity of disease or overall mortality, but this could be due to the lower efficiency with which these compounds inactivate $ONOO^-$ as compared with UA. Alternatively, it could be because these scavengers also inhibit NOS-2 activity, removing the protective influence of NO.

### 7.1.2 Amyotropic Lateral Sclerosis

This results from the progressive death of motor neurons, leading to rapid muscle degeneration and progressive paralysis. Nearly a hundred different mutations have been identified in the SOD1 locus encoding cytosolic Cu/Zn superoxide dismutase. Transgenic mice and rats expressing mutant SOD1 develop motor neuron disease, and there is lipid peroxidation and nitrotyrosine in transgenic mice and in humans. Although those mutant Cu/Zn SODs causing the most rapid forms of ALS form functional proteins with normal SOD activity, they are clearly less stable, rapidly oxidize many intracellular antioxidants like ascorbate and thiols, and operate in reverse to generate $ONOO^-$. However, knocking out the gene for the copper chaperone for Cu/Zn SOD (CCS) was found to not affect disease development in transgenic mice overexpressing mutant Cu/Zn SODs (Subramaniam et al., 2002). While the zinc-deficiency hypothesis can explain the role of Cu/Zn SOD in promoting the death of motor neurons, the disease is progressive and causes the death of the few hundred thousand neurons that control all voluntary muscle contraction. Recently, $ONOO^-$ has been shown to provoke a long-lasting reactive phenotype in spinal cord astrocytes, and astrocytes can cause motor neurons in coculture to undergo apoptosis (Cassina et al., 2002). A consistent finding in ALS spinal cord, and in transgenic mice, is an extremely strong immunoreactivity for NT associated with reactive astrocytes. By provoking surrounding astrocytes, a cluster of dying motor neurons may initiate the progressive death of neighbor motor neurons (Ischiropoulos and Beckman, 2003).

### 7.1.3 Parkinson's Disease

The degenerative condition illustrates how RNS can be central to but not an initiating event of (Hirsch and Hunot, 2000). The initiating event may include mutations of α-synuclein or parkin, responsible for familial PD. Using of antibodies directed against nitrated α-synuclein reveals that most Lewy bodies and protein inclusions contain nitrated α-synuclein, making these resistant to proteolysis and promoting insoluble aggregates (Giasson et al., 2000). The loss of dopamine-containing neurones in the substantia nigra is associated with astrogliosis and activated microglia. Liberatore et al. (1999) suggest that NO produced by glial cells participates in the cascade of events that leads to the degeneration of neurones in mice that are rendered parkinsonian, as this was reduced by 50% when the production of NO by glial cells was abolished. Data obtained from several post-mortem studies of patients are compatible with the hypothesis that NO, secreted by glia, participates in pathophysiology. It is possible that a primary neuronal injury induces activation of glial NO, which plays a deleterious role in dopamine-containing neurones. A time-limited insult to the nigrostriatal system can set in motion a self-perpetuating process of neurodegeneration that involves glial cells. The mechanism by which NO could be involved in the degeneration of dopamine-containing neurones is not known but there are several possible explanations. NO alters iron homeostasis, releasing iron from ferritin and leading to the accumulation of free iron, which can induce oxidative damage to cells. In addition, NO interferes with the regulation of transferrin receptor mRNA and ferritin mRNA, which prevents a further rise in the concentration of iron. Thus, an increase in the concentration of NO in the parkinsonian substantia nigra might result in the higher concentrations of iron that are observed in this disease (for review see Hirsch and Faucheux, 1998).

## 7.2 Acute Injury

### 7.2.1 Cerebral Ischemia

This invokes excitotoxicity, inflammation, cell death, and neurogenesis (Danton and Dietrich, 2003), and there is an increase in the generation of NO from activation of NOS in both resident and infiltrating cells. Experimental cerebral ischemia leads to the upregulation of all three isoforms of NOS, although the patterns of expression differ both temporally and spatially post injury. The classic gene knockout studies in acute experimental stroke indicate that the activation of nNOS is detrimental, while the NO derived from eNOS is beneficial (Chan, 2001).

The expression of iNOS is induced in both resident and infiltrating cells in response to experimental cerebral ischemia (Gibson et al., 2005) and in stroke in humans (Forster et al., 1999). This induction occurs at a later time point than that of either nNOS or eNOS, suggesting that iNOS does not contribute to the early stages of pathology. Indeed, mice lacking a functional iNOS gene do not show alterations in infarct volume compared with wild-type mice when measured 24 h after permanent occlusion of a middle cerebral artery (Iadecola et al., 1997; Loihl et al., 1999). At later time points, iNOS-deficient male mice do show a significant reduction in infarct volume, and there is a gene-dosing effect (Zhao et al., 2000). Furthermore, the infusion of arginine increases ischemic injury in wild-type but not in iNOS-deficient mice (Zhao et al., 2003). An increase in iNOS protein is also found in glia following ischemic proliferative retinopathy, a major cause of blindness, and the NO induces local apoptosis and protein nitration (Sennlaub et al., 2001, 2002).

There are significant increases in IL-1β and TNFα mRNA expression within a few hours of ischemia (Schroeter et al., 2003). On the basis of in vitro observations it is assumed that these cytokines trigger transcriptional activation of the iNOS gene, and direct injection of IL-1β into the cerebral ventricles in the absence of injury does indeed lead to upregulation of iNOS expression along the injection tract (Lopez-Figueroa et al., 2000). The iNOS promoter contains a hypoxia response element and Matrone et al. (2004) provide compelling evidence that HIF-1α can also activate the gene following ischemia. However, transcriptional activation following ischemia may not account for the very rapid appearance of iNOS-positive cells infiltrating the infarct, and a more likely explanation is that these cells express iNOS mRNA constitutively.

While the gene knockout studies in experimental stroke suggest that NO produced by nNOS and iNOS is detrimental and that derived from eNOS is beneficial, studies employing various NOS inhibitors have given conflicting results for effects on lesion size and cerebral blood flow (Willmot and Bath, 2003). Collectively, NOS inhibitors caused a significant reduction in total, cortical, and subcortical infarct volume. Treatment before stroke onset was effective at reducing infarct volume in transient models, while early administration of NOS inhibitors (<1 h of onset) was effective in permanent stroke. Later treatment (<1 h of onset) had a beneficial effect on infarct volume in both types of stroke model. Nonselective inhibitors did not alter infarct volume in permanent ischemia, whereas the "selective" nNOS and iNOS inhibitors reduced lesion size regardless of experimental model. It is likely that the beneficial effects of nonselective inhibitors were limited because they inhibit NOS-3 to a similar degree. Consequently, they may aggravate brain ischemia by increasing platelet aggregation and white cell activity, raising blood pressure, and by restricting penumbral blood supply. Evidence of reduced cerebral blood flow after administration of nonselective inhibitors to permanent stroke models is consistent with this hypothesis. Hence, nonselective inhibitors are not agents of first choice for testing in clinical stroke.

Exogenously applied arginine appears to increase levels of NO partly by the NOS pathway, but also via the release of other vasoactive and arginase enzymes (Kaposzta et al., 2001). However, there are conflicting results as arginine has been shown to decrease, have no effect, or even increase infarct volume. Similar conflicting results have occurred when investigating the effect of arginine on cerebral blood flow which, if enhanced, may rescue salvageable tissue from the spreading ischemic core. This conflicting evidence may be due to the ability of arginine to enhance NO from all three isoforms of NOS. Many NO donors have beneficial effects following experimental ischemia, albeit within a relatively short therapeutic time window. Unopposed high doses of NO donors might be detrimental due to their profound vasodepressant effect, as

seen in a recent study where low doses of sodium nitroprusside were neuroprotective while high doses were neurotoxic (Bath et al., 2002).

There is an increase in the rate of neurogenesis following ischemic brain injury. These new cells arise in the dentate gyrus and subventricular zone, and populate subcortical and to a lesser extent cortical structures. While NO has been reported to be cytostatic, or to promote terminal differentiation of neural stem cells in the uninjured brain (Packer et al., 2003), it appears to be antiapoptotic in the ischemic brain. Zhang et al. (2001) have shown that administration of a NO donor to rats increased neurogenesis after middle cerebral artery occlusion (MCAO), and recipients exhibited significant improvements in neurological outcome. Furthermore, administration of a NO donor in combination with marrow stromal cells significantly enhanced angiogenesis and neurogenesis following cerebral ischemia compared with either treatment alone, and significantly improved functional outcome (Chen et al., 2004). Finally, Zhu et al. (2003) have observed that iNOS(−/−) mice do not display the predicted neurogenic response in the dentate gyrus following ischemia. The mechanism is unknown, but it could be that the resultant inflammatory reaction proceeds unchecked in the absence of iNOS-derived NO.

### 7.2.2 Traumatic Brain Injury

Traumatic injury initiates multiple processes involving resident and infiltrating cells. After traumatic insult, proinflammatory cytokines such as TNFα and IL-1β are rapidly activated. Among other things, these promote expression of iNOS in infiltrating cells and in resident glia, leading to the local production of NO. Disruption of the BBB and subsequent infiltration of circulating immunocompetent cells into the brain parenchyma contribute to inflammation and edematous swelling. Secondary damage occurs through necrotic and apoptotic cell death, leading to neuronal degeneration and loss of brain function, and ultimately physical disability. Interfering with the secondary damage cascade, over which NO has a large influence, could lead to reduced tissue loss and limited disruption of neurological function. Expression of iNOS is reported both in human casualties and in animal models of traumatic brain or spinal cord injury. The detrimental effect of NO produced by iNOS could result from the production of $ONOO^-$. Edema formation is a common trait of traumatic brain injury (TBI) contributing to brain damage, and can be visualized and measured by various techniques such as magnetic resonance imaging (MRI). The extent of edema formation is dependent on the severity of injury, but not on the ability of mice to express iNOS, since no difference in the volume of edema exists between iNOS(−/−) and wild-type mice (Jones et al., 2004). Following TBI, wild-type mice showed a decreased learning ability to locate a hidden platform in the Morris water maze. The iNOS(−/−) mice retained their ability to learn to locate a hidden platform after repeated trials in the Morris water maze task, suggesting functional preservation of cortical/hippocampal neurons after injury. Protection also correlates with a decrease in contralateral limb errors when compared with wild-type animals.

## 7.3 Infection

A common host response to the presence of virulence factors is the transcriptional activation of iNOS, resulting in persistent production of high levels of NO (Nathan, 1997).

### 7.3.1 Meningitis

Meningitis caused by the human pathogen *Neisseria meningitidis* is associated with a relatively high (5–15 %) mortality among children and adults, and a further 10% of those who survive suffer from long-lasting neurological sequelae. In a small percentage of people who harbor meningococcus in the nasopharyngeal mucosa, the bacteria enter the bloodstream to cause a transient bacteremia. This can result in septicemia, or the bacteria may cross the blood–brain and blood–CSF barriers. The disease is rapid at onset, has a

fulminant course in some individuals, and requires rapid antibiotic treatment. The intense host response (edema, increased intracranial pressure, altered cerebral flow) reflects functional alterations in BBB, implying changes in the properties of their constituent cell types (Nau and Bruck, 2002).

There is evidence to support a role for NO in the pathophysiology of meningitis. Patients, and also experimental animals with meningitis, have elevated levels of nitrite in cerebrospinal fluid, and the changes in cerebral blood flow seen early in pathogenesis appear to be mediated by NO (Faraci and Heistad, 1998). This NOS-2 response in the host, while antimicrobial, may also contribute to the ensuing pathophysiology via a bystander effect. The sites and mechanisms by which meningococcus gains entry to the blood, and then the CNS, are starting to become clear (Nassif et al., 2002). Adhesion is an essential step in meningococcal infection. Bacteria first adhere through pili to CD46, an ERM-binding protein, and overexpressing CD46 in mice makes them highly susceptible to meningococcal disease (Johansson et al., 2003). Adhesion of meningococcus is associated with the formation of cortical plaques, which result from the polymerization of actin and recruitment of ezrin and other proteins. This is then followed by elongation of host cell microvilli toward the bacteria, leading to their engulfment and internalization. The human NOS-2 protein has been shown to interact with the PDZ domains of EBP50 (EBP-binding phosphoprotein 50) via a C-terminal S-A-L motif (Glynne et al., 2002). This association promotes NOS-2 targeting to the apical domain of epithelial cells, within a submembranous protein complex tightly bound to cortical actin. This localization of iNOS is believed to direct the production of NO in an appropriate "vectorial" fashion. While meningococcus clearly elicits NO production from cells of host epithelium and endothelium, the bacterium must, in some way, be able to evade the potentially toxic effects of NO. Anjum et al. (2002) have suggested that meningococcus can express NO and nitrite reductases, which would confer protection against nitrosative stress. Another possibility is that through direct interaction of meningococcus with barrier cells, the bacterium modifies the iNOS response and reduces exposure to toxic levels of NO.

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## References

Abu-Soud HM, Hazen SL. 2000. Nitric oxide modulates the catalytic activity of myeloperoxidase. J Biol Chem 275: 5425-5430.

Ahern GP, Klyachko VA, Jackson MB. 2002. cGMP and *S*-nitrosylation. Trends Neurosci 25: 510-517.

Aktan F. 2004. iNOS-mediated nitric oxide production and its regulation. Life Sci 75: 639-653.

Alderton WK, Cooper CE, Knowles RG. 2001. NO synthase: structure, function and inhibition. Biochem J 357: 593-615.

Anjum MF, Stevanin TM, Read RC, Moir JWB. 2002. Nitric oxide metabolism in *Neisseria meningitidis*. J Bacteriol 184: 2987-2993.

Arnt-Ramos LR, O'Brien WE, Vincent SR. 1992. Immunohistochemical localization of arginosuccinate synthetase in the rat brain in relation to NOS-containing neurons. Neuroscience 51: 773-789.

Baek KJ, Thiel BA, Lucas S, Stuehr DJ. 1993. Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. J Biol Chem 268: 21120-21129.

Bath PMW, Beech J, Williams S, Meldrum B. 2002. Dose-dependent neuroprotection and toxicity with the nitric oxide donor sodium nitroprusside in a model of transient ischaemic stroke in the rat. Cerebrovasc Dis 13: 15-30.

Beltran B, Mathur A, Duchen MR, Erusalimsky JD, Moncada S. 2000. The effect of nitric oxide on cell respiration. Proc Natl Acad Sci USA 97: 14602-14607.

Bogdan C. 2001. Nitric oxide and the immune response. Nat Immunol 2: 907-916.

Boullerne AI, Rodriguez JJ, Touil T, Brochet B, Schmidt S, et al. 2002. Anti-*S*-nitrosocysteine antibodies are a

predictive marker for demyelination in experimental autoimmune encephalomyelitis: implications for multiple sclerosis. J Neurosci 22: 123-132.

Bredt DS. 2003. Nitric oxide signaling specificity. J Cell Sci 116: 9-15.

Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, et al. 1991. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature 351: 714-718.

Bredt DS, Snyder SH. 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci USA 87: 682-685.

Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, et al. 1996. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell 84: 757-767.

Brookes PS, Salkinas EP, Darley-Usmar K, Eiserich JP, Freeman BA, et al. 2000. Concentration-dependent effects of NO on mitochondrial permeability transition and cytochrome *c* release. J Biol Chem 275: 20474-20479.

Brune B, Lapetina EG. 1991. Phosphorylation of nitric oxide synthase by PKA. Biochem Biophys Res Commun 181: 921-926.

Calabrese V, Scapagnini G, Ravagna A, Bella R, Foresti R, et al. 2002. NO synthase is present in the CSF of patients with active MS and is associated with increases in CSF protein nitrotyrosine and *S*-nitrosothiols with changes in glutathione levels. J Neurosci Res 70: 580-587.

Cassina T, Peluffo H, Pehar M, Martinez-Palma L, Ressia A, et al. 2002. Peroxynitrite triggers a phenotypic transformation in spinal cord astrocytes that induces motor neuron apoptosis. J Neurosci Res 67: 21-29.

Chan PH. 2001. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab 21: 2-14.

Chen J, Li Y, Zhang R, Katakowski M, Gautam SC, et al. 2004. Combination therapy of stroke in rats with a nitric oxide donor and human bone marrow stromal cells enhances angiogenesis and neurogenesis. Brain Res 1005: 21-28.

Chen PF, Tsai AL, Wu KK. 1995. Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. Biochem Biophys Res Commun 215: 1119-1129.

Cirino G, Fiorucci S, Sessa WC. 2003. Endothelial nitric oxide synthase: the Cinderella of inflammation? Trends Pharmacol Sci 24: 91-95.

Connelly L, Palacios-Callender M, Ameixa C, Moncada S, Hobbs AJ. 2001. Biphasic regulation of NF-κB activity underlies the pro- and anti-inflammatory actions of NO. J Immunol 166: 3873-3881.

Contestabile A, Ciani E. 2004. Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation. Neurochem Int 45: 903-914.

Danton GH, Dietrich WD. 2003. Inflammatory mechanisms after ischemia and stroke. J Neuropathol Exp Neurol 62: 127-136.

Dedio J, Konig P, Wohlfart P, Schroeder C, Kummer W, et al. 2001. NOSIP, a novel modulator of endothelial nitric oxide synthase activity. FASEB J 15: 79-89.

Duda DG, Fukumura D, Jain RK. 2004. Role of eNOS in neovascularization. Trends Mol Med 10: 143-145.

Dunbar AY, Kamada Y, Jenkins GJ, Lowe ER, Billecke SS, et al. 2004. Ubiquitination and degradation of neuronal nitric-oxide synthase in vitro. Mol Pharmacol 66: 964-969.

Eissa NT, Strauss AJ, Haggerty CM, Choo EK, Chu SC, et al. 1996. Alternative splicing of human inducible nitric-oxide synthase mRNA. J Biol Chem 43: 27184-27187.

Eissa NT, Yuan JW, Haggerty CM, Choo EK, Palmer CD, et al. 1998. Cloning and characterisation of human inducible nitric oxide synthase splice variants. Proc Natl Acad Sci USA 95: 7625-7630.

Elfering SL, Sarkela TM, Giulivi C. 2002. Biochemistry of mitochondrial nitric-oxide synthase. J Biol Chem 277: 38079-38086.

Fan J-S, Zhang Q, Li M, Tochio H, Yamazaki T, et al. 1998. Protein inhibitor of neuronal nitric oxide synthase, PIN, binds to a 17 amino acid residue fragment of the enzyme. J Biol Chem 273: 33472-33481.

Faraci FM, Heistad DD. 1998. Regulation of the cerebral circulation. Physiol Rev 78: 53-97.

Fischmann TO, Hruza A, Niu XD, Fossetta JD, Lunn CA, et al. 1999. Structural characterization of nitric oxide synthase isoforms reveals striking active site conservation. Nat Struct Biol 6: 233-242.

Forster C, Clark HB, Ross ME, Iadecola C. 1999. Inducible nitric oxide synthase expression in human cerebral infarcts. Acta Neuropathol 97: 215-220.

Förstermann U, Boissel J-P, Kleinert H. 1998. Expressional control of the constitutive isoforms of nitric oxide synthase. FASEB J 12: 773-790.

Foster MW, McMahon TJ, Stamler JS. 2003. *S*-nitrosylation in health and disease. Trends Mol Med 9: 160-168.

Furchgott RF, Zawadski JV. 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288: 373-376.

García-Cardeña G, Fan R, Stern DF, Liu J, Sessa WC. 1996. Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. J Biol Chem 271: 27237-27240.

Garthwaite J, Charles SL, Chess-Williams R. 1988. Endothelium-derived relaxing factor release on activation of NMDA

receptors suggests role as intercellular messenger in the brain. Nature 336: 385-388.

Ghosh DK, Stuehr DJ. 1995. Macrophage NO synthase: characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction. Biochemistry 34: 801-807.

Ghosh S, Wolan D, Adak S, Crane BR, Kwon NS, et al. 1999. Mutational analysis of the tetrahydrobiopterin-binding site in inducible nitric-oxide synthase. J Biol Chem 274: 24100-24112.

Giasson BI, Duda JE, Murray IVJ, Chen Q, Souza JM, et al. 2000. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. Science 290: 985-989.

Gibson CL, Coughlan TC, Murphy S. 2005. Glial nitric oxide and ischemia. Glia 15: 417-426.

Glynne PA, Darling KE, Picot J, Evans TJ. 2002. Epithelial inducible nitric-oxide synthase is an apical EBP50-binding protein that directs vectorial nitric oxide output. J Biol Chem 277: 33132-33138.

Gold DP, Schroder K, Powell HC, Kelly CJ. 1997. Nitric oxide and the immunomodulation of EAE. Eur J Immunol 27: 2863-2869.

Gonzalez E, Kou R, Lin AJ, Golan DE, Michel T. 2002. Subcellular targeting and agonist-induced site-specific phosphorylation of endothelial nitric-oxide synthase. J Biol Chem 277: 39554-39560.

Goodwin DC, Landino LM, Marnett LJ. 1999. Effects of nitric oxide and nitric oxide-derived species on prostaglandin endoperoxide synthase and prostaglandin biosynthesis. FASEB J 13: 1121-1136.

Green LC, Tannenbaum SR, Goldman P. 1981. Nitrate synthesis in the germfree and conventional rat. Science 212: 56-58.

Griscavage JM, Wilk S, Ignarro LJ. 1996. Inhibitors of the proteosome pathway interfere with induction of nitric oxide synthase in macrophages by blocking activation of NF-κB. Proc Natl Acad Sci USA 93: 3308-3312.

Hayashi Y, Nishio M, Naito Y, Yokokura H, Nimura Y, et al. 1999. Regulation of neuronal nitric oxide synthase by calmodulin kinases. J Biol Chem 274: 20597-20602.

Hemish J, Nakaya N, Mittal V, Enikolopov G. 2003. Nitric oxide activated diverse signalling pathways to regulate gene expression. J Biol Chem 278: 42321-42329.

Hemmens B, Goessler W, Schmidt K, Mayer B. 2000. Role of bound zinc in dimer stabilization but not enzyme activity of neuronal nitric-oxide synthase. J Biol Chem 275: 35786-35791.

Hirsch EC, Faucheux BA. 1998. Iron metabolism and Parkinson's disease. Mov Disord 13 (Suppl. 1): 39-45.

Hirsch EC, Hunot S. 2000. Nitric oxide, glial cells and neuronal degeneration in parkinsonism. Trends Pharmacol Sci 21: 163-165.

Hurst JK. 2002. Whence nitrotyrosine? J Clin Invest 109: 1287-1289.

Iadecola C, Zhang F, Casey R, Nagayama M, Ross ER. 1997. Delayed reduction of ischemic brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. J Neurosci 17: 9157-9164.

Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA 84: 9265-9269.

Ischiropoulos H, Beckman JS. 2003. Oxidative stress and nitration in neurodegneration. J Clin Invest 111: 163-169.

Jaffrey SR, Benfenati F, Snowman AM, Czernik AJ, Snyder SH. 2002. Neuronal nitric oxide synthase localized by a ternary complex with synapsin and CAPON. Proc Natl Acad Sci USA 99: 3199-3204.

Janssens SP, Shimouchi A, Quertermous T, Bloch DB, Bloch KD. 1992. Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. J Biol Chem 267: 14519-14522.

Johansson L, Rytkonen A, Bergman P, Albiger B, Kallstrom H, et al. 2003. CD46 in meningococcal disease. Science 301: 373-375.

Jones NC, Constantin D, Gibson CL, Prior MJW, Morris PG, et al. 2004. A detrimental role for NOS-2 in the pathology resulting from acute cerebral injury. J Neuropathol Exp Neurol 63: 708-720.

Kaposzta Z, Baskerville PA, Madge D, Fraser S, Martin JF, et al. 2001. L-Arginine and *S*-nitrosoglutathione reduce embolization in humans. Circulation 103: 2371-2375.

Kim YM, Ko CB, Park YP, Kim YJ, Paik SG. 1999. Octamer motif is required for the NF-κB-mediated induction of the inducible nitric oxide synthase gene expression in RAW 264.7 macrophages. Mol Cell 28: 99-109.

Kolodziejski PJ, Musial A, Koo J-S, Eissa NT. 2002. Ubi-quitination of inducible nitric oxide synthase is required for its degradation. Proc Natl Acad Sci USA 99: 12315-12320.

Kuncewicz T, Balakrishnan P, Snuggs MB, Kone BC. 2001. Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages. Am J Physiol Renal Physiol 28: F326-F336.

Lamas S, Marsden PA, Li GK, Tempst P, Michel T. 1992. Endothelial nitric oxide synthase. Proc Natl Acad Sci USA 89: 6348-6352.

Lee CM, Robinson LJ, Michel T. 1995. Oligomerization of endothelial nitric oxide synthase. Evidence for a dominant negative effect of truncation mutants. J Biol Chem 270: 27403-27406.

Lee J, Ryu H, Ferrante RJ, Morris Jr, SM Ratan RR. 2003. Translational control of inducible nitric oxide synthase

expression by arginine can explain the arginine paradox. Proc Natl Acad Sci USA 100: 4843-4848.

Li H, Brodsky S, Basco M, Romanov V, De Angelis DA, et al. 2001. Nitric oxide attenuates signal transduction: possible role in dissociating caveolin-1 scaffold. Circ Res 88: 229-236.

Liberatore GT. 1999. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. Nat Med 5: 1403-1409.

Loihl AK, Asensio V, Campbell IL, Murphy SP. 1999. Expression of nitric oxide synthase (NOS)-2 following permanent focal ischemia and the role of nitric oxide in infarct generation in male, female and NOS-2 gene-deficient mice. Brain Res 830: 155-164.

Lopez-Figueroa MO, Day HEW, Lee S, Rivier C, Akil H, et al. 2000. Temporal and anatomical distribution of NOS mRNA expression and NO production during CNS inflammation. Brain Res 852: 239-246.

Lowenstein CJ, Glatt CS, Bredt DS, Snyder SH. 1992. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. Proc Natl Acad Sci USA 89: 6711-6715.

Luss H, Disilvio M, Litton AL, Vedia LMY, Nussler AK, et al. 1994. Inhibition of nitric oxide synthesis enhances the expression of inducible nitric oxide synthase mRNA and protein in a model of chronic liver inflammation. Biochem Biophys Res Commun 204 (2): 635-640.

Lyons CR, Orloff GJ, Cunningham JM. 1992. Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. J Biol Chem 267: 6370-6374.

Marsden PA, Schappert KT, Chen HS, Flowers M, Sundell CL, et al. 1992. Molecular cloning and characterization of human endothelial nitric oxide synthase. FEBS Lett 307: 287-293.

Matrone C, Pignataro G, Molinaro P, Irace C, Scorziello A, et al. 2004. HIF-1α reveals a binding activity to the promoter of iNOS gene after permanent MCAO. J Neurochem 90: 368-378.

Matsubara M, Hayashi N, Jing T, Titani K. 2003. Regulation of endothelial nitric oxide synthase by protein kinase C. J Biochem 133: 773-781.

Meffert MK, Premack BA, Schulman H. 1994. Nitric oxide stimulates $Ca^{2+}$ independent synaptic vesicle release. Neuron 12: 1235-1244.

Murphy S. 2005. Free radicals and EAE. Experimental models of multiple sclerosis. Lavi E, Constantinescu CS, editors. New York: Springer.

Murphy S, Pearce BR. 2005. Second messenger systems. Neuroglia. Kettenmann H, Ransom BR, editors. New York: Oxford University Press: pp. 216-228.

Musial A, Eissa NT. 2001. iNOS is regulated by the proteosome degradation pathway. J Biol Chem 276: 24268-24273.

Nassif X, Bourdoulous S, Eugene E, Couraud PO. 2002. How do extracellular pathogens cross the blood–brain barrier? Trends Microbiol 10: 227-232.

Nathan C. 1997. Inducible nitric oxide synthase: what difference does it make? J Clin Invest 100: 2417-2423.

Nau R, Bruck W. 2002. Neuronal injury in bacterial meningitis. Trends Neurosci 25: 38-44.

Navarro-Lerida I, Corvi MM, Barrientos AA, Gavilanes F, Berthiaume LG, et al. 2004. Palmitoylation of inducible nitric oxide synthase at cys-3 is required for proper intracellular traffic and nitric oxide synthesis. J Biol Chem 279 (53): 55682-55689.

Nishida K, Harrison D, Navas JP, Fisher AA, Dockery SP, et al. 1992. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. J Clin Invest 90: 2092-2096.

O'Banion MK. 1999. Cyclooxygenase-2: molecular biology, pharmacology and neurobiology. Crit Rev Neurobiol 13: 45-82.

Ogilvie P, Schilling K, Billingsley ML, Schmidt HHHW. 1995. Induction and variants of neuronal nitric oxide synthase type I during synaptogenesis. FASEB J 9: 799-806.

Osawa Y, Lowe E. R. Everett AC, Dunbar AY, Billecke SS. 2003. Proteolytic degradation of nitric oxide synthase: effect of inhibitors and role of hsp90-based chaperones. J Pharmacol Exp Therap 304: 493-497.

Packer MA, Stasiv Y, Benraiss A, Chmielnicki E, Grinberg A, et al. 2003. Nitric oxide negatively regulates mammalian adult neurogenesis. Proc Natl Acad Sci USA 100: 9566-9571.

Palmer RMJ, Ferrige AG, Moncada S. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327: 524-526.

Pan J, Burgher KL, Szczepanik AM, Ringheim GE. 1996. Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential post-translational regulation. Biochem J 314: 889-894.

Panda K, Adak S, Aulak KS, Santolini J, McDonald JF, et al. 2003. Distinct influence of N-terminal elements on neuronal nitric oxide synthase structures and catalysis. J Biol Chem 278: 37122-37131.

Park SK, Lin HL, Murphy S. 1994. Nitric oxide limits transcriptional induction of nitric oxide synthase in CNS glial cells. Biochem Biophys Res Commun 201: 762-768.

Park SK, Lin HL, Murphy S. 1997. Nitric oxide regulates nitric oxide synthase-2 gene expression by inhibiting NF-κB binding to DNA. Biochem J 322: 609-613.

Peng HB, Libby P, Liao JK. 1995. Induction and stabilization of IκBa by NO mediates inhibition of NF-κB. J Biol Chem 270: 14214-14219.

Perez-Sala D, Cernuda-Morollon E, Diaz-Cazorla M, Rodriguez-Pascual F, Lamas S. 2001. Posttranscriptional

regulation of human iNOS by the NO/cGMP pathway. Am J Physiol Renal Physiol 280: F466-F473.

Peunova N, Enikolopov G. 1995. Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells. Nature 375: 68-73.

Poluha W, Schonhoff CM, Harrington KS, Lachyankar MB, Crosbie NE, et al. 1997. A novel, nerve growth factor-activated pathway involving nitric oxide, p53, and p21WAF1 regulates neuronal differentiation of PC12 cells. J Biol Chem 272: 24002-24007.

Pow DV. 1994. Immunocytochemical evidence for a glial localization of arginine, and a neuronal localization of citrulline in the rat neurohypophysis. Neurosci Lett 181: 141-144.

Radi R. 2004. Nitric oxide, oxidants, and protein tyrosine nitration. Proc Natl Acad Sci USA 101: 4003-4008.

Rao KM. 2000. Molecular mechanisms regulating iNOS expression in various cell types. J Toxicol Environ Health B: Crit Rev 3: 27-58.

Riefler GM, Firestein BL. 2001. Binding of nNOS to CtBP changes the localization of CtBP from the nucleus to the cytosol. J Biol Chem 276: 48262-48268.

Rizzo V, McIntosh DP, Oh P, Schnitzer JE. 1998. In situ flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association. J Biol Chem 273: 34724-34729.

Rodriguez-Pascual F, Hausding M, Ihrig-Biedert I, Furneaux H, Levy AP, et al. 2000. Complex contribution of the 3′-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene. J Biol Chem 275: 26040-26049.

Saitoh F, Tian QB, Okano A, Sakagami H, Kondo H, et al. 2004. NIDD, a novel DHHC-containing protein, targets neuronal nitric-oxide synthase (nNOS) to the synaptic membrane through a PDZ-dependent interaction and regulates nNOS activity. J Biol Chem 279: 29461-29468.

Salvemini D. 1997. Regulation of cyclooxygenase enzymes by nitric oxide. Cell Mol Life Sci 53: 576-582.

Schmidt HHHW, Wilke P, Evers B, Bohme E. 1989. Enzymatic formation of nitrogen oxides from L-arginine in bovine brain cytosol. Biochem Biophys Res Commun 165: 284-291.

Schroeter M, Kury P, Jander S. 2003. Inflammatory gene expression in focal cortical brain ischemia: differences between rats and mice. Mol Brain Res 117: 1-7.

Scott GS, Kean RB, Southan GJ, Szabo C, Hooper DC. 2001. Effect of mercaptoethylguanidine scavengers of peroxynitrite on the development of experimental allergic encephalomyelitis in PLSJL mice. Neurosci Lett 311: 125-128.

Sennlaub F, Coutois Y, Goureau O. 2001. Inducible nitric oxide synthase mediates the change from retinal to vitreal neovascularization in ischemic retinopathy. J Clin Invest 107: 717-725.

Sennlaub F, Coutois Y, Goureau O. 2002. Inducible nitric oxide synthase mediates retinal apoptosis in ischemic proliferative retinopathy. J Neurosci 10: 3987-3993.

Sessa WC, Harrison JK, Barber CM, Zeng D, Durieux ME. 1992. Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. J Biol Chem 267: 15274-15276.

Shaul PW. 2002. Regulation of eNOS. Annu Rev Physiol 64: 749-774.

Sheehy AM, Burson MA, Black SM. 1998. Nitric oxide exposure inhibits endothelial NOS activity but not gene expression: a role for superoxide. Am J Physiol 274: L833-L841.

Sheta EA, McMillan K, Masters BS. 1994. Evidence for a bidomain structure of constitutive cerebellar nitric oxide synthase. J Biol Chem 269: 15147-15153.

Stuehr DJ, Marletta MA. 1985. Mammalian nitrate biosynthesis. Proc Natl Acad Sci USA 82: 7738-7742.

Stuehr DJ, Santolini J, Wang Z-Q, Wei C-C, Adak S. 2004. Update on mechanism and catalytic regulation in the NO synthases. J Biol Chem 279: 36167-36170.

Subramaniam JR. 2002. Mutant SOD1 causes motor neuron disease independent of copper chaperone-mediated copper loading. Nat Neurosci 5: 301-307.

Tanaka M, Yoshida S, Yano M, Hanaoka F. 1994. Roles of endogenous nitric oxide in cerebellar cortical development in slice cultures. Neuroreport 5: 2049-2052.

Thomas DD, Espey MG, Ridnour LA, Hofseth LJ, Mancardi D, et al. 2004. HIF1α, extracellular signal-regulated kinase, and p53 are regulated by distinct threshold concentrations of NO. Proc Natl Acad Sci USA 101: 8894-8899.

Venema VJ, Marrero MB, Venema RC. 1996. Bradykinin-stimulated protein tyrosine phosphorylation promotes endothelial nitric oxide synthase translocation to the cytoskeleton. Biochem Biophys Res Commun 226: 703-710.

Wang W, Nakayama T, Inoue N, Kato T. 1998. Quantitative analysis of nitric oxide synthase expressed in developing and differentiating rat cerebellum. Dev Brain Res 111: 65-75.

Wang J, Stuehr DJ, Rousseau DL. 1995. Tetrahydrobiopterin-deficient nitric oxide synthase has a modified heme environment and forms a cytochrome P-420 analogue. Biochemistry 34: 7080-7087.

Watanabe Y, Song T, Sugimoto K, Horii M, Araki N, et al. 2003. Post-synaptic density-95 promotes calcium/calmodulin-dependent protein kinase II-mediated Ser847 phosphorylation of neuronal nitric oxide synthase. Biochem J 372: 465-471.

Wiesinger H. 2001. Arginine metabolism and the synthesis of NO in the nervous system. Prog Neurobiol 64: 365-391.

Willmot MR, Bath PMW. 2003. The potential of nitric oxide therapeutics in stroke. Expert Opin Investig Drugs 12: 455-470.

Xie Q, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, et al. 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256: 225-228.

Yao X, Huang Y. 2003. From NO to endothelial cytosolic calcium. Trends Pharmacol Sci 24: 263-266.

Yoshizumi M, Perrella MA, Burnett Jr, JC Lee ME. 1993. Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res 73: 205-209.

Zemojtel T, Penzkofer T, Dandekar T, Schultz J. 2004. A novel conserved family of nitric oxide synthase? Trends Biochem Sci 29: 224-226.

Zhang R, Zhang L, Zhang Z, Wang Y, Lu M, et al. 2001. A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats. Ann Neurol 50: 602-611.

Zhao X, Haensel C, Araki E, Ross ME, Iadecola C. 2000. Gene-dosing effect and persistence of reduction in ischemic brain injury in mice lacking iNOS. Brain Res 872: 215-218.

Zhao X, Ross ME, Iadecola C. 2003. L-Arginine increases ischemic injury in wild-type mice but not in iNOS-deficient mice. Brain Res 966: 308-311.

Zhu D-Y, Liu SH, Dun HS, Lu YM. 2003. Expression of inducible nitric oxide synthase after focal cerebral ischemia stimulates neurogenesis in the adult rodent dentate gyrus. J Neurosci 23: 223-229.

Zielasek J, Jung S, Gols R, Liew FY, Toyka KV, et al. 1995. Administration of nitric oxide inhibitors in experimental autoimmune neuritis and EAE. J Neuroimmunol 58: 81-88.

# 12 14-3-3 Proteins in Brain Function

*Y. Takahashi*

**Abstract:** 14-3-3 proteins were discovered by Moore and Perez in the soluble extract of bovine brain in 1967. These proteins are highly abundant in the brain. In this chapter, the discovery of 14-3-3 protein, the structure of 14-3-3, the cloning of 14-3-3 complementary DNA (cDNA), the nucleotide sequence of 14-3-3 cDNA, the structure of the 14-3-3 gene, the occurrence of 14-3-3 messenger RNA (mRNA) in the brain, the function and regulation of 14-3-3 protein, the binding of 14-3-3 protein to other proteins, the effects of 14-3-3 on the binding of one protein to another, the effects of 14-3-3 on protein kinase, and the neuropathology of 14-3-3 are described concisely.

**List of Abbreviations:** AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ASK, apoptosis signal-regulating kinase; BAD, Bel-xL/Bel-2-associated death promoter; cAMP, cyclic adenosine monophosphate; CBP, CREB-binding protein; Cdc, cell division cycle; *C. elegans*, *Caenorhabditis elegans*; CRE, cAMP response element; CJD, Creutzfeldt–Jakob disease; CSF, cerebrospinal fluid; cTAK, Cdc25c-associated kinase; DEAE cellulose, diethylaminoethyl cellulose; DLBD, diffuse Lewy body disorder; FISH, fluorescence in situ hybridization; FKALR1, forkhead transcription factor; GSK3β, glycogen synthase kinase-3β; HPLC, high-performance liquid chromatography; JNK, C-JunNH(2) terminal kinase; KLC, kinesin light chain; KSR, kinase suppressor of ras; LTP, long-term potentiation; MS/MS, tandem mass spectrometry; mRNA, messenger ribonucleic acid; NFTs, neurofibrillary tangles; PCR, polymerase chain reaction; PC12, pheochromocytoma cell 12; Poly(A), polyadenylic acid; PD, Parkinson's disease; PKC, protein kinase C; SCA1, spinocerebellar ataxia type 1; SDK1, sphingosine-dependent protein kinase 1; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TAB, TATA-binding protein; TAFII, TAB-associated factor II

## 1 Introduction

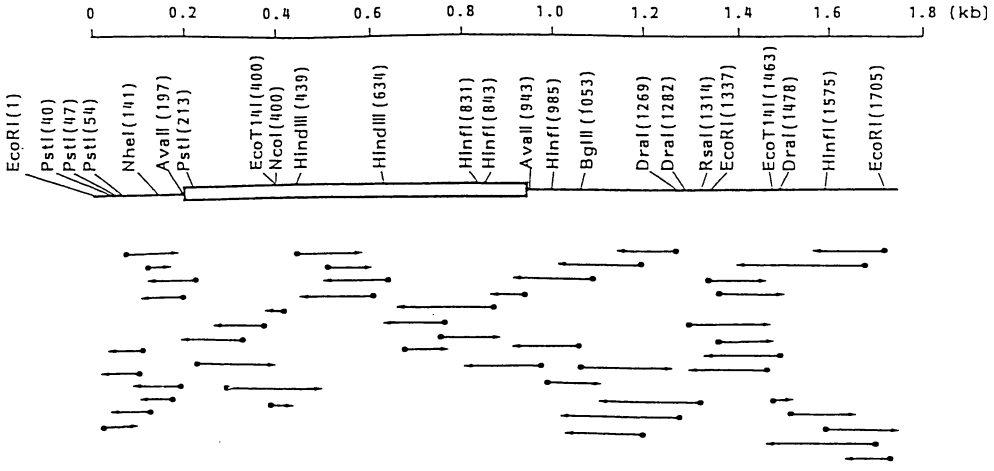
Moore and Perez (1968) reported attempts to fractionate and purify the acidic proteins from the bovine brain. This was subsequently discussed in *Advances in Neurochemistry* (Moore, 1975), and summarized in the second edition of the *Handbook of Neurochemistry* (Moore, 1983). The topic of brain proteins was also reviewed by Bogoch (1969). In 1982, Boston et al. (1982a) reported the axonal flow of these proteins into nerve endings and their separation into two fractions. In 1987, research into these proteins was started in our laboratory. Ichimura et al. (1988) found that 14-3-3 proteins are the regulatory factors of tyrosine and tryptophan hydroxylases, whose activities are dependent on $Ca^{2+}$/calmodulin-dependent protein kinases. They reported that these proteins comprise a family of seven subtypes. The nucleotide sequences of the precursor nucleic acids were analyzed and the amino acid sequences were deduced. Following these results, the structure of 14-3-3 proteins, the immunohistochemistry of 14-3-3 proteins, the interaction of 14-3-3 with other proteins, and their functions were examined (Morrison, 1994; Aitken, 1996, 2002; Ferl, 1996; Pawson and Scott, 1997; Skoulakis and Davis, 1998; Finnie et al., 1999; Fu et al., 2000; Tzivion and Avruch, 2002; Berg et al., 2003a; Klein et al., 2003; Dougherty and Morrison, 2004). In view of the recent advances on 14-3-3, it is time to include this protein in the new edition of *Handbook of Neurochemistry and Molecular Neurobiology.*

## 2 Isolation and Cloning of 14-3-3 Protein

In 1968, Moore and Perez attempted to fractionate soluble proteins of bovine brain and liver using chromatography and electrophoresis and succeeded in purifying S-100 protein using ammonium sulfate precipitation, diethylaminoethyl (DEAE) cellulose chromatography, Sephadex G-100 gel filtration and DEAE Sephadex chromatography (Moore and Perez, 1968). They also partially purified 14-3-3 proteins from the soluble fraction of bovine brain using DEAE cellulose chromatography and starch gel electrophoresis. In Chapter 4 of *Advances of Neurochemistry*, Vol. 1, Moore summarized the fractionation and purification methods for S-100, 14-3-2 protein, and glial fibrillary protein (Moore, 1975). In his preparation chart, 14-3-3 protein was almost separated in the final step of the preparation of 14-3-2. In this step, Moore et al. could separate the protein fraction into 14-3-2 and 14-3-3 using DEAE Sephadex A50 chromatography

(Grasso et al., 1977) and subsequently they were able to separate 14-3-2 protein and 14-3-3 protein using Sephadex G-150 gel filtration and DEAE Sephadex chromatography (Erickson and Moore, 1980). In 1982, Boston et al. described the axonal flow of these proteins into nerve endings and their resolution into two fractions (Boston et al., 1982a, b). Finally, Ichimura et al. (1987, 1988) in our laboratory found that 14-3-3 proteins are the regulatory factors of tyrosine and tryptophan hydroxylases that depend on $Ca^{2+}$/calmodulin-dependent protein kinases. Further, they reported that these proteins are a family composed of seven subtypes (α, β, γ, δ, ε, ζ, and η) by analysis using reverse-phase high-performance liquid chromatography (HPLC) (Ichimura et al., 1988; Isobe et al., 1991). Later the θ type of 14-3-3 was reported. Complementary DNA (cDNA) cloning was carried out for each subtype (*Figure 12-1*). Nucleotide and amino acid sequences of each protein were examined and clarified (*Figure 12-2*). The methodology for molecular

**Figure 12-1**
**cDNA cloning of the gene for human brain 14-3-3 protein η**



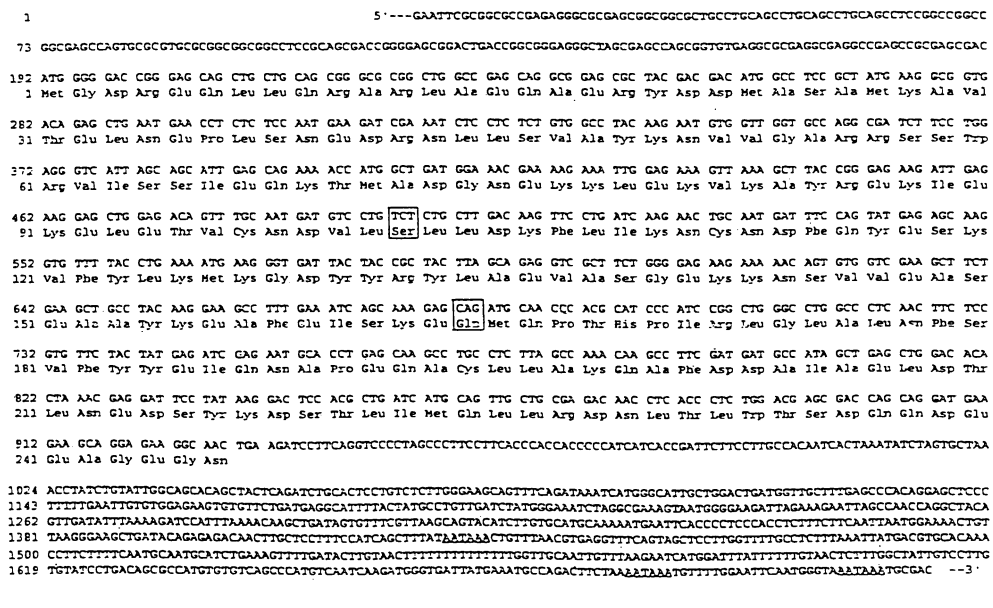

cloning is described in detail in a monograph (Sambrook and Russell, 2001). Following these results, the structure of 14-3-3 proteins, the immunohistochemistry, and the interaction between the 14-3-3 and other proteins and other functions were examined. The structure of the gene for 14-3-3 and gene expression were also studied (Muratake et al., 1995, 1996). Recently, a number of reviews and many papers about 14-3-3 protein were published. In February 2004, a Gordon Research Conference on "Biology of 14-3-3 proteins in the brain" took place in Ventura, California, USA (Gordon Research Conference, 2004) during one week. About 20 speakers gave a lecture about 14-3-3 proteins.

## 3 cDNA and Amino Acid Sequence

As early as 1975, Moore described the separation of 14-3-2 and 14-3-3 using DEAE Sephadex chromatography. Erickson and Moore (1980) reported the isolation of 14-3-3 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). They reported a half-life of 10 days based on the incorporation of $^{3}$H-leucine into the protein. Boston et al. (1982a, b) described the axonal flow of these proteins into nerve endings and the resolution of human brain 14-3-3 proteins into two fractions. In 1987, we succeeded in fractionating 14-3-3 proteins into seven subtypes and in cloning cDNA for each 14-3-3 protein (Ichimura et al., 1988) (*Figure 12-1*). We then determined the amino acid sequences of each 14-3-3 protein by determining the nucleotide sequences of each cloned cDNA (*Figure 12-2*). Since another 14-3-3 subtype was discovered, we felt that there was evidence for the existence of eight subtypes. However, according to other reports, α and δ may be the phosphorylated species of β and ζ (Aitken, 2002).

**Figure 12-2**

**Nucleotide sequence and the deduced amino acid sequence of human 14-3-3 protein η**




In plants, five subtypes of 14-3-3 were found in *Arabidopsis saliana*. Roberts (2000) found 12 tomato 14-3-3 genes. In *Saccharomyces cerevisiae* and *Schizosaccharomyces ponte*, two subtypes (BMHI and BMHII and rad 24 and rad 25, respectively) were found (Ferl, 1996; Finnie et al., 1999). After 1987, we cloned cDNA of the β, γ, ζ, and θ isoforms of 14-3-3 but not of the η isoforms and determined their nucleotide sequences and amino acid sequences (Isobe et al., 1991; Watanabe et al., 1991, 1993a, b, 1994). These 14-3-3 cDNAs were classified into several groups and the nucleotide homology for each subtype was calculated, with evidence of high homology (about 70–90%) among them. cDNA of 14-3-3 from plants and yeast also showed nucleotide sequences similar to those of the subtypes from the animal source, which in turn showed high homology (about 70%) to those of human ε subtype.

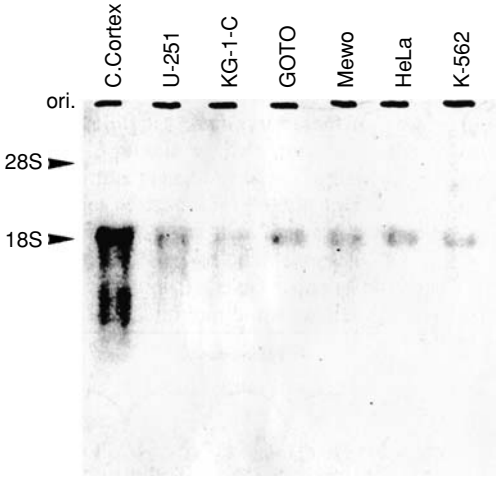
The 14-3-3 subtypes have about 250 amino acid residues: the β chain is composed of 246 amino acids, and the γ chain of 247 amino acids. The η chain consists of 246 amino acids, ζ of 245 amino acids, and θ of 245 amino acids. These residues do not contain a hydrophobic amino acid region such as is found in transmembrane proteins. Further, there is no modification by carbohydrate and fatty acids in this 14-3-3 protein. These findings suggest that 14-3-3 protein is a cytoplasmic protein. There are some conserved, invariant domains in all subtypes and some specific unique regions in each subtype. These domains and regions are mosaically distributed in the 14-3-3 sequence. Each subtype has a specific acidic carboxy-terminal region, which contains the box1 for binding to tryptophan hydroxylase. Each polypeptide is organized into nine α-helices in an antiparallel array and contains a region including Ser for cyclic adenosine monophosphate (cAMP)- and $Ca^{2+}$-dependent phosphorylation. It was reported that 14-3-3 sequences from plants contain EF hand type Ca-binding sequences such as the sequences in calmodulin and S-100.

The above amino acid sequence was largely deduced from the nucleotide sequence of 14-3-3 cDNA (*Figure 12-2)*. 14-3-3 η cDNA from our cloning was the first 14-3-3 cDNA to be reported. This cDNA is composed of 1,793 bases that contain an open reading frame from the translation start site, the ATG codon at 166 bases to the TGA stop codon at 904 bases. Further, all nucleotide sequences contain 165 noncoding bases at the 5-end and 793 noncoding hands at the 3-end. The 3′-noncoding region contains a polyadenylation signal (AATAAA) and a part of the poly(A) tail. Similar cDNA cloning of rat 14-3-3 β, γ, ζ, and θ subtypes was carried out and their nucleotide sequences and amino acid sequences were determined.

In addition to cloning 14-3-3 η in mice, rats, and bovines, the cloning of human 14-3-3 η cDNA was also performed (Ichimura-Ohshima et al., 1992). The amino acid sequence of the human 14-3-3 η was almost similar to those of rat and bovine proteins. By using the human cDNA as a probe, the expression of 14-3-3 η messenger RNA (mRNA) was detected in the human cultured nervous cell lines such as the U-251 astroglioma cell line and KG-1-C oligodendroglioma cell line (Ichimura-Ohshima et al., 1992) (*Figure 12-3)*. Further, the influence of the added methamphetamine on these cells was examined. We found the stimulating effect of methamphetamine on the 14-3-3 mRNA level at $10^{-6}$ M in the U-251 cells. Next, we had to clarify the structure of the 14-3-3 gene. In 1996, we succeeded in cloning the human 14-3-3 η gene (Muratake et al., 1995, 1996). Furthermore, in 2002, we isolated the mouse 14-3-3 η gene and clarified the structure and sequence of this gene (Toyooka et al., 2002).

**Figure 12-3**

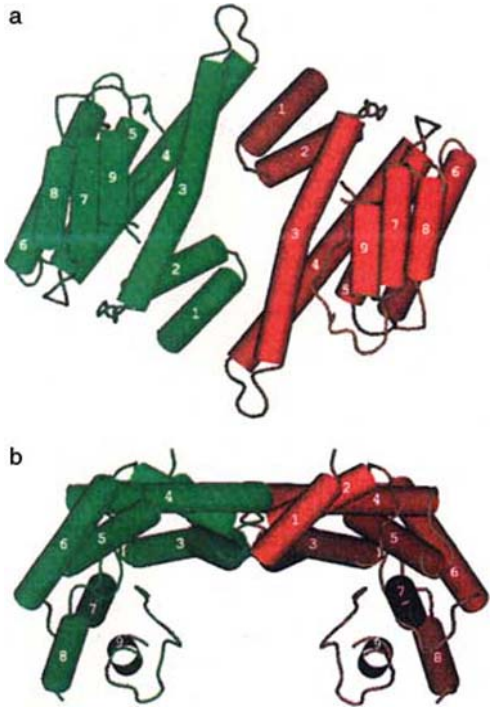
**Northern blot analysis. Total RNA (10 μg) for human cerebral cortex and cell lines were hybridized with the Dra I (1478)–EcoR1 (1705) fragment. The lanes contain RNAs from human cerebral cortex; astroglioma cells, U-251; oligodendroglioma cells, KG-1-C; neuroblastoma cells, GOTO; melanoma cells, Mewo; cervical carcinoma cells, HeLa; and leukemia cells, K-562. Ori. indicates origin**




## 4 Three-Dimensional Structure of 14-3-3 Protein

In 1995, the crystal structures of 14-3-3 τ and 14-3-3 ζ revealed markedly similar dimeric structures by X-ray analysis (Liu et al., 1995; Xiao et al., 1995). Views of the 14-3-3 dimer are shown by Liu et al. (*Figure 12-4)*. The following summary is mainly based on the findings of Skoulakis and Davis. All 14-3-3 isoforms form dimers, in the form of both homodimers and heterodimers. Dimerization appears to involve primarily hydrophobic interactions mediated by highly conserved residues within the amino-terminal part (Liu et al., 1995; Xiao et al., 1995). This suggests that all 14-3-3 isoforms may be able to heterodimerize. Each subunit of the dimer is composed of nine α-helices in an antiparallel array, each separated by a short loop as described previously. α-Helices are perpendicular to the axis of dyad symmetry, and form a palisade around a central negatively charged groove composed mostly of invariant amino acids. This gives a binding surface conserved during evolution and suggests recognition of common features in target proteins. The amino terminal helix mediates dimerization and the conserved residues in this interface may support the

**Figure 12-4**
**Views of the 14-3-3 dimer. a, View down the molecular twofold axis, with belices as cylinders, and the two monomers in green and red. b, As a, but rotated by 90°. These figures were used with the permission of the Nature Editorial Office**




heterodimer formation. The remainder of this unusual structural arrangement can be subdivided into two domains of three-helix bundles each, both contributing to the formation of the acidic pocket (Liu et al., 1995; Xiao et al., 1995).

The width of the groove corresponds to the length (about 55 Å) of α3 and α4, the longest helices. Each subunit of the dimer is arranged in a symmetric position and forms a groove-like structure with a characteristic groove within the inside of the whole molecule. About 100 amino acid residues in the N terminal regions containing α1–α4 in each subunit participate in forming of the dimer. Thus, the dimer structure is stabilized mainly by ionic binding of α1 and α2 to α3 and α4. As a whole molecule, dimer subunits bind by contacting at two points holding a central hole (diameter: 6–8 Å) rich in ionic amino acid side chain. A mutual contacting area of each subunit (620 Å) has a size in which 14-3-3 could be in existence as a stable dimer in the cell. The inside of the most characteristic groove-like structure for 14-3-3 molecule has a size suitable for holding one typical α-helix.

In the inside wall, the hydrophobic side chain (Leu 172, Leu 216, Leu 220, Leu 227) from α7 and α9, the basic side chain (Lys 49, Arg 56, Arg 60) from α3, and the polic side chain (Lys 120, Asp 124, Arg 127, Tyr 128) from α5 are exposed, and Arg 56, Arg 60, Lys 120, and Arg 127 from the above chains form the basic cluster in the groove. This cluster is stabilized by interaction between this and a part of the acidic loop composed of C-terminal 15 amino acids (231–245). These polar/inpolar partial structures become independent and are localized in the inner wall of the groove, and as a whole body may constitute bipolar binding sites against the helix originating from the target protein.

An annexin-like sequence, as described previously (Lys 122 to Asp 136 in the ζ chain), is localized in the latter half of α, which constitutes a part of the groove. Furthermore, Ser 58 and Ser 65, which are expected to

be phosphorylated by cAMP- and $Ca^{2+}$-dependent reactions, are localized in the linker part, which combines α7 and α8 in a form that can be buried in the hydrophobic binding site in order to form the dimer. Ser 184, which is expected to be phosphorylated at proline, is localized in the same part, exposing the molecular surface.

On the other hand, amino acid sequences of 14-3-3 protein in various organisms contain conserved, invariant domains and some specific unique regions in relation to the crystal structure of 14-3-3 protein. The amino acids in the conserved domains are localized on the inside of the groove that 14-3-3 proteins form, whereas the amino acids in the specific regions are localized on the outside of the groove. These data suggest that the inside structure of the groove is essential for its function and that this function is conserved from yeast to mammals.

## 5 Localization and Detection of the 14-3-3 Proteins

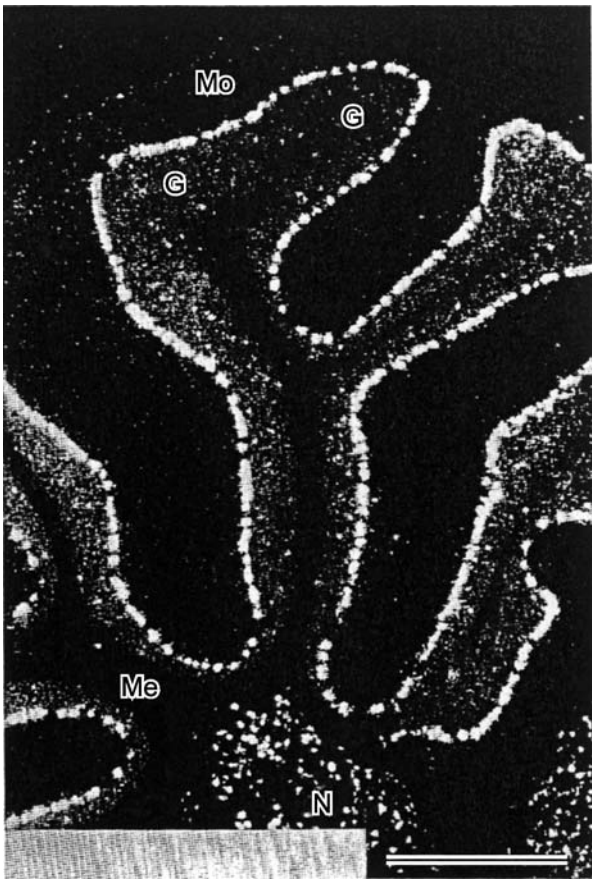
### 5.1 In Situ Hybridization

14-3-3 proteins were fractionated into eight polypeptide subtypes by using reverse-phase HPLC (Ichimura et al., 1988, 1991; Isobe et al., 1991). The polypeptides were named α, β, γ, δ, ε, ζ, and η, respectively. Later, the τ isoform was found, which occurs only in glia-like cells of white matter. The other seven subtypes are found in rat brain gray matter. The ε isoform is highly enriched in the pineal gland. The β, γ, and η isoforms are enriched in the Purkinje cells of the cerebellum. An oligonucleotide probe (45 mer), corresponding to part of the 3′-noncoding region of 14-3-3 η mRNA, was synthesized using a DNA synthesizer (ABI, USA). Another probe (350b) was synthesized by polymerase chain reaction (PCR) with mouse genomic DNA as the template. These probes were labeled with [α-$^{35}$S]dATP and used for in situ hybridization and developmental research (Watanabe et al., 1991; Toyooka et al., 2002).

The in situ hybridization studies of 14-3-3 in rat brain tissue with a cDNA probe were carried out by Watanabe et al. (1991, 1993a, b, 1994) (*Figure 12-5)*. Recently, the in situ hybridization of 14-3-3 in mouse brain tissue was also examined by Toyooka et al. (2002).

In humans, bovines, rats, and mice, abundant 14-3-3 proteins were found in most areas of the central nervous system. Particularly, it is very important that isoforms of 14-3-3 are highly expressed in the pyramidal cells of the hippocampus, the neurons of the cerebral cortex, olfactory bulb neurons, and Purkinje cells of the cerebellum as described above (*Figure 12-5)*. These results were confirmed by Northern blot hybridization (*Figure 12-3)*. Apart from being abundant in the nervous system, 14-3-3 is relatively abundant in the suprarenal gland, intestines, and liver. Furthermore, as already described, 14-3-3 proteins are composed of at least eight subtypes. It is important that each subtype shows different distributions and localizations. The ζ isoform is present at high levels in the gray matter of the rat brain and β, γ, η, and τ are also localized in similar brain areas. The γ isoform is specifically expressed in the nervous system. Variation is also observed among brain areas. The β, γ, and η isoforms are enriched in the Purkinje cells of the cerebellum (*Figure 12-5)*. The ε isoform is highly enriched in the pineal gland and in significant amounts in the retina. The τ isoform is only found in glia-like cells of the white matter (Watanabe et al., 1993a, b). The variations in distribution and amount of the 14-3-3 subtypes may reflect functional differences or participation of distinct signal transduction pathways by different cell groups. Further, in a particular cell, homodimers and heterodimers of these isoforms might be involved in specific neuronal function. In Drosophila, Skoulakis and Davis (1998) described in detail the 14-3-3 isoform expression in the nervous system. The Drosophila ζ isoform, Leonardo, shows preferential expression in the nervous system. In fact, the gene was isolated because of its preferential expression in a specific area of the brain, the mushroom body, a center for learning and memory in insects. Leonardo is also expressed ubiquitously at low levels. Although the expression pattern of Drosophila 14-3-3 ε is not known, it is likely that it is expressed in the retina in a manner similar to its mammalian counterpart. 14-3-3 ε may be important in the function of the retina, because mutation in the gene disturbs development of retinal cells. Finally, the preferential expression of mammalian and Drosophila 14-3-3 isoforms in the nervous system is consistent with their roles in signal transduction pathways, processes essential for nervous system function.

**Figure 12-5**
**In situ hybridization histochemistry of 14-3-3η mRNA in the rat cerebellum. Bar = 1 mm**




## 5.2 Immunohistochemistry

The specific antibody against each subtype of the 14-3-3 family was used for the immunohistological study (Martin et al., 1994; Baxter et al., 2002). There was significant staining of the cytoplasm, including neuronal axons and dendrites. This result was confirmed by the ultracentrifugal cellular fractionation method, indicating that 14-3-3 is mainly localized in the neuronal cytoplasm and that a portion of 14-3-3 may be bound to the plasma membrane, endoplasmic reticulum, and Golgi membrane. More extensive studies about the function of membrane-bound 14-3-3 are required. The identification of the receptor on the membrane would be of particular importance. 14-3-3 is expressed in animals such as mammals, Xenopus, Drosophila, *Caenorhabditis elegans*, and amoeba, and in plants such as barley, *Arabidopsis saliana*, and yeast. These cells are all eukaryotes. However, 14-3-3 is not present in prokaryotes, such as *Escherichia coli*. In plants such as barley, 14-3-3 expression increases as a result of virus infection. Therefore 14-3-3 may share the signal transduction system in the plant (Tzivion et al., 2001; Tzivion and Avruch, 2002). Immunohistochemical studies of protein kinase C (PKCγ) in mouse brain were also carried out, and it was discovered that the areas with high activity of PKCγ almost correspond to those containing 14-3-3 η. This result is very important, considering the functional relation between 14-3-3 and PKC. In the first stage of a study of 14-3-3, Aitken et al. showed that 14-3-3 could regulate PKC activity (Aitken, 1996; Fu et al., 2000).

### 5.3 Northern Blot Analysis

During development of the mammalian central nervous system, the expression of 14-3-3 proteins was also observed (Takahashi, 1992, 2003). Although there are considerable variations in each area, some neurons exhibit high levels of mRNA between embryonic day 13 and postnatal day 1, with a subsequent rapid increase during development. In some cases a high level is maintained and in other cases rapid decrease is observed. After 1987, we studied the biosynthesis of 14-3-3 in the brain tissue in vivo and in vitro (Ichimura-Ohshima et al., 1992; Muratake et al., 1995). Ichimura-Ohshima et al. (1992) extracted total RNA from an astroglioma cell line, U-251, an oligodendroglioma cell line, KG-1-C, a neuroblastoma cell line, GOTO, and a melanoma cell, MEMO, and purified poly(A)RNA from the total RNA. They found 14-3-3 mRNA in this poly(A)RNA using Northern blot analysis (Ichimura-Ohshima et al., 1992) (❯ *Figure 12-3)*. Muratake et al. (1995) also found the expression of 14-3-3 mRNA in human cultured cells U-251 and KG-1-C. From these experiments it is concluded that 14-3-3 proteins except the τ isoform are localized in gray matter neurons and are highly concentrated in cultured glioma cells. Furthermore, if normal cells become malignant, the cells may be able to express the 14-3-3 gene.

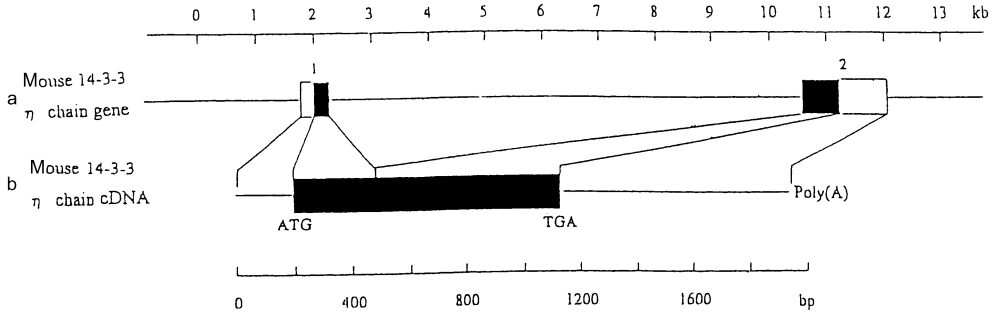
## 6 Gene and Gene Expression of 14-3-3

As previously described, in 1988 Ichimura et al. (1988) succeeded in cloning cDNA for the bovine 14-3-3 protein. Then using this cDNA as a probe, Watanabe et al. examined the distribution of 14-3-3 proteins in rat and bovine brains by in situ hybridization. The results show that 14-3-3 proteins are localized in almost all areas of the central nervous system. Particularly, 14-3-3 proteins are highly expressed in the pyramidal cells of the hippocampus, the neurons of the cerebral cortex, olfactory bulb neurons, and the Purkinje cells of the cerebellum (❯ *Figure 12-5)*. These results were confirmed by Northern blot hybridization and immunohistochemistry (❯ *Figure 12-3)*.

However, there were very few studies about the gene of 14-3-3 protein. In 1996, Muratake et al. (1996) succeeded in cloning the human 14-3-3 gene. Furthermore, in 2002, Toyooka et al. isolated the mouse 14-3-3 η gene and clarified the structure and nucleotide sequence of this gene (Toyooka et al., 2002) (❯ *Figures 6*

**Figure 12-6**

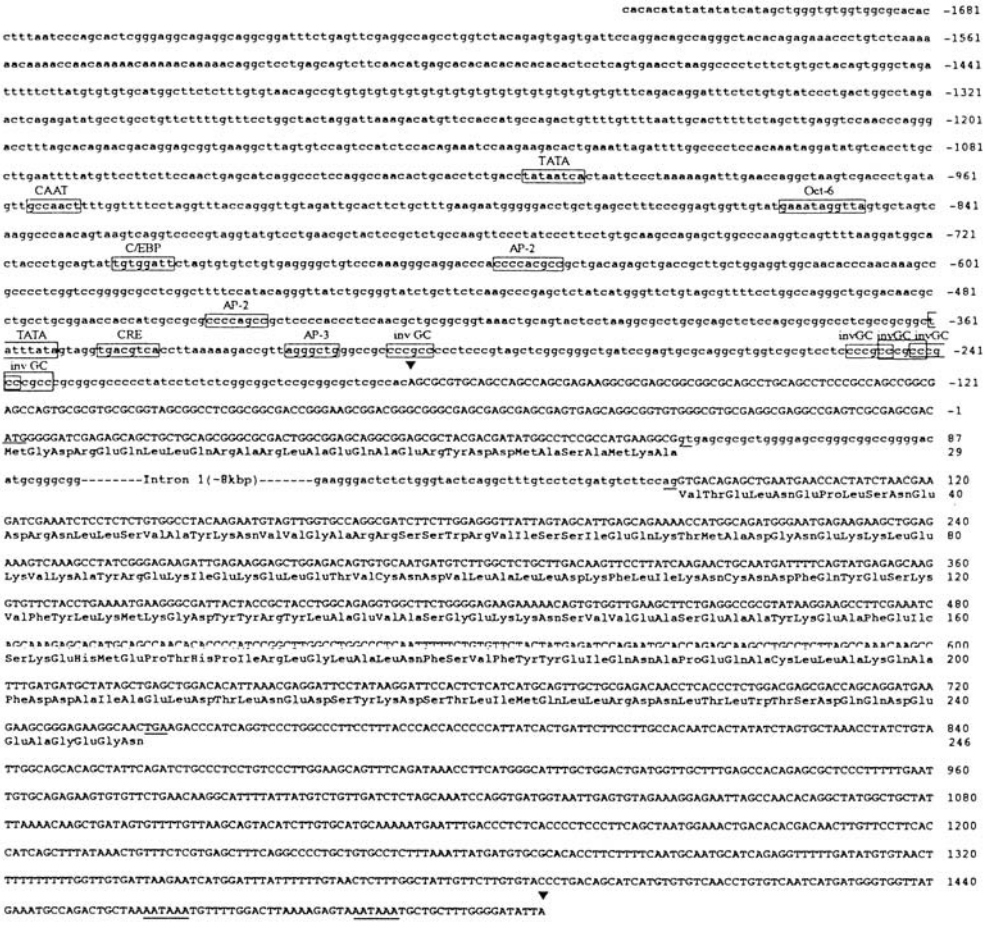
**Schematic representation of the mouse 14-3-3 η gene. a, Structure of the mouse 14-3-3 η gene. Open boxes show the noncoding regions of the exons. Solid boxes show the coding region of the exons. Thick bars show the intron and the flanking regions. b, Structure of mouse 14-3-3 η cDNA**




and ❯ *7*). The human 14-3-3 η gene is about 10 kb long and is composed of two exons separated by an 8 kb-long intron. The translation start site was identified in exon 1, and the stop codon was found in exon 2 of the human gene. Further, in the 5′-upstream sequence, Toyooka et al. found several *cis*-elements, including a cAMP response element (CRE) sequence, a TATA-like sequence, and a GC box. Fluorescence in situ

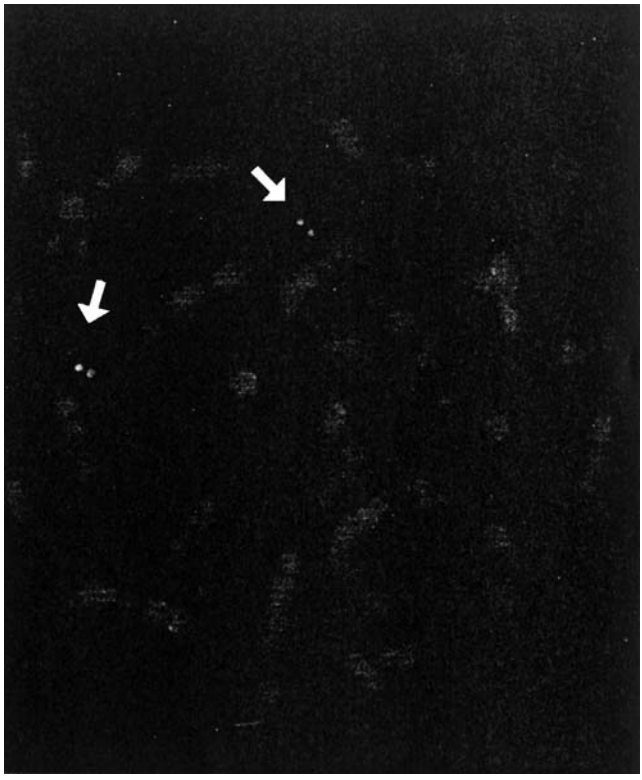
**Figure 12-7**

**Nucleotide sequence of the mouse 14-3-3 η gene. Exon sequences are shown in uppercase letters. Intron and flanking sequences are in lower case letters**




hybridization (FISH) with DNA probes of the human 14-3-3 η gene mapped the 14-3-3 gene to chromosome 22q12.1–q13.1 (*Figure 12-8)*. In the noncoding region of exon 1 of the 14-3-3 η gene, a 7-bp repeat sequence was found. Using this repeat sequence as a probe, Toyooka et al. (1999) examined the relation between the 14-3-3 η gene and schizophrenia and found evidence for genetic association of the 14-3-3 η gene with schizophrenia. Recently, Toyooka et al. (2002) isolated the mouse 14-3-3 η gene and clarified its structure, showing similarity between the human and mouse 14-3-3 η genes. Further studies are planned to construct a knockout mouse using the mouse 14-3-3 gene. If a 14-3-3 η gene knockout mouse can be constructed, the function of 14-3-3 η in the central nervous system could be clarified. However, recently Skoulakis and Davis (1998) expressed doubt about the effectiveness of the knockout experiment of the 14-3-3 gene. In 1999, Chan et al. carried out a knockout experiment of the human 14-3-3 gene (Chan et al., 1999). This report is very important. After DNA knockout damage, human colorectal cells were unable to maintain cell cycle arrest. In these cells, both 14-3-3 δ alleles are inactivated. Recently, Ichimura et al. (2004) reported transcriptomic and proteomic analysis of a 14-3-3 gene-deficient yeast. They compared the transcriptomic and proteomic profiles of the wild type and a BMH 1/2-deficient *S. cerevisiae* mutant (bmh Δ) using DNA microarrays and two-dimensional polyacrylamide gel electrophoresis. A subset of

**Figure 12-8**
**Localization of the human 14-3-3 η (YWHAH) to chromosome 22q12.1–q13.1 by fluorescence in situ hybridization. Two pairs of YWHAH signals were localized on the two Q-banded chromosome 22 (arrows)**

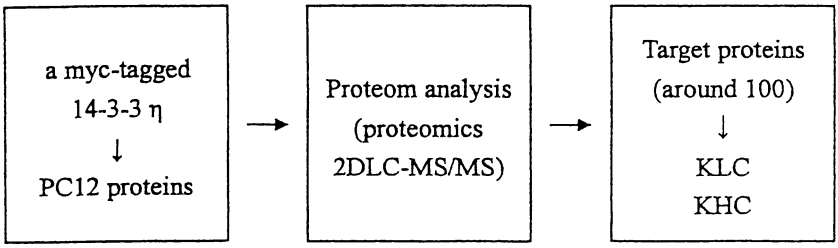


genes and proteins (a total of 220 genes) is significantly induced or reduced in the absence of Bmh 1/2p. The presence of the CRE sequence in the 5′-noncoding region of the mouse 14-3-3 η gene may be related to the effect of methamphetamine. Further, the presence of the CRE sequence and cAMP response element-binding protein (CREB) protein may be related to inducing and maintaining of long-term potentiation (LTP) into the animal (Nestler et al., 2001). According to some recent papers, 14-3-3 proteins regulate potassium channel activity (O'Kelly et al., 2002; Rajan et al., 2002; Rishi et al., 2003; Zhou et al., 2003).

## 7 Target Protein Binding of 14-3-3

In this section we are going to describe the target protein binding of 14-3-3, a study being carried out currently. Recently, rapid progress has occurred in the research methods of protein, and proteome analysis (proteomics; 2DLC–MS/MS) is widely being used (Itagaki et al., 1999; Beranova-Giorgianni et al., 2002; Ichimura et al., 2002) (*Figure 12-9)*. This procedure may not only be very useful for purification of 14-3-3 itself, but also be widely applicable for analysis of many target proteins bound to 14-3-3 proteins, as described later. Recently Powell et al. (2003) reported proteomic identification of 14-3-3 ζ as a mitogen-activated protein kinase (MAPK)-activated protein kinase 2 substrate. Many proteins (more than 100–200) were discovered as target proteins (Yaffe, 2004). Several reports indicate that these are at least 100 and possibly more than 200 target proteins for 14-3-3 (Aitken, 2002; Pozuelo Rubio et al., 2004; Yaffe, 2004). It is likely that additional target proteins with very important functions will be found in the future. Two recent

**Figure 12-9**
**Target protein assay. The research method of targeting protein for 14-3-3 in PC12 cells is concisely summarized in the figure. Protein technology was used**




monographs discuss the methodology used in protein and proteomics research (Simpson, 2003, 2004). Ichimura et al. (2002) applied this protein analysis using a myc-tagged 14-3-3 η isoform to the proteins in PC12 cells and found that kinesin heavy chain and kinesin light chain-2 (KLC2) were important target proteins of 14-3-3. In particular, KLC2 is very important. Recently, yeast species that survived after knocking out the 14-3-3 gene were isolated.

Target proteins such as tyrosine and tryptophan hydroxylases (Ichimura et al., 1988, 1995; Furukawa et al., 1993), Raf, Bcr (Michaud et al., 1995), Bel-xL/Bel-2-associated death promoter (BAD) (Zah et al., 1996), and keratin K18 (Lias and Omary, 1996) must generally be phosphorylated for binding of 14-3-3. Using synthetic phosphopeptides, Muslin et al. (1996), Yaffe et al. (1997), and Rittingers et al. (1999) provided evidence for two distinct 14-3-3 binding motifs: RSXpSXP (mode 1) and RXXXpSXP (mode 2). The binding of 14-3-3 to the mode 1 motif is favored by an aromatic or positively charged amino acid at position −1. The 14-3-3 binding to the mode 2 motif exhibits a preference for aromatic residues at position −2, positive residues at position −1, and Leu, Glu, Ala, or Met at position +1. It was found that most target proteins contain either a mode 1 motif or a mode 2 motif. However, some proteins that bind to 14-3-3 depending on phosphorylation do not contain either of these motifs. Further, the stable association of most cellular partner proteins with 14-3-3 requires a 14-3-3 dimer, but proteins such as C-Raf-1 that contain a high-affinity 14-3-3 binding site can bind monomeric 14-3-3 in a stable manner. 14-3-3 can bind well to a variety of nonphosphorylated proteins such as exoenzyme S and Cdc25B, in addition to nonphosphorylated synthetic or recombinant peptides.

## 8 14-3-3 Modes of Action

The modes of action of 14-3-3 were summarized by Fu et al. (2000), Tzivion et al. (2001), Tzivion and Avruch (2002), and Aitken (2002). Fu et al. (2000) reported in detail recent investigations involving three 14-3-3 ligands: Raf-1, Bad, and Cdc25. ❯ *Table 12-1* simply shows different roles of 14-3-3 proteins. We

**Table 12-1**
**Functions of 14-3-3 proteins**

| Types of function | Target proteins |
|---|---|
| Signal transduction regulation | PKC |
| Cell cycle regulation | KSR 1 |
| Differentiation regulation | Raf-1 |
| Apoptosis regulation | BAD, ASK1 |
| Ion channel regulation | K channel |

will describe these modes mainly according to Tzivion et al. The mechanisms by which 14-3-3 binding participates in the regulation of target protein function can be grouped into six major types.

1. Binding of 14-3-3 may change the ability of the target protein to interact with other partner proteins. For example, the binding of 14-3-3 to BAD competes with Bcl2 binding and causes an inhibition of apoptosis and the promotion of cell survival. BAD is the target for both antiapoptotic and proapoptotic signals (Hsu et al., 1997; Datta et al., 2000). Protein kinase A, Akt/protein kinase B, PKC, and Raf-1 are capable of phosphorylating BAD in vitro. A proline-rich Akt substrate was recently identified as a 14-3-3 binding partner (Kovacinn et al., 2003).
2. Binding to 14-3-3 can modify the localization (cytoplasmic/nuclear partition) of the target protein by increasing the nuclear export rate, decreasing the nuclear import rate, or both (Muslin and Xing, 2000). Proteins shown to be subject to this mode are the cell cycle protein phosphatase Cdc25c (Dalal et al., 1999; Kumagai and Durphy, 1999; Lopez-Girona et al., 1999), telomerase (Seimiya et al., 2000), the insulin-regulated transcription factors FKALR1 (forkhead transcription factor) and DAF-16 (Cahill et al., 2001), etc. In particular, Cdc25c is a major cell cycle regulator that dephosphorylates and activates PKCdc2 to trigger entry into mitosis. Recent papers reported that 14-3-3 regulates mitosis by interaction with Cdc25b and Cdc25c (Bialkowska et al., 2003; Giles et al., 2003). Further, Müller et al. (2003) reported that Cdc25c-associated kinase (c-TAK1) has been implicated in cell cycle regulation and Ras signaling through its interactions with two putative substrates, Cdc25c phosphatase and MAPK scaffold kinase suppressor of ras-1 (KSR1). They concluded that c-TAK1 is a regulator of 14-3-3 binding. Uchida et al. (2004) reported that binding of 14-3-3 beta but not 14-3-3 sigma controls the cytoplasmic localization of Cdc25b. Recent findings demonstrated that FKALR1 and DAF-16 are phosphorylated by PKB (Aitken, 2002; Woods and Rena, 2002).
3. 14-3-3 can serve to bridge two target proteins as a phosphorylation-dependent adaptor/scaffold. Ligation between Raf-Bcr (Brasselman and McCormick, 1995), Raf-A20 (Vincenz and Dixit, 1996), and Raf-PKC (Van Der Hoeven et al., 2000) is 14-3-3-dependent. Further, the identification of 14-3-3 as a Raf-1-binding protein added a new component to the regulatory machinery of Raf-1.
4. Binding of 14-3-3 can either increase or inhibit the intrinsic catalytic activity of the target protein. As an example of the former, after phosphorylation of tryptophan and tyrosine hydroxylases by calmodulin kinase II, the subsequent binding of 14-3-3 increases their activity. In the latter example, apoptosis signal-regulating kinase 1 (ASK-1) kinase activity is inhibited by binding of 14-3-3 (Zhang et al., 1999; Liu et al., 2001). Recently Subramanian et al. (2004) reported the interaction of ASK-1 with various isoforms of 14-3-3 proteins. 14-3-3 proteins also interact with the C-terminal end of exoenzyme S (Hallberg, 2002).
5. The binding of 14-3-3 can protect the target protein from proteolysis and dephosphorylation. The protection by 14-3-3 of Raf (Dent et al., 1995; Thorson et al., 1998), histone (Chen and Wagner, 1994), and BAD from dephosphorylation and plant nitrate reductase (Weiner and Kaiser, 1999) and several proteins in *Arabidopsis* (Cotelle et al., 2000) from proteolysis are examples.
6. Sometimes 14-3-3 exhibits more than one function in the regulation of a target. For example, 14-3-3 regulates both transcription factor DAF-16 localization and its intrinsic DNA-binding activity (Cahill et al., 2001).

## 9 Regulation of 14-3-3 in the Cell

The objective of many studies on the role of 14-3-3 in cellular regulation were the changes in target protein phosphorylation as the first regulatory event. The 14-3-3 protein was considered to be the passive element (Tzivion and Avruch, 2002). However, several potential types of 14-3-3 regulation such as isoform-specific expression, subcellular localization, phosphorylation regulation, and differential target protein-binding specificity may also be important (Hsu et al., 1997; Aitken, 2002). The 14-3-3 proteins are abundant, but it is doubtful whether there are adequate amounts of total 14-3-3 protein in vivo.

A recent idea about a mechanism for 14-3-3 regulation suggests that association of 14-3-3 with intermediate filaments such as vimentin may take place and may serve to isolate 14-3-3 proteins and to

modulate their function by limiting their availability to other target proteins. This raises the possibility that the 14-3-3 proteins may be limited despite their high level of expression in the cell, and that the changing distribution of its association with different target proteins may serve as one regulatory mechanism for modulating 14-3-3 availability.

Here we describe 14-3-3 regulation, laying stress on isoform-specific expression, phosphorylation, and subcellular localization.

## 9.1 Isoform-Specific Regulation

The functions of 14-3-3 isoforms were discussed by Skoulakis and Davis (1998), Fu et al. (2000), Tzivion and Avruch (2002), and Aitken (2002) in their reviews. According to their findings, the various 14-3-3 isoforms exhibit similar binding specificities. Since the residues lining the phosphopeptide-binding groove of the various 14-3-3 isoforms are markedly conserved, considerable overlap specificity would be predicted from the structure. However, differences in the abilities of the 14-3-3 isoforms to bind peptides and proteins have been described (Martin et al., 1994; Vincenz and Dixit, 1996; Tang et al., 1998; Van Der Hoeven et al., 2000; Muslin and Xing, 2000). There are also several examples of isoform-specific biological effects. For example, as Fu et al. described, overexpression of 14-3-3 σ caused a G2 cell cycle arrest in colorectal carcinoma cells, whereas 14-3-3 β overexpression did not. This result may be a consequence of preferential interaction of 14-3-3 with the Cdc2/cyclin B/complex, and may be due to differences in binding specificity and subcellular localization. However, the selective increase in the expression of the 14-3-3 σ isoform in the response to DNA damage highlights the physiological significance of this difference with respect to 14-3-3 σ and 14-3-3 β (Hermeking et al., 1997; Chan et al., 1999; Ferguson et al., 2000; Laronga et al., 2000; Suzuki et al., 2000). Most isoforms, except 14-3-3 σ, are almost equally expressed; however, 14-3-3 θ expression is different and under stimulus-dependent regulation (Perego and Berruti, 1997; Rosenquist et al., 2000).

Considering the existence of seven independently regulated 14-3-3 genes, the differential expression of 14-3-3 isoforms during development, the isoform-specific 14-3-3 levels in various subcellular localizations, and the moderate tendency toward heterodimerization in addition to homodimerization, one may suggest that small differences in binding specificity among isoforms can combine and generate a potentially active regulatory machine, when combined with heterodimerization and differential regulation of the level of expression of individual 14-3-3 isoforms (Dubois et al., 1997a, b).

## 9.2 Regulation by Phosphorylation

Aitken (2002) reported that phosphorylation of specific 14-3-3 isoforms can also regulate the interactions. Phosphorylation of 14-3-3 may be another regulatory mechanism (Dubois et al., 1997a, b), and 14-3-3 proteins were reported to be phosphorylated by several kinases (sphingosine-dependent protein kinase 1 (SDK 1) (Megidish et al., 1995, 1998), casein kinase 1 (Dubois et al., 1997a, b), and PKCs). As described previously, 14-3-3 α and δ are the phosphorylated forms of β and ζ, respectively. According to Aitken (2002) casein kinase 1 phosphorylates 14-3-3 ζ and τ isoforms. In 14-3-3 ζ, three phosphorylation sites have been determined. Recently Tsuruta et al. (2004) demonstrated that activated C-JunNH(2)-terminal kinase (JNK) promotes Bax translocation to mitochondria through phosphorylation of 14-3-3, a cytoplasmic anchor of Bax. However, the role of these phosphorylations in the physiologic regulation of 14-3-3 function is not yet clear.

## 9.3 Regulation by Subcellular Localization

Localization of 14-3-3 in the cytoplasm, nucleus, various membranes, and centrosome structures has been described (Ferl, 1996). However, except for the cytoplasmic/nuclear partition, the significance of such differential localization for 14-3-3 functions remains unclear (vanZeijl et al., 2002). Recently, Muslin and

Xing reported in a review that 14-3-3 proteins promote the cytoplasmic localization of many binding partner proteins (Muslin and Xing, 2000). Skoulakis and Davis (1998) described the role of 14-3-3 proteins in exocytosis in their review.

## 10 14-3-3 Proteins in Neuropsychiatric Diseases

Recently two reviews about 14-3-3 proteins were published (Berg et al., 2003a; Klein et al., 2003). These reviews summarized the studies on the relation between 14-3-3 proteins and some neurological diseases. *Table 12-2* in the review of Berg et al. (2003a, b) is very useful for examining the isotypes of 14-3-3

**Table 12-2**
**Known isotypes of 14-3-3 and results of isotype-specific investigations**

| 14-3-3 isotype | Gene symbol | Genomic localization | Isotype-specific CSF disorders | Isotype-specific histology | Animal models and experimental results |
|---|---|---|---|---|---|
| β and its phosphorylated isotype α | YWHAB | 20q13.1 | CJD (β) | Present in tangles in AD and associated with tau proteins (β)− | |
| Γ | YWHAG | 7q11.23 | CJD | Elevated in AD, no association with tau, reduction in Down's syndrome and present in LBs in PD | Increased in rat carotid arteries after injury |
| E | YWHAE | 17p13.3 | CJD | Elevated in AD, no association with tau and present in LBs in PD | |
| Z and its phosphorylated isotype δ | YWHAZ | 8q23.1 | | Present in tangles in AD, associated with tau and present in LBs in PD (ζ) | Upregulated in rat hypoglossal motor neurons after injury (ζ) |
| η | YWHAH | 22q12.3 | CJD, AD, other dementias, and herpes encephalitis | | |
| σ | SFN | 1p35.3 | | Only expressed in T cells and epithelial cells | Expression induced in response to DNA damage, might function as main tumor suppressor |
| T/θ | YWHAQ | 2p25.1 | | Present in LBs in PD | Upregulated in rat hypoglossal motor neurons after injury |

*AD* Alzheimer's disease
*CJD* Creutzfeldt–Jakob disease
*CSF* cerebrospinal fluid
*LBs* Lewy bodies
*PD* Parkinson's disease
Table 2 was used with the permission of Dr. D. Berg.

proteins and the results of isotype-specific investigation (❯ *Table 12-2)*. Berg et al. described detection of 14-3-3 proteins in the cerebrospinal fluid (CSF) of patients with the following disorders: meningoencephalitis, aseptic meningitis, central nervous system vasculitis, multiple sclerosis, stroke, subarachinoid hemorrhage, cerebral amyloid angiopathy, vascular dementia, frontotemporal dementia, amyotrophic lateral sclerosis (ALS) with dementia, brain tumors, paraneoplastic disorders, central intravascular lymphoma, carcinomatous meningitis, anoxic encephalopathy, Hashimoto's encephalopathy, mitochondrial encephalomyopathy. Furthermore, schizophrenia, Creutzfeldt–Jakob disease (CJD), Alzheimer's disease (AD), and neurodegenerative disorders with Lewy bodies, polyglutamine disorders, ALS, and motor neuron injury were shown to have some relation to 14-3-3 proteins as described below.

## 10.1 Schizophrenia

Two genomic clones that cover 15 kb of the 14-3-3 η chain gene were mixed and used as a probe. The hybridization signal appeared on band q12.1–q13.1 of chromosome 22. It was suggested that schizophrenia is related to chromosome 22; thus we examined the relation between the 14-3-3 η gene and schizophrenia using a 7-bp repeat sequence in the noncoding region of exon 1 of the 14-3-3 gene as a probe. We found evidence for a genetic association of the 14-3-3 η gene with schizophrenia (Toyooka et al., 1999). However, Bell et al. (2000) and Hayakawa et al. (1998) could not obtain evidence for polymorphism of the coding region in the 14-3-3 gene of schizophrenic patients. Thus, no definite conclusion has been obtained to date.

## 10.2 Creutzfeldt–Jakob Disease

The clinical symptoms of CJD are a rapidly progressive dementia and a combination of neurological symptoms, but the definite diagnosis can only be made using histopathological methods. Two proteins in the CSF of CJD patients were found to be potential markers. This result suggested that the presence of 14-3-3 proteins in the CSF may be due to destruction of brain tissue and leakage of cellular protein like 14-3-3 into CSF (Zerr et al., 1998).

The β, γ, ε, and η isotypes are present in the CSF of patients of CJD; however, the ζ isotype does not seem to be elevated. The ζ isotype is unequivocally immunostained in amyloid plaque of sporadic and variant CJD. Subcellularly, the β, γ, ε, η, and ζ isotypes have been found in synaptic vesicle membranes and some isotypes (γ, ε, and η) might be located at the synaptic junction and bind to the synaptic membrane.

## 10.3 Alzheimer's Disease

AD is neuropathologically characterized by cortical and perivascular amyloid plaques and neurofibrillary tangles (NFTs). The tangles are composed of paired helical filaments and microtubule-associated protein tau, which regulates normal assembly and disassembly of microtubules. One mechanism controlling microtubule structure and function is the phosphorylation of tau, which reduces its affinity for microtubules. In AD, abnormally phosphorylated tau associates with paired helical filaments, thus preventing normal microtubule-related functions.

14-3-3 γ and ε are increased in several regions of patient brains with AD. Further, according to Layfield et al. (1996), all cases of AD that they examined by immunohistochemistry showed positive 14-3-3 staining of NFTs. However, there was almost no staining with anti-14-3-3 in amyloid plaques. Recently, a "tau-phosphorylation complex" has been described. In bovine brain, tau, glycogen synthase kinase-3β (GSK3β), and 14-3-3 ζ are integral parts of this complex.

## 10.4 Neurodegenerative Disorders with Lewy Bodies

Lewy bodies are a pathological hallmark of Parkinson's disease (PD) and diffuse Lewy body disorder (DLBD) (Kawamoto et al., 2002; Ubl et al., 2002). Lewy bodies of PD are found in the brainstem (particularly in substantia nigra). Lewy bodies of DLBD are present in cortical and subcortical regions.

Immunohistochemical investigation of Lewy bodies showed positive 14-3-3 staining in PD and DLBD. Recently, colocalization of the ε, γ, ζ, and θ isotypes with Lewy bodies in PD was found.

One of the main features of PD is neuronal death of dopaminergic neuronal cells accompanied by a reduction of brain dopamine. The rate-limiting enzyme in dopamine synthesis is tyrosine hydroxylase (Ichimura et al., 1988), an enzyme reported to bind 14-3-3 proteins. Binding of 14-3-3 proteins to tyrosine hydroxylase is required for its optimal activation by phosphorylation. Recently, α-synuclein, one of the main components of Lewy bodies, was shown to bind to 14-3-3 proteins. Normally, α-synuclein reduces the activity of tyrosine hydroxylase by binding to dephosphorylated tyrosine hydroxylase. However, the binding of 14-3-3 proteins to phosphorylated tyrosine hydroxylase enhances dopamine synthesis, as described above. Xu et al. (2002) showed a selective increase of the 14-3-3/α–synuclein complex in the substantia nigra of PD patients.

## 10.5 Polyglutamine Disease

Recently an involvement of 14-3-3 proteins in neurodegenerative diseases caused by the expansion of polyglutamine stretches was shown in spinocerebellar ataxia type 1 (SCA1) (Chen et al., 2003). In the case of SCA1, ataxin 1 is stabilized by 14-3-3 proteins. Probably, ataxin 1 and 14-3-3 form soluble complexes in vivo. Binding of 14-3-3 to ataxin 1 needs ataxin 1 phosphorylation (Paulson et al., 1997).

A second connection between neurodegeneration and 14-3-3 proteins in polyglutamine disease comes from the following result (McCampbell et al., 2000). Transcription factors like CREB-binding protein (CBP), TATA-binding protein (TAB), and TAB-associated factor II-130 (TAFII130) are recruited into the nuclear aggregates. Some of these molecules are known to have histone acetyltransferese activity, an activity that is reversed by histone deacetylases. 14-3-3 proteins bind to histone deacetylases, sequestering them in the cytoplasm. Histone deacetylase inhibitors, such as valproate, might be effective in treating polyglutamine diseases.

## 10.6 ALS and Motor Neuron Injury

Pathological changes after motor neuron injury are obscure in many respects. However, 14-3-3 proteins seem to be involved in these processes. For example, a substantial increase of the mRNAs of 14-3-3 ζ and θ after hypoglossal nerve injury in rats could be shown in the injured motor neurons. Since one of the functions of 14-3-3 proteins is the regulation of Raf 1, the stimulation of genes involved in cell division through Raf 1 activation might be the result of 14-3-3 upregulation (Namikawa et al., 1998).

ALS is a fatal disorder that is characterized by the progressive selective death of upper and lower motor neurons. An upregulation of 14-3-3 isoforms was found in the spinal cord of ALS patients.

## 10.7 Neuronal Migration Defect

The importance of 14-3-3 proteins in the development of neuronal photoreceptors has been established in Drosophila. Disruption of neuronal differentiation and synaptic plasticity came from mutations in some 14-3-3 Drosophila gene. In humans, decreased levels of 14-3-3 γ in the cortex of an embryo with Down's syndrome were detected, indicating a possible impairment of neuronal differentiation, synaptic plasticity, and signal pathways. In some patients with severe lissencephaly, deletion of 17p13.3, the chromosomal segment containing 14-3-3 ε, has been observed.

Another review of neuropathological studies of the pineal body has been published by Klein et al. (2003). They reported the photoreception, neurotransmission, signal transduction, and the biosynthesis of melatonin from tryptophan as the function of the pineal body. Then they described in detail the regulatory effect of 14-3-3 on these pineal functions and the pathological changes of these functions.

## 11 Conclusions

This review summarizes the results of our research on 14-3-3 over the last 10 years, along with studies from other laboratories. Our work was mainly confined to cDNA cloning and nucleotide sequencing, the structure and expression of 14-3-3 genes, and in situ distribution of 14-3-3 mRNA in the brain. Work from other laboratories cited here include work on the binding of 14-3-3 to other proteins, the influence of 14-3-3 on the binding among other proteins, and the effects of 14-3-3 on protein kinase activities. At present, more than 100–200 target proteins of 14-3-3 are known. As a result of the recent rapid development of proteomic technology, many more target proteins of 14-3-3 will likely be discovered. In this review, I also describe some pathological conditions in which 14-3-3 proteins are implicated.

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## References

Aitken A. 1996. 14-3-3 and its possible role in co-ordinating multiple signaling pathways. Trends Cell Biol 6: 341-347.

Aitken A. 2002. 14-3-3 proteins in cell regulation. Biochem Soc Trans 30: 351-421.

Baxter HC, Liu WG, Forster JL, Aitken A, Fraser JR. 2002. Immunolocalization of 14-3-3 isoforms in normal and scrapie-infected murine brain. Neuroscience 109: 5-14.

Bell R, Munro J, Russ C, Powel JF, Bruinvels A, et al. 2000. Systematic screening of the 14-3-3 eta (η) chain gene for polymorphic variants and case-control analysis in schizophrenia. Am J Med Genet 96: 736-743.

Beranova-Giorgianni S, Pabst MJ, Russel TM, Giorgianni F, Goldowitz D, et al. 2002. Preliminary analysis of the mouse cerebellum proteome. Mol Brain Res 98: 135-140.

Berg D, Holzmann C, Riess O. 2003a. 14-3-3 proteins in the nervous system. Nature Rev Neurosci 4: 752-762.

Berg D, Riess O, Bornemiann A. 2003b. Specification of 14-3-3 proteins in Lewy bodies. Ann Neurol 54:135.

Bialkowska K, Zaffran Y, Meyer SC, Fox JEB. 2003. 14-3-3 ζ mediates integrin-induced activation of Cdc42 and Rac. J Biol Chem 278: 33342-33350.

Bogoch S. 1969. Proteins. *Handbook of neurochemistry.* Lajtha. A, editor. New York: Plenum Press; Vol. 1 pp. 75-92

Boston PF, Jackson P, Kynoch PAM, Thompson RJ. 1982a. Purification, properties and immunohistochemical localization of human brain 14-3-3 protein. J Neurochem 38: 1466-1474.

Boston PF, Jackson P, Thompson RJ. 1982b. Human 14-3-3 protein: radioimmunoassay, tissue distribution and cerebrospinal fluid levels in patients with neurological disorders. J Neurochem 38:1475-1482.

Brasselman S, McCormick F. 1995. BCR and RAF form a complex in vivo via 14-3-3 proteins. EMBO J 14: 4839-4848.

Cahill CM, Tzivion G, Nasrin N, Ogg S, Dore J, et al. 2001. Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3 dependent and 14-3-3 independent pathways. J Biol Chem 276: 13402-13410.

Chan TA, Hermeking H, Lenganer C, Kingler KW, Vogelstein B. 1999. 14-3-3 is required to prevent mitotic catastrophe after DNA damage. Nature 401: 616-620.

Chen HK, Fernandez-Funez P, Acevedo SF, Lam YC, Kaytor MD, et al. 2003. Interaction of Akt-phosphorylated

ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. Cell 113: 457-468.

Chen F, Wagner PD. 1994. 14-3-3 proteins bind to histone and affect both histone phosphorylation and dephosphorylation. FEBS Lett 347: 128-132.

Cotelle V, Meck SE, Provan F, Milne FC, Morrice N, et al. 2000. 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved Arabidopsis cells. EMBO J 19: 2869-2876.

Dalal SN, Schweitzer CM, Gan J, De- Caprio GA. 1999. Cytoplasmic localization of human cdc25c during interphase requires an intact 14-3-3 binding site. Mol Cell Biol 19: 4465-4479.

Datta SR, Katsov A, Hu L, Petros A, Fesik SW, et al. 2000. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell 6: 41-51.

Dent P, Jelinek T, Morrison DK, Weber MJ, Sturgill TW. 1995. Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. Science 268: 1902-1906.

Dougherty MK, Morrison DK. 2004. Unlocking the code of 14-3-3. J Cell Sci 117: 1875-1884.

Dubois T, Howell S, Amess B, Kerai P, Learmouth M, et al. 1997a. Structure and sites of phosphorylation of 14-3-3 protein: role in coordinating signal transduction pathways. J Protein Chem 16:513-522.

Dubois T, Rommel C, Howell S, Steinhussen U, Soneji Y, et al. 1997b. 14-3-3 is phosphorylated by casein kinase 1 on residue 233. Phosphorylation at this site in vivo regulates Raf/14-3-3 interaction. J Biol Chem 272: 28882-28888.

Erickson PF, Moore BW. 1980. Investigation of the axonal transport of three acidic, soluble proteins (14-3-2, 14-3-3 and S-100) in the rabbit visual system. J Neurochem 35: 232-241.

Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, et al. 2000. High frequency of hypermethylation at the 14-3-3 θ locus leads to gene silencing in breast cancer. Proc Natl Acad Sci USA 97: 6049-6054.

Ferl R. 1996. 14-3-3 proteins and signal transduction. Annu Rev Plant Physiol Plant Mol Biol 47: 49-73.

Finnie C, Borch J, Collinge DB. 1999. 14-3-3 proteins: eukaryotic regulatory proteins with many functions. Plant Mol Biol 40: 545-554.

Fu H, Subramanian RR, Masters SC. 2000. 14-3-3 proteins: structure, function and regulation. Annu Rev Pharmacol Toxicol 40: 617-647.

Furukawa Y, Ikuta N, Omata S, Yamauchi T, Isobe T, et al. 1993. Demonstration of the phosphorylation-dependent interaction of tryptophan hydroxylase with the 14-3-3 protein. Biochem Biophys Res Commun 194: 144-149.

Giles N, Forrest A, Gabrielli B. 2003. 14-3-3 acts as an intramolecular bridge to regulate Cdc25B. Localization and activity. J Biol Chem 278: 28580-28587.

Gordon Research Conference, 2004. In February 2004, a Gordon Research Conference on "Biology 14-3-3 proteins in the brain" took place in Ventura, CA, USA during 1 week. About 20 speakers gave a lecture about 14-3-3 proteins.

Grasso A, Roda G, Hogue-Angeletti RA, Moore BW, Perez VJ. 1977. Preparation and properties of the brain specific protein 14-3-2. Brain Res 124: 497-507.

Hallberg B. 2002. Exoenzyme S binds its cofactor 14-3-3 through a non-phosphorylated motif. Biochem Soc Trans 30: 401-405.

Hayakawa T, Ishiguro H, Toru M, Hamaguchi H, Arinami T. 1998. Systematic search for mutations in the 14-3-3 η chain gene on chromosome 22 in schizophrenia. Psychiatr Genet 8: 33-36.

Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, et al. 1997. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell 1: 3-11.

Hsu SY, Kaipia A, Zhu L, Hsueh AJ. 1997. Interference of BAD (Bcl-xL/Bcl-2-associated death promoter)-induced apoptosis in mammalian cells by 14-3-3 isoforms and P11. Mol Endocrinol 11: 1858-1867.

Ichimura T, Isobe T, Okuyama T, Takahashi N, Araki K, et al. 1988. Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. Proc Natl Acad Sci USA 85: 7084-7088.

Ichimura T, Isobe T, Okuyama T, Yamauchi T, Fujisawa H. 1987. Brain 14-3-3 protein is an activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of $Ca^{2+}$, calmodulin-dependent protein kinase II. FEBS Lett 219: 79-82.

Ichimura T, Kubota H, Goma T, Mizushima N, Ohsumi Y, et al. 2004. Transcriptomic and proteomic analysis of a 14-3-3 gene-deficient yeast. Biochemistry 43: 6149-6158.

Ichimura T, Sugano H, Kuwano R, Sunaya T, Okuyama T, et al. 1991. Widespread distribution of the 14-3-3 protein in vertebrate brains and bovine tissues: correlation with the distributions of calcium-dependent protein kinases. J Neurochem 56: 1449-1451.

Ichimura T, Uchiyama J, Kunihiro O, Ito M, Horigome T, et al. 1995. Identification of the site of interaction of the 14-3-3 protein with phosphorylated tryptophan hydroxylase. J Biol Chem 270: 28515-28518.

Ichimura T, Wakamiya-Tsuruta A, Itagaki C, Taoka M, Hayano T, et al. 2002. Phosphorylation-dependent interaction of kinesin light chain 2 and the 14-3-3 protein. Biochemistry 41: 5566-5572.

Ichimura-Ohshima Y, Morii K, Ichimura T, Araki K, Takahashi Y, et al. 1992. cDNA cloning and chromosome assignment of the gene for human brain 14-3-3 protein η chain. J Neurosci Res 31: 600-605.

Isobe T, Ichimura T, Sunaya T, Okuyama T, Takahashi N, et al. 1991. Distinct forms of the protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. J Mol Biol 217: 125-132.

Itagaki C, Isobe T, Taoka M, Natsume T, Nomura N, et al. 1999. Stimulus-coupled interaction of tyrosine hydroxylase with 14-3-3 protein. Biochemistry. 38: 15673-15680.

Kawamoto Y, Akiguchi I, Nakamura S, Honjo Y, Shibasaki H, et al. 2002. 14-3-3 proteins in Lewy bodies in Parkinson's disease and diffuse Lewy body disease brains. J Neuropathol Exp Neurol 61: 245-253.

Klein DC, Ganguly S, Coon SL, Shi Q, Gaildrat P, et al. 2003. 14-3-3 proteins in pineal photoneuroendocrine transduction: how many roles? J Neuroendocrinol 15:370-377.

Kovacinn KS, Park GY, Bae SS, Guzzetta AW, Schaefer E, et al. 2003. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. J Biol Chem 278: 10184-10194.

Kumagai A, Durphy WG. 1999. Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. Genes Dev 13: 1067-1072.

Laronga C, Yang HY, Neal C, Lee MH. 2000. Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. J Biol Chem 275: 23106-23112.

Layfield R, Fergusson J, Aitken A, Lowe J, London M, et al. 1996. Neurofibrillary tangles of Alzheimer's disease brains contain 14-3-3 proteins. Neurosci Lett 209: 57-60.

Lias J, Omary MB. 1996. 14-3-3 proteins associate with phosphorylated simple epithelial keratins during cell cycle progression and act as a solubility cofactor. J Cell Biol 133: 345-357.

Liu D, Bienkowska I, Petasa C, Collier RJ, Fu H, et al. 1995. Crystal structure of the zeta isoform of the 14-3-3 protein. Nature 376: 191-194.

Liu Y, Yin G, Suprapisichat J, Berk BC, Min W. 2001. Laminar flow inhibits TNF-induced ASK1 activation by preventing dissociation of ASK1 from its inhibitor 14-3-3. J Clin Invest 107: 917-923.

Lopez-Girona A, Furnari B, Mondesert O, Russell. P. 1999. Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature 397: 172-175.

Martin H, Rostas J, Patel Y, Aitken A. 1994. Subcellular localization of 14-3-3 isoforms in rat brain using specific antibodies. J Neurochem 63: 2259-2265.

McCampbell A, Taylor JP, Tage AA, Robitschek J, Li M, et al. 2000. CREB-binding protein sequestration by expanded polyglutamine. Hum Mol Genet 9: 2197-2202.

Megidish T, Corper J, Zhang L, Fu H, Hakomori S. 1998. A novel sphingosine-dependent protein kinase (SDK 1) specifically phosphorylates certain isoforms of 14-3-3 protein. J Biol Chem 273: 21834-21845.

Megidish T, White T, Takio K, Titani K, Igarashi Y, et al. 1995. The signal modulator protein 14-3-3 is a target of sphingosine- or *N*, *N*-dimethyl-sphingosine-dependent kinase in 3T3(A31) cells. Biochem Biophys Res Commun 216: 739-747.

Michaud NR, Fabian JR, Mathes KD, Morrison DK. 1995. 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-3-3 and Ras-independent manner. Mol Cell Biol 15: 3390-3391.

Moore BE. 1975. Brain-specific protein: S-100 protein, 14-3-2 protein, and glial fibrillary protein. *Advances in neurochemistry.* Agranoff BW, Aprison MH, editors. New York: Plenum Press; pp. 137-155.

Moore BE. 1983. Acidic proteins. *Handbook of neurochemistry.* Lajtha A, editor. Plenum Press; New York: Vol. I, pp. 93-99.

Moore BE, Perez VJ. 1968. Specific acidic proteins of the nervous system. *Physiological and biochemical aspects of nervous integration.* Carlson FD, editor. Englewood Cliffs, New Jersey: Prentice-Hall; pp. 343-359.

Morrison D. 1994. 14-3-3: modulators of signaling proteins? Science 266:56-57.

Müller J, Ritt DA, Copeland TD, Morrison DK. 2003. Functional analysis of c-TAK1 substrate binding and identification of PKP2 as a new c-TAK1 substrate. EMBO J 22: 4431-4442.

Muratake T, Hayashi S, Ichikawa T, Kumanishi T, Ichimura Y, et al. 1996. Structural organization and chromosomal assignment of the human 14-3-3 η chain gene (YWHAH). Genomics 36: 63-69.

Muratake T, Hayashi S, Ichimura Y, Morii K, Kuwano R, et al. 1995. The effect on methamphetamine on the mRNA level for 14-3-3 η chain in the human cultured cells. Mol Neurobiol 11: 223-230.

Muslin AJ, Tanner JW, Allen PM, Shaw AS. 1996. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell 84: 889-897.

Muslin AJ, Xing H. 2000. 14-3-3 proteins: regulation of subcellular localization by molecular interference. Cell Signal 12: 703-709.

Namikawa K, Su Q, Kiryu-Seo S, Kiyama H. 1998. Enhanced expression of 14-3-3 family members in injured motoneurons. Brain Res Mol Brain Res 55: 315-320.

Nestler EG, Hyman SE, Malenka RC. 2001. *Molecular neuropharmacology.* New York: McGraw-Hill.

O'Kelly I, Butler MH, Zilberborg N, Goldstein SAN. 2002. Forward transport: 14-3-3 binding overcomes retention in endoplasmic reticulum by dibasic signals. Cell 111: 577-588.

Paulson HL, Perez MK, Trottior Y, Trojonowski JQ, Subramony SH, et al. 1997. Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. Neuron 19: 333-344.

Pawson T, Scott JD. 1997. Signaling through scaffold, anchoring, and adaptor proteins. Science 278: 2075-2080.

Perego L, Berruti G. 1997. Molecular cloning and tissue-specific expression of the mouse homologue of the rat brain 14-3-3 θ protein: characterization of its cellular and developmental pattern if expression in male germ line. Mol Reprod Dev 47:370-379.

Powell DW, Rane MJ, Joughin BA, Kalmukova R, Hong J-H, et al. 2003. Proteomic identification of 14-3-3 ζ as a mitogen-activated protein kinase-activated protein kinase 2 substrate: role in dimer formation and ligand binding. Mol Cell Biol 23: 5376-5387.

Pozuelo Rubio M, Geraghty KM, Wong BHC, Wood NT, Campbell DG, et al. 2004. 14-3-3 affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. Biochem J 379: 395-408.

Rajan S, Preisig- Muller R, Wischmeyer E, Nehring R, Hanley PJ, et al. 2002. Interaction with 14-3-3 proteins promotes functional expression of potassium channels TASK-1 and TASK-3. J Physiol 545: 13-26.

Rishi AK, Zhang L, Boyanapolli M, Wali A, Mohammad RM, et al. 2003. Identification and characterization of a cell cycle and apoptosis signaling by retinoid CD437. J Biol Chem 278: 33422-33435.

Rittingers K, Budman G, Xu J, Volinia S, Cantley LC, et al. 1999. Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for nuclear export signal of 14-3-3 in ligand binding. Mol Cell 4: 153-166.

Roberts MR. 2000. Regulatory 14-3-3 protein–protein interactions in plant cells. Curr Opin Plant Biol 3: 400-405.

Rosenquist M, Seinke P, Ferl RJ, Sommarin M, Larsson C. 2000. Evolution of the 14-3-3 protein family: does the large number of isoforms in multicellular organisms reflect functional specificity? J Mol Evol 51:446-458.

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor.

Seimiya H, Sawada H, Muramatsu Y, Shimizu M, Ohko K, et al. 2000. Involvement of 14-3-3 proteins in nuclear localization of telomerase. EMBO J 19: 2652-2661.

Simpson RJ. 2003. Proteins and proteomics: a laboratory manual. New York: Cold Spring Harbor.

Simpson RJ. 2004. Purifying proteins for proteomics: a laboratory manual. New York: Cold Spring Harbor.

Skoulakis EM, Davis RL. 1998. 14-3-3 proteins in neuronal development and function. Mol Neurobiol 16: 269-284.

Subramanian RR, Zhang H, Wang H, Ichijo H, Miyashita T, et al. 2004. Interaction of apoptosis signal-regulating kinase 1 with isoforms of 14-3-3 proteins. Exp Cell Res 294: 581-591.

Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H, et al. 2000. Inactivation of the 14-3-3 θ gene is associated with 5′ CpG island hypermethylation in human cancer. Cancer Res 60: 4353-4357.

Takahashi Y. 1992. Gene expression in cells of the central nervous system. Prog Neurobiol 38: 523-569.

Takahashi Y. 2003. The 14-3-3 proteins: gene, gene expression and function. Neurochem Res 28: 1265-1273.

Tang SJ, Suen TC, McInnes RR, Buchwald M. 1998. Association of the TLX-2 homeodomain and 14-3-3 eta signaling proteins. J Biol Chem 273: 25356-25363.

Thorson JA, Yu LW, Hsu AL, Shih NY, Graves PR, et al. 1998. 14-3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. Mol Cell Biol 18: 5229-5238.

Toyooka K, Muratake T, Tanaka T, Igarashi S, Watanabe H, et al. 1999. 14-3-3 protein η chain gene (YWHAH) polymorphism and its genetic association with schizophrenia. Am J Med Genet 88: 164-167.

Toyooka K, Muratake T, Watanabe H, Hayashi S, Ichikawa T, et al. 2002. Isolation and structure of the mouse 14-3-3 η mRNA in the mouse brain. Mol Brain Res 100: 13-20.

Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, et al. 2004. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. EMBO J 23: 1889-1899.

Tzivion G, Avruch J. 2002. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem 277: 3061-3064.

Ubl A, Berg D, Holzmann C, Krüger R, Berger K, et al. 2002. 14-3-3 protein is a component of Lewy bodies in Parkinson's disease—mutation analysis and association studies of 14-3-3 eta. Mol Brain Res 108: 33-39.

Uchida S, Kuma A, Ohtsubo M, Shimura M, Hirata M, et al. 2004. Binding of 14-3-3 beta but not 14-3-3 sigma controls the cytoplasmic localization of CDC25B: binding site preference of 14-3-3 subtypes and the subcellular localization of CDC25B. J Cell Sci 117: 3011-3020.

Van Der Hoeven PC, Wal Van Der JC, Ruurs P, Van Dijk MC, Van Blitterswijk J. 2000. 14-3-3 isotypes facilitate coupling of protein kinase C-ζ to Raf-1: negative regulation by 14-3-3 phosphorylation. Biochem J 345: 297-306.

van Zeijl MJ, Testerink C, Kijne JW, Wang M. 2002. Subcellular differences in post-translational modification of barley 14-3-3 proteins. FEBS Lett 473: 292-296.

Vincenz C, Dixit VM. 1996. 14-3-3 proteins associate with A20 in an isoform-specific manner and function both as chaperone and adapter molecules. J Biol Chem 271: 20029-20034.

Watanabe M, Isobe T, Ichimura T, Kuwano R, Takahashi Y, et al. 1993a. Developmental regulation of neuronal expression for the η subtype of the 14-3-3 protein, a putative regulatory protein for protein kinase C. Dev Brain Res 73: 225-235.

Watanabe M, Isobe T, Ichimura T, Kuwano R, Takahashi Y, et al. 1993b. Molecular cloning of rat cDNAs for β and γ subtypes of 14-3-3 protein and developmental changes in expression of their mRNAs in the nervous system. Mol Brain Res 17: 135-146.

Watanabe M, Isobe T, Ichimura T, Kuwano R, Takahashi Y, et al. 1994. Molecular cloning of rat cDNAs for the ξ and θ mRNA in the brain. Mol Brain Res 25: 113-121.

Watanabe M, Isobe T, Okuyama T, Ichimura T, Kuwano R, et al. 1991. Molecular cloning of cDNA to rat 14-3-3 η chain polypeptide and the neuronal expression of the mRNA in the central nervous system. Mol Brain Res 10: 151-158.

Weiner H, Kaiser WM. 1999. 14-3-3 proteins control proteolysis of nitrate reductase in spinach leaves. FEBS Lett 455: 75-78.

Woods YL, Rena G. 2002. Effect of multiple phosphorylation events on the transcription factors FKHR, FKHDL1 and AFX. Biochem Soc Trans 30: 391-397.

Xiao B, Smerdon SJ, Jones DH, Dodson GG, Soneji Y, et al. 1995. Structure of a 14-3-3 protein and implications for coordination of multiple signaling pathways. Nature 376: 188-191.

Xu J, Kao S-Y, Lee FJS, Song W, Jin L-W, et al. 2002. Dopamine-dependent neurotoxicity of α-synuclein: a mechanism for selective neurodegeneration in Parkinson's disease. Nature Med 8:600-606.

Yaffe MB. 2004. Master of all things phosphorylated. Biochem J 379 (Pt 2): e1-e2.

Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, et al. 1997. The structural basis for 14-3-3: phosphopeptide binding specificity. Cell 91: 961-971.

Zah MB, Harada H, Yang E, Juckel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X (L). Cell 87: 619-628.

Zerr I, Bodemer M, Gefeller O, Otto M, Poser S, et al. 1998. Detection of 14-3-3 protein in the cerebrospinal fluid supports the diagnosis of Creutzfeldt-Jakob disease. Ann Neurol 43: 32-40.

Zhang L, Chen J, Fu H. 1999. Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins. Proc Natl Acad Sci USA 96: 8511-8515.

Zhou Y, Reddy S, Murrey H, Fei H, Levitan IB. 2003. Monomeric 14-3-3 protein is sufficient to modulate the activity of the Drosophila slowpoke calcium-dependent potassium channel. J Biol Chem 278: 10073-10080.

# 13 Heat Shock Proteins in Brain Function

*P. Csermely*

**Abstract:** Heat shock proteins are ubiquitous, highly conserved proteins helping the formation and repair of the correct conformation of other protein molecules. Cellular stress leads to heat shock protein (stress protein, molecular chaperone) induction, reflecting their protective role in cell survival. Heat shock proteins have a key importance in neuronal repair after brain damage, like trauma or stroke and in neurodegenerative diseases, such as in Alzheimer's, Parkinson's, and Huntington's type diseases. Because of the increasing amount of damaged proteins, heat shock proteins become overloaded during the aging process. This may lead to the release of heat shock protein-buffered, silent mutations, leading to the phenotypic exposure of previously hidden features and contributing to the onset of polygenic diseases such as neurodegenerative diseases. Heat shock protein induction and inhibition are promising pharmacological tools to protect neurons or to fight against brain tumors, respectively.

**List of Abbreviations:** ALS, amyotrophic lateral sclerosis; DnaJ, a co-chaperone of the 70-kDa heat shock protein, Hsp70; ER, endoplasmic reticulum; G-protein, small GTP-binding protein; Grp, glucose regulated protein; Hsc70, the constitutively expressed form of the 70-kDa heat shock protein, Hsp70; Hsp, heat shock protein; PU3, a purine-based inhibitor of the 90-kDa heat shock protein, Hsp90

## 1 Introduction

Protein folding has numerous steps, which need assistance in vivo. Heat shock proteins are required for many proteins to fold, or refold into native structures, for their oligomeric assembly and transport to their final destination inside the cell. This function is called chaperone function and, therefore, most heat shock proteins are also molecular chaperones. Heat shock proteins and their counterparts in the endoplasmic reticulum (and in mitochondria), glucose-regulated proteins form an ancient, primary system for "intracellular self-defense." Heat shock proteins have a profound importance in medical practice (Latchman, 1991; Welch, 1992; Hartl, 1996; Thirumalai and Lorimer, 2001). Their function is necessary for the homeostasis of the living cell, and becomes especially important in disease when our cells have to cope with a stressful environment. In damaged cells (such as in cells after heat shock), heat shock proteins will be up-regulated, which is an adaptive response of the cell to repair the increased amount of damaged proteins.

This chapter will briefly summarize and explain the role of heat shock proteins in cell survival, list a few of their recently uncovered specific functions, describe their role in neuroprotection, in the aging brain, in neurodegenerative diseases, and highlight some novel advances of heat shock protein-related medical therapies.

## 2 Heat Shock Proteins

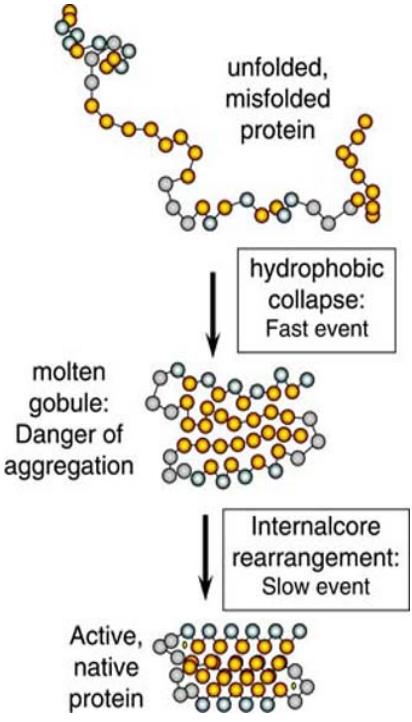
### 2.1 Definition and General Functions

Heat shock proteins help protein folding. In ❯ *Figure 13-1* the two major steps of a usual folding process is shown. First, a fast collapse of the nascent or misfolded protein structure occurs, which leads to the development of the hydrophobic core. Here the hydrophobic amino acids become buried and thus their disturbing effect on the hydrogen-bonded water structure is prevented. However, this process is often incomplete and results in a molten globule, having hydrophobic amino acids on its surface, which make it prone to aggregation. When aggregation occurs, the hydrophobic amino acids on the surface of the partially folded protein are forced to bind to each other since this is the only way by which their energetically costly interaction with the water structure can be avoided. The second step of protein folding is usually slow. Here the hydrophobic core is rearranged, which results in the formation of the native protein structure.

However, protein folding is not a straightforward process. Dead-end pathways, reverse reactions, futile cycles are all characteristic of protein folding. A minor amount of fully folded, native protein always coexists with various forms of molten globules and with traces of remaining unfolded molecules. This unordered flow of events needs a lot of help. Aggregation of unfolded proteins and of molten globules is a great danger

**Figure 13-1**

**Major steps of protein folding in vitro. First, a fast collapse of the protein structure occurs, which leads to the development of the hydrophobic core. However, this process is often incomplete and results in a molten globule, which is prone to aggregation. The second step is usually slow. Here the hydrophobic core is rearranged**




that would drive the majority of folding intermediates to a nonproductive side-reaction, much before reaching their fully folded, competent state. Heat shock proteins serve to prevent this. They recognize and cover hydrophobic surfaces, successfully competing with the aggregation process. However, there is an important difference here. Unlike aggregating partners, heat shock proteins can leave their complex with misfolded proteins, utilizing the energy of ATP-hydrolysis-driven conformational changes. This function is called as chaperone function; therefore most of heat shock proteins are often called molecular chaperones.

Heat shock proteins (1) protect other proteins against aggregation, (2) solubilize initial, loose protein aggregates, (3) assist in folding of nascent proteins or in refolding of damaged proteins, (4) target severely damaged proteins to degradation, and (5) in case of excessive damage, sequester damaged proteins to larger aggregates. Heat shock proteins are ubiquitous, highly conserved proteins, which utilize a cycle of ATP-driven conformational changes to re-fold their targets and which probably played a major role in the molecular evolution of modern enzymes (Hartl, 1996; Csermely, 1997, 1999; Thirumalai and Lorimer, 2001).

Cellular stress leads to the expression of heat shock proteins. Stress can be any sudden change in the cellular environment to which the cell is not prepared to respond, such as heat shock. However, almost all types of cellular stress induce heat shock proteins. Because of the generality of this phenomenon, heat shock proteins are often called stress proteins. The rationale behind this phenomenon is that after stress, there is an increased need for the chaperone function of heat shock proteins, which triggers their induction (Morimoto, 1998).

Heat shock proteins are best classified by their molecular weights, as there is significant overlap in their functions. Besides the major classes of heat shock proteins listed in *Table 13-1*, which generally target all

**Table 13-1**
**Major classes of Hsp-s**

| Most important representatives[a] | Recent reviews |
|---|---|
| Hsp25[b], Hsp27, crystallins, small heat shock proteins | Arrigo, 1998; Haslbeck, 2002; Ganea, 2001 |
| Hsp60, chaperonins | Bukau and Horwich, 1998 Hartl, 1996; Thirumalai and Lorimer, 2001 |
| Hsp70, Hsc70, Grp78 | Bukau and Horwich, 1998 Hartl, 1996; Ohtsuka and Suzuki, 2000 |
| Hsp90, Grp94 | Csermely et al., 1998; Picard, 2002; Pratt and Toft, 2003; Richter and Buchner, 2001; Sreedhar et al., 2004a; Young et al., 2001 |
| Hsp104 | Porankiewicz et al., 1999 |

[a]Neither the co-chaperones (chaperones which help the function of other chaperones listed), nor the so-called folding catalysts, the peptidyl-prolyl isomerases (immunophilins) and protein disulfide isomerases were included in this table, albeit almost all of these proteins also possess a ''traditional'' chaperone activity in their own right. Several chaperones of the endoplasmic reticulum (e.g. calreticulin, calnexin, etc.), which do not belong to any of the major chaperone families, as well as some heat shock proteins (e.g. ubiquitin), which do not possess chaperone activity were also not mentioned
[b]The abbreviation ''Hsp'' and ''Grp'' refer to heat shock proteins, and glucose-regulated proteins, chaperones induced by heat shock or glucose deprivation, respectively. Numbers refer to their molecular weight in kDa

misfolded proteins with hydrophobic surfaces, there are also specialized heat shock proteins, like Hsp47, which is the procollagen-chaperone (Nagata, 1998). Heat shock proteins usually increase only the yield but not the speed of protein folding. However, special chaperones, called "folding catalysts," may accelerate certain steps of protein folding, such as the isomerization of peptide bonds besides prolyl residues (peptidyl–prolyl *cis*/*trans* isomerases, or immunophilins) or the formation of disulfide bridges (protein disulfide isomerases) (Hartl, 1996; Bukau and Horwich, 1998).

Heat shock proteins never work alone. They always form a complex with each other and recruit various smaller proteins, called co-chaperones, which regulate their ATP-ase cycle, therefore increase the rate of heat shock protein-assisted refolding. A central chaperone complex of the cytoplasm is assembled around the 90-kDa heat shock protein, Hsp90, and is called as the foldosome.

Nascent proteins have to fold when they are not even ready yet. The first protein segment, which leaves the ribosome, has a different energy minimum than the whole protein. In many cases, in vivo protein folding has to be delayed. Heat shock proteins are attached to the ribosomes "waiting" for the nascent protein chain. When it appears, the chaperones "sit on it," preventing premature protein folding before the rest of the protein is synthesized (Kim and Baldwin, 1990; Matthews, 1993).

Heat shock proteins also direct proteins inside the cell. Pores of the mitochondria or of the endoplasmic reticulum are too small to accommodate fully folded, globular proteins. Proteins have to unfold to get through and refold in the lumen of the organelle (Chirico et al., 1988).

Heat shock proteins help both the folding and degradation of damaged proteins. After a few futile refolding attempts—most probably due to the extension of the transit time of the unfolded target protein with the heat shock protein molecule—heat shock proteins (such as Hsp90 or Hsp70) recruit novel co-chaperones (like CHIP or the neuronal DnaJ proteins, HSJ1a and HSJ1b) and present their target to the proteasome (Chapple et al., 2004; Urushitani et al., 2004; Whittier et al., 2004). The proteasomal system, in fact, degrades a large amount of newly folded proteins in eukaryotic cells, accomplishing a very tight quality control during the translational process (Turner and Varshavsky, 2000). The proteasome itself is also behaving as a molecular chaperone, since its "cap" has to unfold damaged proteins to be able to insert them to the tight cavity of the protease domain (Braun et al., 1999). Heat shock proteins are also involved in lysosome-related protein degradation, such as autophagocytosis (Chiang et al., 1989). In case of massive protein damage, when the amount of degradable proteins exceeds the capacity of the intracellular proteolytic systems, chaperones help to form inclusion bodies to segregate damaged proteins (Mayer et al., 1991).

## 2.2 Nonconventional Roles: Dustmen of the Cells

Heat shock proteins are regarded as molecular chaperones and their major cellular function is thought to be associated with their role in protein folding. However, most protein folding experiments are conducted in an in vitro environment. When protein folding is studied in vitro, the experimenter has to use rather diluted conditions to prevent unwanted aggregation. Dilution also helps to make the kinetic analysis easier and spares precious research materials. On the contrary to these usual experimental conditions, the cellular environment is crowded (Zimmerman and Minton, 1993). Molecular crowding promotes protein aggregation and thus calls for an enhanced need for chaperone action. On the other hand, bona fide chaperones are not the only cellular solutions for aggregation-protection. Several "innocent bystanders," such as tubulin (Guha et al., 1998) or even small molecules (lipids, other amphiphyles, sugars, a class of compounds called as chemical chaperones; Welch and Brown, 1996) may assist folding and prevent aggregation albeit at much higher concentrations than the efficient concentration of heat shock proteins. Though we have several important lines of evidence, which undoubtedly show the necessity of chaperones in folding of numerous protein kinases, receptors, actin, tubulin, etc. (Hartl, 1996) we do not really know to what an extent heat shock proteins are really used for protein folding in the eukaryotic cell, which is mostly settled to degrade and not to repair its cellular proteins due to the energy surplus obtained from the acquired mitochondria (Frydman et al., 1994).

With the above statements I do not want to question the importance of heat shock proteins in assisting protein folding. Nevertheless, I would like to stress that there is enough room to think about other important functions of heat shock proteins related to, but not equal to their participation in protein folding. One of these possibilities lies in the peptide-binding properties of heat shock proteins. Heat shock proteins may behave as the "dustmen" of our cells. The proteasomal apparatus is most probably linked with oligo- and dipeptidases and therefore the peptide-endproducts of proteasomal degradation (Kisselev et al., 1998) are usually cleaved further into single amino acids. However, the coupled protein–peptide degradation can leak, which may especially happen under stressed conditions like in oxidative stress. Released peptide segments may often contain elements of important binding sites and thus may efficiently interfere with signaling and metabolic processes. If this happened at a massive scale, this would be a disaster for the cell. Peptides need to be eliminated, and safeguarding mechanisms must exist to correct the occasional "sloppiness" of degradative processes. Heat shock proteins are excellent candidates for this purpose and their role in the collection of "peptide-rubbish" must be considered besides their well-established function in peptide presentation for the immune system (Srivastava et al., 1998). Heat shock protein-mediated sequestration of bioactive molecules can be especially important in the brain where neuropeptides play a prominent role in interneuronal signaling.

## 2.3 Nonconventional Roles: Organization of the Cytoarchitechture

As another important and nonconventional aspect of heat shock proteins lies in their incredibly high affinity for complex formation. Chaperones often form dimers and tend to associate to tetramers, hexamers, octamers, and to even higher oligomers (Benaroudj et al., 1996; Trent et al., 1997; Csermely et al., 1998). Oligomerization usually affects only a few percent of the total protein; but by addition of divalent cations and certain nucleotides, heat treatment enhances oligomer formation. It is important to note that oligomerization studies were usually performed under "normal" in vitro experimental conditions using a few μg/ml of purified chaperone. The in vivo concentration of chaperones is estimated to be around a 100- or 1000-fold higher. This may significantly enhance the in vivo oligomerization tendencies of these proteins. Oligomer formation of chaperones might be further promoted by the large excluded volume effect of the "molecularly crowded" cytoplasm (Zimmerman and Minton, 1993).

Different chaperones also associate with each other. The Hsp90-organized foldosome may contain almost a dozen independent chaperones, or co-chaperones. The stoichiometry and affinity of these associations dynamically varies, and the variations are affected by the folding state of the actual target (or targets), which associate with the extensive folding machinery (Kamal et al., 2003).

Besides binding to themselves, to their sibling-chaperones, and to their targets, many chaperones bind to actin filaments, tubulin, and other cellular filamentous structures such as intermediate filaments. There is a chaperone complex associated with the centrosome (Wigley et al., 1999) and several chaperones, especially Hsp90, were considered to be involved in the direction of cytoplasmic traffic (Pratt and Toft, 2003).

The above model describing chaperones as a highly dynamic "appendix" of various, and often quite poorly identifiable, cytoplasmic filamentous structures is reminiscent of the early view (Wolosewick and Porter, 1979; Schliwa et al., 1981) about the microtrabecular lattice of the cytoplasm. Although later studies efficiently questioned the validity of the original electronmicroscopic evidence of the microtrabeculae, pointing out many possibilities for artefact formation during sample preparation, several indirect evidence, such as diffusion anomalies support the existence of a cytoplasmic mesh-like structure (Clegg, 1984; Jacobson and Wojcieszyn, 1984; Luby-Phelps et al., 1988). The major cytoplasmic chaperones (Hsp90, TCP1/Hsp60 and their associated proteins) may well form a part of this network in cells (Csermely, 2001a).

Our experiments showing the acceleration of the efflux of cytoplasmic constituents after the inhibition of the major cytoplasmic heat shock protein, Hsp90, both in case of numerous cell lines (Pato et al., 2001; Csermely et al., 2003; Sreedhar et al., 2003, 2004b) suggest the involvement of the 90-kDa molecular chaperone, Hsp90, in the maintenance of the cytoarchitecture. Interestingly, we did not see an acceleration of cytoplasmic release in *Escherichia coli*, which is in agreement with the lower level of cytoplasmic organization of prokaryotes compared with eukaryotes. We cannot ascertain at the moment that the faster release of cytoplasmic proteins after the disruption of Hsp90 complexes by Hsp90 inhibitors or anti-Hsp90 ribozyme treatment is a consequence of a disrupted cytoplasmic meshwork or shows the involvement of Hsp90 in the stabilization of the "traditional" cytoskeleton. However, future experiments analyzing the distribution of Hsp90 in the cytoplasm after these treatments as well as changes in the intracellular diffusion rates might answer this question.

The possible involvement of heat shock proteins in the organization of the cytoplasm were interesting in neural cells all the more since these cells utilize the cytoarchitecture in all important aspects of their signaling, contacts, and memory formation.

## 2.4 Nonconventional Roles: Buffering of Silent Mutations

In the last few years, several experiments were published, which suggested that chaperones behave as "buffers of evolutionary changes." Chaperones seem to correct the conformational changes caused by various mutations and make the genetic changes phenotypically silent in various organisms studied (Rutherford and Lindquist, 1998; Roberts and Feder, 1999; Fares et al., 2002; Queitsch et al., 2002). However, if a large stress occurs, the suddenly increased amount of damaged proteins may cause a "chaperone-overload," and may prevent the conformational repair of misfolded mutants. Therefore many previously hidden genotypical changes may appear in the phenotype, resulting in a "boom" of genetical variations in the whole population. This may help the selection of a beneficial change, which, in turn, may help the adaptation of the population to changed environmental conditions. Nevertheless, most of the exposed mutations are disadvantageous and tend to disappear from the population by natural selection.

Changes in living conditions and the significantly better medical care throughout life in the last 150 years have significantly reduced the occurrence of large physiological stresses that would normally result in significant intracellular proteotoxicity. There is little "chaperone overload" during reproductive years in the present times. Even major stressful events such as critical infections and extreme and unexpected changes in the environment that do cause a massive "chaperone overload" can be mitigated by improved medical care, thus saving lives that would otherwise have been lost. More people harboring deleterious mutations survive today and transmit their genes to later generations. Thus improved medical care may have led to a rise in phenotypically silent mutations in the human genome. As a consequence we may be carrying more and more chaperone-buffered, silent mutations from generation to generation (Csermely, 2001b).

The chance of the phenotypic manifestation of these mutations becomes especially large in aged subjects, where protein damage is abundant, and both chaperone induction and chaperone function are

impaired (Sőti and Csermely, 2000, 2002). Here the background of misfolded proteins increases and by competition prevents the chaperone-mediated buffering of silent mutations. Phenotypically exposed mutations may contribute to a more abundant manifestation of multigene diseases, such as atherosclerosis, autoimmune-type diseases, cancer, diabetes, hypertensive cardiovascular disease, and several psychiatric illnesses (Alzheimer disease, schizophrenia, etc.). Chaperone overload might be even more pronounced in neuronal cells, where selective apoptosis and clonal expansion cannot play a kind of natural selection as it is the case with other somatic cells. Aging neurons may begin to display more and more unexpected features. For quite a while the robust behavior of the neural system covers these deleterious changes; however system resistance is gradually lost and dysfunction occurs (Csermely, 2001b, 2004).

Recently, several other proteins, such as yeast prions, p53, and many others were shown to buffer genetic changes (Scharloo, 1991; True and Lindquist, 2000; Aranda-Anzaldo and Dent, 2003). On basis of theoretical studies it was proposed that the number of buffering proteins is even larger (Bergman and Siegal, 2003). However, these proteins are not all chaperones. What can be common property? Comparing the known examples with other information on complex systems, it was suggested that the formation of low-affinity, weak links is the most important common feature of these proteins. Indeed, weak links were shown to stabilize many systems from single macromolecules up to the human society (Csermely, 2004; 2006).

## 3 Heat Shock Proteins and Brain Function

Heat shock proteins have a complex role in most cellular functions. To have a comprehensive survey on their involvement in brain function needs further investigations. However, a few elements of their putative brain function have been already uncovered. Hsc70, together with the synaptic vesicle cysteine string protein, a DnaJ homologue, forms a chaperone complex of synaptic vesicles and is involved in neurotransmitter release. Targets for this chaperone machine include the vesicle protein VAMP/synaptobrevin and the plasma membrane protein syntaxin 1 (Chamberlain and Burgoyne, 2000; Tobaben et al., 2001). Another major chaperone, Hsp90, is necessary for the efficient neurotransmitter release at the presynaptic terminal. Moreover, Hsp90 is a critical component of the cellular machinery that constitutively delivers glutamate receptors into the postsynaptic membrane (Gerges et al., 2004).

## 4 Heat Shock Proteins in Neuroprotection

If heat shock proteins are generally cytoprotective, their beneficial effects should be observed in various cases of neuronal damage. Indeed, overexpression of the 70-kDa heat shock protein protected neuronal cells from ischemic damage in an experimental stroke model (Hoehn et al., 2001), which was also observed in epileptic models (Yenari et al., 1998). A heat shock protein coinducer molecule protected both motor and sensory neurons after damage, where the beneficial effect was most probably due to the enhanced expression of heat shock proteins (Kalmar et al., 2002, 2003).

## 5 Heat Shock Proteins and the Aging Brain

### 5.1 Protein Damage During Aging

During the lifespan of a stable protein, various posttranslational modifications occur (Harding et al., 1989). These include deamidation of asparaginyl and glutaminyl residues and the subsequent formation of isopeptide bonds (Wright, 1991), protein glycation, methionine oxidation (Sun et al., 1999), etc. In several cases, age-related posttranslational modifications induce conformational changes and impair protein function: aging-induced inactivation of isocitrate-lyase (Reiss and Rothstein, 1974) or phosphoglycerate kinase (Yuh and Gafni, 1987) could be associated with the accumulation of a nonnative, heat labile conformation of the enzymes. In a refolding study, the increased helical content of "old" aldolase was

preserved after refolding of the enzyme, which suggested that the conformational changes were mostly induced by the various posttranslational modifications during the life of the protein (Demchenko et al., 1983).

## 5.2 Protein Degradation in Aging

Accumulating misfolded proteins due to their vulnerability for aggregation pose a great danger to the aging cell. Since the reason for the folding anomaly is mostly a posttranslational modification, the change becomes irreversible and cannot be reversed by molecular chaperones. Chaperones may only accompany these proteins, and by a stable association with their hydrophobic surfaces, prevent their aggregation. Thus the only solution to protect the cell from these misfolded proteins is their elimination and not their repair. Protein degradation is mostly accomplished by the proteasome and helped by various chaperones. Aging leads to a decrease in the activity of the major cytoplasmic proteolytic apparatus, the proteasome (Conconi et al., 1996; Heydari et al., 1994). Besides the decline in the activation of protease systems, some oxidized, glycated, and aggregated proteins are much poorer substrates, but highly effective inhibitors of the proteasome (Friguet et al., 1994; Bence et al., 2001; Bulteau et al., 2001). Autophagic lysosomal protein degradation is also impaired in aged rats (Cuervo and Dice, 2000), probably due to the lipofuscin-mediated inhibition of autophagy (Terman et al., 1999). All these events cause a massive accumulation of post-translationally modified, misfolded proteins. In most tissues, cells die after a large proteotoxic damage and other cells start to proliferate to take over their functions (Sőti et al., 2003). However, in the brain, it is much more difficult than in other tissues and due to the increased cell loss, malfunction develops.

## 5.3 Heat Shock Proteins in Aging Brain

Accumulation of misfolded proteins in aged organisms requires an increased amount of heat shock proteins to prevent protein aggregation. This may be the reason why some aged species develop a constitutively increased level of several chaperones, such as small heat shock proteins or Hsc70. On the other hand, a large number of reports demonstrate that the induction of various chaperones is impaired in aged organisms (Sőti and Csermely, 2000, 2002). Interestingly, while heat-induced synthesis of Hsp70 is impaired in aged rats, exercise in the same animal is able to induce a significant amount of Hsp70 (Kregel and Moseley, 1996).

The above general statements can be applied to chaperone levels and chaperone inducibility in the brain of aged organisms. Level of several chaperones, such as small heat shock proteins and Hsc70 is elevated, while the inducibility of Hsp70 is impaired (*Table 13-2*). In contrast to ad libitum fed rats, Hsc70 elevation could not be observed in food-restricted rats (Unno et al., 2000). Moreover, the brain of aged, food-restricted rats did not display a loss of capacity to accumulate Hsp70 in response to heat stress (Walters et al., 2001). This shows that calorie restriction, a well-known method to increase longevity

**Table 13-2**
**Chaperone expression in aging brain**

| Chaperone | Change | References |
|---|---|---|
| Chaperone levels | | |
| ubiquitin, Hsp27, αB-crystallin | Elevated in pallido-nigral spheroid bodies | Schultz et al., 2001 |
| Hsc70[a] | Elevated in pons, medulla, striatum, and thalamus | Unno et al., 2000 |
| Chaperone induction | | |
| Hsp70 | Heat induction is impaired | Rogue et al., 1993 |
| Hsp70 | Heat induction is maintained in food-restricted rats | Walters et al., 2001 |

[a]Hsc70 denotes the noninducible (cognate) form of Hsp70

(Hall et al., 2000; Ramsey et al., 2000), maintains the brain chaperone system in a "young state". On the other hand, rats maintained on a dietary restriction schedule exhibited increased resistance of hippocampal neurons and striatal neurons to excitotoxic and metabolic stress (Bruce-Keller et al., 1999). Calorie restriction also attenuated the degeneration of dopaminergic neurons in mouse Parkinson models (Duan and Mattson, 1999).

## 6 Heat Shock Proteins and Neurodegenerative Diseases

Accumulation of misfolded proteins in aged organisms is especially pronounced in postmitotic cells, such as in neurons. The threat of damaged proteins becomes even greater if the protein is protease-resistant. The difficulties of protein degradation, together with an impaired protease activity and chaperone action in aging neurons, lead to a massive accumulation of these proteins and cause neurodegeneration (Macario and Conway de Macario, 2001).

Oxidative damage and inflammatory processes are more prevalent during aging, accompany, and aggravate neurodegeneration (Goodman and Mattson, 1994; Gibson et al., 2000; Hemmer et al., 2001). Several molecular chaperones are involved in the maintenance of cellular redox status (Arrigo, 1998) and protect neurons against oxidative stress (Lee et al., 1999; Yu et al., 1999). However, a direct effect of chaperones on aging- or neurodegeneration-induced redox changes has not been demonstrated yet.

### 6.1 Alzheimer's Disease

The best-known example of folding-related neurodegenerative diseases is Alzheimer's disease. Several studies showed the induction of small heat shock proteins (Hsp27, crystallin), Hsp70 and ubiquitin (a 6-kDa heat shock protein, which labels damaged proteins and directs them for proteolytic degradation), in neurons affected by Alzheimer's disease and in surrounding astrocytes. The accumulation of Hsp90 and (to a smaller extent) Hsp60 was shown in the choroids plexus of brains affected with Alzheimer's disease. Neuronal chaperones were localized in neuritic plaques and neurofibrillary tangles (Hamos et al., 1991; Perez et al., 1991; Cisse et al., 1993; Renkawek et al., 1993; Shinohara et al., 1993; Anthony et al., 2003; Lukiw, 2004).

Accumulated chaperones participate in the heroic attempts of the affected neuron to sequester the β-amyloid and other damaged proteins in Alzheimer's disease (Hamos et al., 1991; Kouchi et al., 1999). However, the small heat shock protein, αB-crystallin, enhanced the neurotoxicity of the β-amyloid 1–40 peptide probably by keeping it in a nonfibrillar, highly toxic form (Stege et al., 1999). Cytoplasmic Hsp60, a specific chaperone for actin and tubulin, is decreased in Alzheimer's disease-affected neurons, leaving the cytoskeletal proteins deficient and aggregated (Schuller et al., 2001). Nonaffected nerve cells of Alzheimer victims, such as olfactory neurons (Getchell et al., 1995), also showed a decreased expression of Hsp70.

The pathologically hyperphosphorylated tau protein is often associated with β-amyloid fibers. Hsp27 has been shown to bind the hyperphosphorylated tau protein preferentially. The formation of this complex altered the conformation of pathological, hyperphosphorylated tau and reduced its concentration by facilitating its degradation and dephosphorylation. Hsp27 also prevented pathological hyperphosphorylated tau-mediated cell death (Shimura et al., 2004).

Since the amyloid precursor is an integral protein of the plasma membrane, which is usually processed in the endoplasmic reticulum (ER), the ER might be an especially important site for the fight for cell survival. Indeed, calreticulin, an abundant ER chaperone was shown to participate in the quality control of the amyloid precursor protein (Johnson et al., 2001) and the ER-homologue of Hsp70, Grp78, had an increased expression in successfully surviving neurons (Hamos et al., 1991). There are reports to show that mutant presenilin-1, an ER transmembrane protein being the most prevalent cause of early-onset familial Alzheimer's disease, impairs the ER chaperone response and thus sensitizes the affected neuron to apoptosis. However, this latter finding could not be confirmed in other systems (Lee, 2001).

### 6.2 Parkinson's Disease

Parkinson's disease is an age-related disorder characterized by a progressive degeneration of dopaminergic neurons in the substantia nigra and shows a corresponding motor deficit. An increasing number of evidence shows that besides oxidative stress and mitochondrial dysfunction, protein folding defects are also key elements of Parkinson's disease etiology. Glial and astroglial cells of Parkinson's disease victims showed the expression of αB-crystallin, as seen in Alzheimer's disease, and aggregated proteins in Lewy bodies had a large content of various heat shock proteins, as observed in neurofibrillary tangles (Jellinger, 2000). Dietary restriction induced an expression of Hsp70 and Grp78 parallel with a protection in a Parkinson's disease model (Duan and Mattson, 1999). Interestingly, parkin, the protein whose mutations cause the autosomal recessive juvenile parkinsonism was identified as an ubiquitin-ligase, playing a key role in the degradation of ER-misfolded proteins, such as a G-protein-coupled membrane receptor, called Pael, and synphilin, an α-synuclein interacting protein (Chung et al., 2001; Imai et al., 2001). This gives us one more example for the similarities of the protein-folding homeostasis in Parkinson's and Alzheimer's diseases.

### 6.3 Huntington's Disease

Polyglutamine repeats make proteins vulnerable for aggregation. Diseases such as Huntington's disease, Kennedy spinal bulbar muscular atrophy, spinocerebral ataxia, Machado-Joseph disease all develop due to an expansion of polyglutamine segments in the respective proteins. Chaperones colocalize with the aggregates of these polyglutamine-containing proteins and increased chaperone levels such as that of Hsp40, Hsp60, Hsp70, Hsc70, Hsp100 inhibit polyglutamine-containing protein aggregation and slow down the progress of the disease (Cummings et al., 1998; Krobitsch and Lindquist, 2000; Carmichael et al., 2000; Hughes and Olson, 2001).

## 7 Heat Shock Protein-Related Therapeutic Approaches

The beneficial role of heat shock proteins in neuronal survival and their protection against various insults including ischemia, excitatory damage as well as as various forms of neurodegeneration make them prominent therapeutic targets. However, heat shock proteins are not only protecting damaged neurons, but they also protect malignantly transformed neural cells. Here the inhibition of neural heat shock proteins might be a good approach to fight against brain tumors.

### 7.1 Heat Shock Protein Inhibition: Brain Tumors

When heat shock proteins protect our malignant cells, they are not really beneficial. Still the inhibition of proteins, which have a profound role in the survival of all cells, seems to be a wild idea. However, if we consider that heat shock proteins are necessary for the folding of cyclin-dependent kinases and numerous other proteins which are upregulated in cancer (Neckers, 2003; Workman, 2004) and some of the heat shock protein inhibitors are selectively enriched in tumor cells (Chiosis et al., 2003) as well as selectively interact with tumor-specific forms of heat shock proteins (Kamal et al., 2003), we begin to believe that heat shock protein inhibition might be a valid pharmacological intervention against tumors. Indeed, heat shock protein inhibitors are currently in clinical trials against various forms of cancer (Neckers, 2003; Workman, 2004).

Since the 90-kDa molecular chaperone (Hsp90) has the most specific and most cell-permeable inhibitors and since this chaperone is the center of the kinase-related chaperone machinery, in most cases chaperone-based inhibition is achieved by using Hsp90 inhibitors. The first Hsp90 inhibitor drug was geldanamycin, a natural product isolated from *Streptomyces hygroscopicus*. Though the antitumor effects of geldanamycin were initially thought to be due to specific tyrosine kinase inhibition, later studies revealed

that the antitumor potential relies on depletion of oncogenic protein kinases via the proteasome (Whitesell et al., 1994). The major regulatory signaling proteins, which are affected by geldanamycin, include the protooncogene kinases ErbB2, EGF, v-Src, Raf-1, and Cdk4 (Neckers, 2003; Workman, 2004). Radicicol, another Hsp90 inhibitor (Soga et al., 1998), is a macrocyclic antibiotic isolated from *Monosporium bonorden*. As a recent development, PU3, a purine-based Hsp90 inhibitor was designed using X-ray crystallographic data. PU3 behaves like geldanamycin in inhibiting Hsp90 client protein degradation, and possesses a robust antitumor potential (Chiosis et al., 2002). Recently it was shown that Hsp90 contains a second nucleotide-binding site at the C-terminal domain (Marcu et al., 2000; Garnier et al., 2002; Sőti et al., 2002), which opens up new possibilities to develop Hsp90 inhibitors.

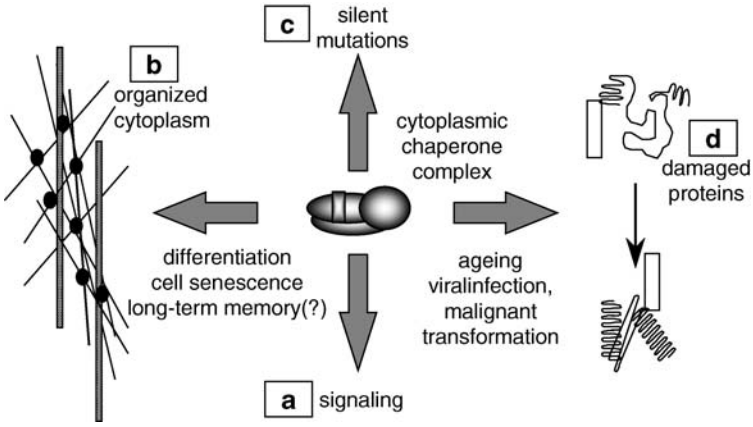
In agreement with the above considerations, the overexpression of Hsp27 and other small heat shock proteins has been found in gliomas and in other types of brain tumors (Hitotsumatsu et al., 1996; Zhang et al., 2003). Geldanamycin was shown to be effective in the treatment of medulloblastomas (Calabrese et al., 2003), gliomas, and glioblastomas (Yang et al., 2001; Zagzag et al., 2003).

## 7.2 Heat Shock Protein Induction: Brain Damage and Neurodegenerative Diseases

Induction of heat shock proteins is a part of cellular self-defense, which is mobilized in most disease states, e.g. by fever. However, heat shock protein induction may not be enough or the chronic disease may attenuate the level of induction. Heat shock protein induction becomes especially aggravated in aged organisms as described before. Because of these reasons, it was highly beneficial to help the expression of heat shock proteins. Several common drugs, such as aspirin (Jurivich et al., 1992), promote the induction of heat shock proteins; however, recently a specific heat shock protein coinducer drug family (Vígh et al.,

**Figure 13-2**

**Competition for chaperone occupancy and its changes in the ageing process. Clockwise from bottom: *a*, Cytoplasmic chaperones of eukaryotic cells participate in the maintenance of the conformation of some selected protein substrates. Most of these unstable proteins are parts of various signaling cascades (Csermely et al., 1998; Pratt et al., 1999). *b*, Chaperones form low-affinity and highly dynamic extensions of the cytoskeleton participating in cellular traffic and in the organization of the cytoarchitecture (Csermely, 2001a; Pratt et al., 1999). *c*, phenotypically buffered, silent mutations require the assistance of chaperones to rescue them from folding traps (Rutherford and Lindquist, 1998; Csermely, 2001b). *d*, During the aging process, chaperones become more and more occupied by damaged proteins. As a consequence of this (a) signaling is impaired silent, (b) cell architecture becomes disorganized, and (c) mutations escape and contribute to the onset of polygenic diseases. The verification of these – presently largely hypothetical – changes requires further experimentation**



1997; Török et al., 2003) extending the duration of DNA binding by the specific transcription factor inducing heat shock proteins (Hargitai et al., 2003) has also been described. These drug candidates work only in stressed cells, which already started the induction of heat shock proteins themselves. They protected both motor and sensory neurons after damage where the beneficial effect was most probably due to the enhanced expression of heat shock proteins (Kalmar et al., 2002, 2003). Moreover, heat shock protein coinducer drugs were shown to improve the conditions of superoxide dismutase mutant mice, which develop the symptoms of amyotrophic lateral sclerosis (ALS) or Lou Gehrig's disease (Kieran et al., 2004).

## 8 Closing Remarks

Numerous key elements of cellular life are competing with each other for the maintenance and repair function of heat shock proteins (damaged proteins, signaling proteins, silent mutations, and cytoarchitecture, see *Figure 13-2*). Therefore, heat shock proteins emerge as a central switchboard of the integration of cellular homeostasis. Their induction is highly beneficial to protect neurons against oxidative or neuroexcitatory damage. On the contrary, heat shock protein inhibition may be a promising tool to fight against brain tumors. I hope that with this short review I may increase the courage of some fellow scientists to enter this difficult, but very promising path of multidisciplinary research.

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## References

Anthony SG, Schipper HM, Tavares R, Hovanesian V, Cortez SC, et al. 2003. Stress protein expression in the Alzheimer-diseased choroid plexus. J Alzheimers Dis 5: 171-177.

Aranda-Anzaldo A, Dent MA. 2003. Developmental noise, ageing and cancer. Mech Ageing Dev 124: 711-720.

Arrigo AP. 1998. Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. Biol Chem 379: 19-26.

Benaroudj N, Triniolles F, Ladjimi MM. 1996. Effect of nucleotides, peptides, and unfolded proteins on the self-association of the molecular chaperone HSC70. J Biol Chem 271: 18471-18476.

Bence NF, Sampat RM, Kopito RR. 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. Science 292: 1552-1555.

Bergman A, Siegal ML. 2003. Evolutionary capacitance as a general feature of complex gene networks. Nature 424: 549-551.

Braun BC, Glickman M, Kraft R, Dahlmann B, Kloetzel PM, et al. 1999. The base of the proteasome regulatory particle exhibits chaperone-like activity. Nat Cell Biol 1: 221-226.

Bruce-Keller AJ, Umberger G, McFall R, Mattson MP. 1999. Food restriction reduces brain damage and improves behavioral outcome following excitotoxic and metabolic insults. Ann Neurol 45: 8-15.

Bukau B, Horwich AL. 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92: 351-366.

Bulteau A-L, Verbeke P, Petropoulos I, Chaffotte A-F, Friguet B. 2001. Proteasome inhibition in glyoxal-treated fibroblasts and resistence of glycated glucose-6-phosphate dehydrogenase to 20S proteasome degradation in vitro. J Biol Chem 276: 30057-30063.

Calabrese C, Frank A, Maclean K, Gilbertson R. 2003. Medulloblastoma sensitivity to 17-allylamino-17-demethoxygeldanamycin requires MEK/ERKM. J Biol Chem 278: 24951-24959.

Carmichael J, Chatellier J, Woolfson A, Milstein C, Fersht AR, et al. 2000. Bacterial and yeast chaperones reduce both aggregate formation and cell death in mammalian cell models of Huntington's disease. Proc Natl Acad Sci USA 97: 9701-9705.

Chamberlain LH, Burgoyne RD. 2000. Cysteine-string protein: the chaperone at the synapse. J Neurochem 74: 1781-1789.

Chapple JP, van der Spuy J, Poopalasundaram S, Cheetham ME. 2004. Neuronal DnaJ proteins HSJ1a and HSJ1b: a

role in linking the Hsp70 chaperone machine to the ubiquitin-proteasome system? Biochem Soc Trans 32: 640-642.

Chiang H-L, Terlecky SR, Plant CP, Dice JF. 1989. A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science 246: 382-385.

Chiosis G, Lucas B, Shtil A, Huezo H, Rosen N. 2002. Development of a purine-scaffold novel class of Hsp90 binders that inhibit the proliferation of cancer cells and induce the degradation of Her2 tyrosine kinase. Bioorg Med Chem 10: 3555-3564.

Chiosis G, Huezo H, Rosen N, Mimnaugh E, Whitesell L, et al. 2003. 17AAG low target binding affinity and potent cell activity – finding an explanation. Mol Cancer Therap 2: 123-129.

Chirico WJ, Waters MG, Blobel G. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. Nature 332: 805-810.

Chung KK, Zhang Y, Lim KL, Huang H, Gao J, et al. 2001. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med 7: 1144-1150.

Cisse S, Perry G, Lacoste-Royal G, Cabana T, Gauvreau D. 1993. Immunochemical identification of ubiquitin and heat-shock proteins in corpora amylacea from normal aged and Alzheimer's disease brains. Acta Neuropathol (Berl)85: 233-240.

Clegg JS. 1984. Properties and metabolism of the aqueous cytoplasm and its boundaries. Am J Physiol 246: R133-R151.

Conconi M, Szweda LI, Levine RL, Stadtman ER, Friguet B. 1996. Age-related decline of rat liver multicatalytic proteinase activity and protection from oxidative inactivation by heat shock protein 90. Arch Biochem Biophys 331: 232-240.

Csermely P. 1997. Proteins, RNA-s, chaperones and enzyme evolution: a folding perspective. Trends Biochem Sci 22: 147-149.

Csermely P. 1999. The "chaperone-percolator" model: a possible molecular mechanism of Anfinsen-cage type chaperone action. Bioessays 21: 959-965.

Csermely P. 2001a. A nonconventional role of molecular chaperones: involvement in the cytoarchitecture. News Physiol Sci 15: 123-126.

Csermely P. 2001b. Chaperone-overload as a possible contributor to "civilization diseases": atherosclerosis, cancer, diabetes. Trends Genet 17: 701-704.

Csermely P. 2004. Strongs links are important – but weak links stabilize them. Trends Biochem Sci 29: 331-334.

Csermely P. 2006. Weak links: Stabilizers of Complex Systems from Proteins to Social Networks, Springer Verlag, Heidelberg.

Csermely P, Schnaider T, Sőti Cs, Prohászka Z, Nardai G. 1998. The 90 kDa molecular chaperone family: structure, function and clinical applications. A comprehensive review Pharmacol Ther 79: 129-168.

Csermely P, Sőti C, Kalmar E, Papp E, Pato B, et al. 2003. Molecular chaperones, evolution and medicine. J Mol Struct Theochem 666–667: 373-380.

Cuervo AM, Dice JF. 2000. Age-related decline in chaperone-mediated autophagy. J Biol Chem 275: 31505-31513.

Cummings CJ, Mancini MA, Antalfy B, De Franco DB, Orr HT, et al. 1998. Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA 1. Nat Genet 19: 148-154.

Demchenko AP, Orlovska NN, Sukhomudrenko AG. 1983. Age-dependent changes of protein structure. The properties of young and old rabbit aldolase are restored after reversible denaturation. Exp Gerontol 18: 437-446.

Duan W, Mattson MP. 1999. Dietary restriction and 2-deoxyglucose administration improve behavioral outcome and reduce degeneration of dopaminergic neurons in models of Parkinson's disease. J Neurosci Res 57: 195-206.

Fares MA, Riuz-Gonzalez MX, Moya A, Elena SF, Barrio E. 2002. Endosymbiotic bacteria: GroEL buffers against deleterious mutations. Nature 417: 398.

Friguet B, Stadtman ER, Szweda LI. 1994. Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. Formation of cross-linked protein that inhibits the multicatalytic protease. J Biol Chem 269: 21639-21643.

Frydman J, Nimmesgern E, Ohtsuka K, Hartl FU. 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. Nature 370: 111-117.

Ganea E. 2001. Chaperone-like activity of alpha-crystallin and other small heat shock proteins. Curr Protein Pept Sci 2: 205-225.

Garnier C, Lafitte D, Tsvetkov PO, Barbier P, Leclerc-Devin J, et al. 2002. Binding of ATP to heat shock protein 90: evidence for an ATP-binding site in the C-terminal domain. J Biol Chem 277: 12208-12214.

Gerges NZ, Tran IC, Backos DS, Harrell JM, Chinkers M, et al. 2004. Independent functions of hsp90 in neurotransmitter release and in the continuous synaptic cycling of AMPA receptors. J Neurosci 24: 4758-4766.

Getchell TV, Krishna NS, Dhooper N, Sparks DL, Getchell ML. 1995. Human olfactory receptor neurons express heat shock protein 70: age-related trends. Ann Otol Rhinol Laryngol 104: 47-56.

Gibson GE, Park LC, Sheu KF, Blass JP, Calingasan NY. 2000. The alpha-ketoglutarate dehydrogenase complex in neurodegeneration. Neurochem Int 36: 97-112.

Goodman Y, Mattson MP. 1994. Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury. Exp Neurol 128: 1-12.

Guha S, Manna TK, Das KP, Bhattacharya B. 1998. Chaperone-like activity of tubulin. J Biol Chem 273: 30077-30080.

Hall DM, Oberley TD, Moseley PM, Buettner GR, Oberley LW, et al. 2000. Caloric restriction improves thermotolerance and reduces hyperthermia-induced cellular damage in old rats. FASEB J 14: 78-86.

Hamos JE, Oblas B, Pulaski-Salo D, Welch WJ, Bole DG, et al. 1991. Expression of heat shock proteins in Alzheimer's disease. Neurology 41: 345-350.

Harding JJ, Beswick HT, Ajiboye R, Huby R, Blakytny R, et al. 1989. Non-enzymatic post-translational modification of proteins in aging. A review. Mech Ageing Dev 50: 7-16.

Hargitai J, Lewis H, Boros I, Rácz T, Fiser A, et al. 2003. Bimoclomol, a heat shock protein co-inducer acts by the prolonged activation of heat shock factor-1. Biochem Biophys Res Commun 307: 689-695.

Hartl F-U. 1996. Molecular chaperones in cellular protein folding. Nature 381: 571-580.

Haslbeck M. 2002. sHsps and their role in the chaperone network. Cell Mol Life Sci 59: 1649-1657.

Hemmer K, Fransen L, Vanderstichele H, Vanmechelen E, Heuschling P. 2001. An in vitro model for the study of microglia-induced neurodegeneration: involvement of nitric oxide and tumor necrosis factor-alpha. Neurochem Int 38: 557-565.

Heydari AR, Takahashi R, Gutsmann A, You S, Richardson A. 1994. Hsp70 and aging. Experientia 50: 1092-1098.

Hitotsumatsu T, Iwaki T, Fukui M, Tateishi J. 1996. Distinctive immunohistochemical profiles of small heat shock proteins (heat shock protein 27 and alpha B-crystallin) in human brain tumors. Cancer 77: 352-361.

Hoehn B, Ringer TM, Xu L, Giffard RG, Sapolsky RM, et al. 2001. Overexpression of HSP72 after induction of experimental stroke protects neurons from ischemic damage. J Cereb Blood Flow Metab 21: 1303-1309.

Hughes RE, Olson JM. 2001. Therapeutic opportunities in polyglutamine disease. Nat Med 7: 419-423.

Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, et al. 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 105: 891-902.

Jacobson K, Wojcieszyn J. 1984. The translational mobility of substances within the cytoplasmic matrix. Proc Natl Acad Sci USA 81: 6747-6751.

Jellinger KA. 2000. Cell death mechanisms in Parkinson's disease. J Neural Transm 107: 1-29.

Johnson RJ, Xiao G, Shanmugaratnam J, Fine RE. 2001. Calreticulin functions as a molecular chaperone for the beta-amyloid precursor protein. Neurobiol Aging 22: 387-395.

Jurivich DA, Sistonen L, Kroes RA, Morimoto RI. 1992. Effect of sodium salicylate on the human heat shock response. Science 255: 1243-1245.

Kalmar B, Burnstock G, Vrbova G, Urbanics R, Csermely P, et al. 2002. Upregulation of heat shock proteins rescues motoneurones from axotomy-induced cell death in neonatal rats. Exp Neurol 176: 87-97.

Kalmar B, Greensmith L, Malcangio M, Mac Mahon SB, Csermely P, et al. 2003. The effect of treatment with BRX-220, a co-inducer of heat shock proteins, on sensory fibres of the rat following peripheral nerve injury. Exp Neurol 184: 636-647.

Kamal A, Thao L, Sensintaffar J, Zhang L, Boehm MF, et al. 2003. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature 425: 407-410.

Kieran D, Kalmar B, Dick JRT, Riddoch-Contreras J, Burnstock G, et al. 2004. Treatment with Arimoclomol, a co-inducer of heat shock proteins, delays disease progression in ALS mice. Nat Med 10: 402-405.

Kim PS, Baldwin RL. 1990. Intermediates in the folding reactions of small proteins. Annu Rev Biochem 59: 631-660.

Kisselev AF, Akopian TN, Goldberg AL. 1998. Range of sizes of peptide products generated during degradation of different proteins by archaeal proteasomes. J Biol Chem 273: 1982-1989.

Kouchi Z, Sorimachi H, Suzuki K, Ishiura S. 1999. Proteasome inhibitors induced the association of Alzheimer's amyloid precursor protein with Hsc73. Biochem Biophys Res Commun 254: 804-810.

Kregel KC, Moseley PL. 1996. Differential effects of exercise and heat stress on liver hsp70 accumulation with aging. J Appl Physiol 80: 547-551.

Krobitsch S, Lindquist S. 2000. Aggregation of huntingtin in yeast varies with the length of the polyglutamine expansion and the expression of chaperone proteins. Proc Natl Acad Sci USA 97: 1589-1594.

Latchman DS. 1991. Heat shock proteins and human disease. JR Coll Physicians Lond 25: 295-299.

Lee AS. 2001. The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem Sci 26: 504-510.

Lee J, Bruce-Keller AJ, Kruman Y, Chan SL, Mattson MP. 1999. 2-Deoxy-D-glucose protects hippocampal neurons against excitotoxic and oxidative injury: evidence for the involvement of stress proteins. J Neurosci Res 57: 48-61.

Luby-Phelps K, Lanni F, Taylor DL. 1988. The submicroscopic properties of cytoplasm as a determinant of cellular function. Annu Rev Biophys Biophys Chem 17: 369-396.

Lukiw WJ. 2004. Gene expression profiling in fetal, aged, and Alzheimer hippocampus: a continuum of stress-related signaling. Neurochem Res 29: 1287-1297.

Macario AJL, Conway de Macario E. 2001. Molecular chaperones and age-related degenerative disorders. Adv Cell Aging Gerontol 7: 131-162.

Marcu MG, Chadli A, Bouhouche I, Catelli M, Neckers LM. 2000. The heat shock protein 90 antagonist novobiocin

interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. J Biol Chem 275: 37181-37186.

Matthews CR. 1993. Pathways of protein folding. Annu Rev Biochem 62: 653-683.

Mayer RJ, Arnold J, Laszlo L, Landon M, Lowe J. 1991. Ubiquitin in health and disease. Biochim Biophys Acta 1089: 141-157.

Morimoto RI. 1998. Regulation of the heat shock transcriptional response: Cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev 12: 3788-3796.

Nagata K. 1998. Expression and function of heat shock protein 47: a collagen-specific molecular chaperone in the endoplasmic reticulum. Matrix Biol 16: 379-386.

Neckers L. 2003. Development of small molecule Hsp90 inhibitors: utilizing both forward and reverse chemical genomics for drug identification. Curr Med Chem 10: 733-739.

Ohtsuka K, Suzuki T. 2000. Roles of molecular chaperones in the nervous system. Brain Res Bull 53: 141-146.

Pato B, Mihaly K, Csermely P. 2001. Chaperones and cytoarchitecture: geldanamycin induces an accelerated flux of cytoplasmic proteins from detergent-treated cells. Eur J Biochem 268: S107.

Perez N, Sugar J, Charya S, Johnson G, Merril C, et al. 1991. Increased synthesis and accumulation of heat shock 70 proteins in Alzheimer's disease. Brain Res Mol Brain Res 11: 249-254.

Picard D. 2002. Heat-shock protein 90, a chaperone for folding and regulation. Cell Mol Life Sci 59: 1640-1648.

Porankiewicz J, Wang J, Clarke AK. 1999. New insights into the ATP-dependent Clp protease: *Escherichia coli* and beyond. Mol Microbiol 32: 449-458.

Pratt WB, Silverstein AM, Galigniana MD. 1999. A model for the cytoplasmic trafficking of signalling proteins involving the hsp90-binding immunophilins and p50cdc37. Cell Signal 11: 839-851.

Pratt WB, Toft DO. 2003. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med 228: 111-133.

Queitsch C, Sangster TA, Lindquist S. 2002. Hsp90 as a capacitor of phenotypic variation. Nature 417: 618-624.

Ramsey JJ, Colman RJ, Binkley NC, Christensen JD, Gresl TA, et al. 2000. Dietary restriction and aging in rhesus monkeys: the University of Wisconsin study. Exp Gerontol 35: 1131-1149.

Reiss U, Rothstein M. 1974. Heat-labile isozymes of isocitrate lyase from aging Turbatrix aceti. Biochem Biophys Res Commun 61: 1012-1016.

Renkawek K, Bosman GJ, Gaestel M. 1993. Increased expression of heat-shock protein 27 kDa in Alzheimer disease: a preliminary study. Neuroreport 5: 14-16.

Richter K, Buchner J. 2001. Hsp90: chaperoning signal transduction. J Cell Physiol 188: 281-290.

Roberts SP, Feder M. 1999. Natural hyperthermia and expression of the heat-shock protein Hsp70 affect development in Drosophila melanogaster. Oecologia 121: 323-329.

Rogue PJ, Ritz MF, Malviya AN. 1993. Impaired gene transcription and nuclear protein kinase C activation in the brain and liver of aged rats. FEBS Lett 334: 351-354.

Rutherford SL, Lindquist S. 1998. Hsp90 as a capacitor for morphological evolution. Nature 396: 336-342.

Scharloo W. 1991. Canalization: Genetic and developmental aspects. Annu Rev Ecol Syst 22: 65-93.

Schliwa M, van Blerkom J, Porter KR. 1981. Stabilization of the cytoplasmic ground substance in detergent-opened cells and a structural and biochemical analysis of its composition. Proc Natl Acad Sci USA 78: 4329-4333.

Schuller E, Gulesserian T, Seidl R, Cairns N, Lube G. 2001. Brain t-complex polypeptide 1 (TCP-1) related to its natural substrate beta1 tubulin is decreased in Alzheimer's disease. Life Sci 69: 263-270.

Schultz C, Dick EJ, Hubbard GB, Braak E, Braak H. 2001. Expression of stress proteins alpha B-crystallin, ubiquitin, and hsp27 in pallido-nigral spheroids of aged rhesus monkeys. Neurobiol Aging 22: 677-682.

Shimura H, Miura-Shimura Y, Kosik KS. 2004. Binding of tau to heat shock protein 27 leads to decreased concentration of hyperphosphorylated tau and enhanced cell survival. J Biol Chem 279: 17957-17962.

Shinohara H, Inaguma Y, Goto S, Inagaki T, Kato K. 1993. Alpha B crystallin and Hsp28 are enhanced in the cerebral cortex of patients with Alzheimer's disease. J Neurol Sci 119: 203-208.

Soga S, Kozawa T, Narumi H, Akinaga S, Irie K, et al. 1998. Radicicol leads to selective depletion of Raf kinase and disrupts K-Ras-activated aberrant signaling pathway. J Biol Chem 273: 822-828.

Sőti C, Csermely P. 2000. Molecular chaperones and the aging process. Biogerontology 1: 225-233.

Sőti C, Csermely P. 2002. Chaperones and aging: their role in neurodegeneration and other civilizational diseases. Neurochem Int 41: 383-389.

Sőti C, Rácz A, Csermely P. 2002. A nucleotide-dependent molecular switch controls ATP binding at the C-terminal domain of Hsp90. N-terminal nucleotide binding unmasks a C-terminal binding pocket. J Biol Chem 277: 7066-7075.

Sőti C, Sreedhar AS, Csermely P. 2003. Apoptosis, necrosis and cellular senescence: chaperone occupancy as a potential switch. Aging Cell 2: 39-45.

Sreedhar AS, Mihály K, Pató B, Schnaider T, Steták A, et al. 2003. Hsp90 inhibition accelerates cell lysis: anti-Hsp90 ribozyme reveals a complex mechanism of Hsp90

inhibitors involving both superoxide- and Hsp90-dependent events. J Biol Chem 278: 35231-35240.

Sreedhar AS, Kalmar E, Csermely P, Shen YF. 2004a. Hsp90 isoforms: functions, expression and clinical importance. FEBS Lett 562: 11-15.

Sreedhar AS, Nardai G, Csermely P. 2004b. Enhancement of complement-induced cell lysis: a novel mechanism for the anticancer effects of Hsp90 inhibitors. Immunol Lett 92: 157-161.

Srivastava PK, Menoret A, Basu S, Binder RJ, McQuade KL. 1998. Heat shock proteins come of age: primitive functions acquire new roles in an adaptive world. Immunity 8: 657-665.

Stege GJ, Renkawek K, Overkamp PS, Verschuure P, van Rijk AF, et al. 1999. The molecular chaperone alphaB-crystallin enhances amyloid beta neurotoxicity. Biochem Biophys Res Commun 262: 152-156.

Sun H, Gao J, Ferrington DA, Biesiada H, Williams TD, et al. 1999. Repair of oxidized calmodulin by methionine sulfoxide reductase restores ability to activate the plasma membrane Ca-ATPase. Biochemistry 38: 105-112.

Terman A, Dalen H, Brunk UT. 1999. Ceroid/lipofuscin-loaded human fibroblasts show decreased survival time and diminished autophagocytosis during amino acid starvation. Exp Gerontol 34: 943-957.

Thirumalai D, Lorimer GH. 2001. Chaperonin-mediated protein folding. Annu Rev Biophys Biomol Struct 30: 245-269.

Tobaben S, Thakur P, Fernandez-Chacon R, Sudhof TC, Rettig J, et al. 2001. A trimeric protein complex functions as a synaptic chaperone machine. Neuron 31: 987-999.

Török Zs, Tsvetkova NM, Balogh G, Horváth I, Nagy E, et al. 2003. Heat shock protein co-inducers with no effect on protein denaturation specifically modulate the membrane lipid phase. Proc Natl Acad Sci USA 100: 3131-3136.

Trent JD, Kagawa HK, Yaoi T, Olle E, Zaluzec NJ. 1997. Chaperonin filaments: the archaeal cytoskeleton? Proc Natl Acad Sci USA 94: 5383-5388.

True HL, Lindquist S. 2000. A yeast prion provides a mechanism for genetic variation and phenotypic diversity. Nature 407: 477-483.

Turner GC, Varshavsky A. 2000. Detecting and measuring cotranslational protein degradation in vivo. Science 289: 2117-2120.

Unno K, Asakura H, Shibuya Y, Kaiho M, Okada S, et al. 2000. Increase in basal level of Hsp70, consisting chiefly of constitutively expressed Hsp70 (Hsc70) in aged rat brain. J Gerontol A Biol Sci Med Sci 55: B329-B335.

Urushitani M, Kurisu J, Tateno M, Hatakeyama S, Nakayama K, et al. 2004. CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70. J Neurochem 90: 231-244.

Vigh L, Literati Nagy P, Horvath I, Torok Z, Balogh G, et al. 1997. Bimoclomol: a nontoxic, hydroxilamine derivative with stress protein-inducing activity and cytoprotective effects. Nat Med 3: 1150-1154.

Walters TJ, Ryan KL, Mason PA. 2001. Regional distribution of Hsp70 in the CNS of young and old food-restricted rats following hyperthermia. Brain Res Bull 55: 367-374.

Welch WJ. 1992. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiol Rev 72: 1063-1081.

Welch WJ, Brown CR. 1996. Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperones 1: 109-115.

Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. 1994. Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci USA 91: 8324-8328.

Whittier JE, Xiong YE, Rechsteiner MC, Squier TC. 2004. Hsp90 enhances degradation of oxidized calmodulin by the 20S proteasome. J Biol Chem 279: 46135-46142.

Wigley WC, Fabunmi RP, Lee MG, Marino CR, Muallem S, et al. 1999. Dynamic association of proteasomal machinery with the centrosome. J Cell Biol 145: 481-490.

Wolosewick JJ, Porter KR. 1979. Microtrabecular lattice of the cytoplasmic ground substance. Artifact or reality. J Cell Biol 82: 114-139.

Workman P. 2004. Combinatorial attack on multistep oncogenesis by inhibiting the Hsp90 molecular chaperone. Cancer Lett 206: 149-157.

Wright HT. 1991. Nonenzymatic deamination of asparaginyl and glutaminyl residues in proteins. Crit Rev Biochem Mol Biol 26: 1-52.

Yang J, Yang JM, Iannone M, Shih WJ, Lin Y, et al. 2001. Disruption of the EF-2 kinase/Hsp90 protein complex: a possible mechanism to inhibit glioblastoma by geldanamycin. Cancer Res 61: 4010-4016.

Yenari MA, Fink SL, Sun GH, Chang LK, Patel MK, et al. 1998. Gene therapy with Hsp72 is neuroprotective in rat models of stroke and epilepsy. Ann Neurol 44: 584-591.

Young JC, Moarefi I, Hartl FU. 2001. Hsp90: a specialized but essential protein-folding tool. J Cell Biol 154: 267-273.

Yu Z, Luo H, Fu W, Mattson MP. 1999. The endoplasmic reticulum stress-responsive protein GRP78 protects neurons against excitotoxicity and apoptosis: suppression of oxidative stress and stabilization of calcium homeostasis. Exp Neurol 155: 302-314.

Yuh KCM, Gafni A. 1987. Reversal of age-related effects in rat muscle phosphoglycerate kinase. Proc Natl Acad Sci USA 84: 7458-7462.

Zagzag D, Nomura M, Friedlander DR, Blanco CY, Gagner JP, et al. 2003. Geldanamycin inhibits migration of glioma cells in vitro: a potential role for hypoxia-inducible factor (HIF-1alpha) in glioma cell invasion. J Cell Physiol 196: 394-402.

Zhang R, Tremblay TL, McDermid A, Thibault P, Stanimirovic D. 2003. Identification of differentially expressed proteins in human glioblastoma cell lines and tumors. Glia 42: 194-208.

Zimmerman SB, Minton AP. 1993. Macromolecular crowding: biochemical, biophysical and physiological consequences. Annu Rev Biophys Biomol Struct 22: 27-65.

# 14 GFAP and Astrocyte Intermediate Filaments

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**Abstract:** This chapter focuses on the role of glial fibrillary acidic protein (GFAP) and other intermediate filament (IF) proteins expressed in astroglial cells under physiological situations and it also discusses their functions in the context of selected central nervous system (CNS) pathologies. We have paid particular attention to mouse genetic models, which in the last decade have significantly advanced our understanding of the function of IF proteins in many cell types including astroglial cells.

**List of Abbreviations:** CNS, central nervous system; ENU, ethylnitrosourea; GFAP, glial fibrillary acidic protein; IF, intermediate filament

## 1 Introduction

Glial fibrillary acidic protein (GFAP) is a well-known astrocyte marker, even though it can also be found in several cell types outside the central nervous system (CNS). In the CNS, GFAP is the major component of astrocyte intermediate filaments (IFs). IFs are cytosketetal structures that are composed of GFAP and vimentin in mature nonreactive astrocytes, and of GFAP, vimentin, and nestin in reactive astrocytes. Upregulation of IF proteins in reactive astrocytes is a well-known hallmark of so-called reactive gliosis (known also as astrogliosis) that accompanies many pathological situations in the CNS, such as trauma and hypoxia.

## 2 Intermediate Filament Proteins Expressed in Astrocytes

### 2.1 Glial Fibrillary Acidic Protein

GFAP (432 amino acids) was first identified in 1971 when it was isolated from the CNS of patients suffering from multiple sclerosis (Eng et al., 1971). During the last 30 years, GFAP has been used as the primary marker of mature astrocytes in the CNS (Eng et al., 2000). The human GFAP gene is located on chromosome 17 (Reeves et al., 1989), while the mouse gene lies on chromosome 11 (Bernier et al., 1988). Like other genes coding for IF proteins, the GFAP gene is highly conserved among species (Balcarek and Cowan, 1985; Nielsen and Jorgensen, 2003). Transcription of GFAP is controlled through several different regulatory elements, which were described in both humans and rodents (Miura et al., 1990; Besnard et al., 1991; Sarid, 1991; Sarkar and Cowan, 1991; Kaneko and Sueoka, 1993; Brenner, 1994). About 2 kb of both the human and rodent GFAP promoter was shown to be sufficient to direct the expression of reporter genes to astrocytes (Brenner et al., 1994; Johnson et al., 1995; Brenner and Messing, 1996). Constructs containing variable length of the GFAP promoter have been used to direct gene expression to astroglial cells as well as to GFAP-positive cells outside the CNS (for review see Su et al., 2004). Regulatory elements of the GFAP gene were mapped to a distal and to a proximal region within this 2-kb-long sequence (Miura et al., 1990; Sarkar and Cowan, 1991; Masood et al., 1993) and include the consensus AP-1 sequence, a binding site for the Fos and Jun families of transcription factors (Sarid, 1991; Masood et al., 1993; Yu et al., 1995).

The GFAP gene contains several methylation sites, which were suggested to control cell differentiation, and the extent of methylation is lower in neural than in nonneural tissues (Condorelli et al., 1994, 1997; Teter et al., 1994; Barresi et al., 1999). It was suggested that GFAP promoter region at position −1176 is demethylated during development in cells of neuroectodermal origin in the CNS and later remethylated in the mature cells (Teter et al., 1996; Barresi et al., 1999). The demethylation and remethylation of a binding element for transcription factors such as STAT3 might induce differentiation toward a glial lineage (Takizawa et al., 2001). Song and Ghosh (2004) reported that FGF-2 facilitates the access for transcription factor STAT to the promoter binding site by inducing chromatin remodeling at the GFAP promoter through altered methylation of histone H3.

### 2.2 Vimentin

Apart from being found in astroglial cells, vimentin is found mainly in cells of mesenchymal origin (Bernal and Stahel, 1985). The human vimentin gene encodes a 466-amino-acid-long protein and is located on chromosome 10 (Ferrari et al., 1986). Several regulatory elements in the vimentin promoter region have been described (Pieper et al., 1987; Paulin et al., 1990; Kryszke and Vicart, 1998; Izmailova et al., 1999); however, their involvement in astrocytic expression of vimentin remains incompletely understood. Like the GFAP gene, the vimentin promoter region contains the AP-1 sequence that activates vimentin expression through the Jun and Fos pathways (Sommers et al., 1994; Moura-Neto et al., 1996). Silencer elements with binding sites for regulatory factors Sp1 and ZBP-89 (Pieper et al., 1992; van de Klundert et al., 1992; Wieczorek et al., 2000; Zhang et al., 2003) as well as an NF-κB binding site have been identified (Lilienbaum and Paulin, 1993).

### 2.3 Nestin

Nestin is an unusually long IF protein (1618 amino acids) and most of the amino acids belong to the long tail domain (Dahlstrand et al., 1992). The human nestin gene is located on chromosome 1 (Dahlstrand et al., 1992). Nestin is widely expressed during development in the nervous system, in the developing muscle (Sejersen and Lendahl, 1993; Kachinsky et al., 1994), and in the adult CNS, e.g. in neural stem cells, reactive astrocytes, and endothelial cells (Hockfield and McKay, 1985; Lendahl et al., 1990; Dahlstrand et al., 1995; Frisen et al., 1995; Sugawara et al., 2002). Important enhancer elements for the expression of nestin in the embryonic CNS were identified in the second intron of the gene (Zimmerman et al., 1994; Lothian and Lendahl, 1997; Josephson et al., 1998; Lothian et al., 1999; Yaworsky and Kappen, 1999). In the adult CNS, a 636-bp region of the second intron in the rat nestin gene is sufficient for nestin expression in the neurogenic zones, but not for a maximal response in nestin expression in reactive astrocytes upon injury (Johansson et al., 2002).

## 3 IFs and Their Formation in Astroglial Cells

The IFs can be considered the least understood part of the cytoskeleton. The family of IF proteins expressed in vertebrates is large (in humans 65 different IF proteins have been identified (Herrmann et al., 2003; Herrmann and Aebi, 2004) (❯ *Table 14-1*), and there is a complex expression pattern of IF proteins unique for each cell type as well as for different developmental stages. In contrast to the globular subunits of microtubules and actin filaments, the subunits of IFs are highly elongated molecules with a central α-helical rod domain flanked by globular N-terminal head and C-terminal tail domains. The head and tail domains are highly variable in size and sequence (Steinert and Parry, 1985), while the α-helical rod of approximately 300 amino acids has a strictly conserved substructure of heptad repeats $(abcdefg)_n$, where the positions a and d are generally apolar residues, which favors the formation of coiled coils between two α-helices (Steinert and Roop, 1988). The monomers assemble into dimers, which assemble into antiparallel tetramers or larger subunits of polymers that are then incorporated into the IF network (Herrmann et al., 2003).

The dynamic feature of the IF network depends both on the equilibrium between filaments and unassembled subunits and on the regulation of filament assembly/disassembly by phosphorylation of the head domain of the IF proteins.

IFs were at first considered to be static structures primarily responsible for maintaining the cell shape (Rueger et al., 1979; Renner et al., 1981). However, later studies, both in vitro (Angelides et al., 1989; Nakamura et al., 1991) and in vivo (Miller et al., 1991; Wiegers et al., 1991; Vikstrom et al., 1992; Yoon et al., 1998), revealed the rather dynamic nature of IFs and the existence of a dynamic equilibrium between the assembled filaments and the pool of soluble subunits (reviewed in Goldman et al., 1999). Vikström and coworkers assessed the turnover of vimentin subunits in IF fibers in vitro by using rhodamine-labeled vimentin that was injected into fibroblasts and readily incorporated into the endogenous IF network. After

**Table 14-1**
**The main IF proteins and the cell-types expressing them (examples)**

| IF protein type | Molecular weight kDa | Cell-type |
|---|---|---|
| **I** | | |
| Acidic keratins | 40-64 | Epithelial cells |
| **II** | | |
| Basic keratins | 52-68 | Epithelial cells |
| **III** | | |
| Vimentin | 55 | Mesenchyme, Astrocytes |
| Desmin | 53 | Muscle |
| GFAP | 50-52 | Astrocytes |
| Peripherin | 54 | Neurons |
| **IV** | | |
| Neurofilament-L, -M and -H | 68-130 | Neurons |
| Internexin | 56 | Developing CNS |
| **V** | | |
| Lamin A, B and C | 62-68 | Nuclear envelope |
| **VI** | | |
| Nestin | 240 | Neural stem cells, Astrocytes |

bleaching the fluorescent IF fibers with a laser beam, fluorescence returned to the IF fibers throughout their length within only a few minutes, proving the existence of a pool of subunits and unpolymerized IF proteins that are in a dynamic equilibrium with the IF network (Vikstrom et al., 1992). Nonfilamentous IF protein can be rapidly transported along the microtubule tracks (Prahlad et al., 1998; Helfand et al., 2003), implying a complex crosstalk between different cytoskeletal systems (Chou et al., 2001).

Various kinases, such as cdc2 kinase, protein kinase A, protein kinase C, $Ca^{2+}$/calmodulin-dependent protein kinase II and Rho kinase, all phosphorylate GFAP and thereby both increase the disassembly of IFs and inhibit the filament assembly (Matsuoka et al., 1992; Tsujimura et al., 1994; Nakamura et al., 1996; Kosako et al., 1997). These events increase the pool of free, phosphorylated monomers that can readily be reassembled after dephosphorylation by phosphatases. Phosphorylation of several serine and threonine residues, predominantly on the N-terminal head domain of the GFAP molecule, is important for the rearrangements of the IF network in situations such as cell motility (Inagaki et al., 1990; Nishizawa et al., 1991; Nakamura et al., 1992; Inagaki et al., 1994). Even though phosphorylation is a general mechanism that regulates the equilibrium and turnover rate of different pools of IFs, the specificity seems to be achieved by the existence of distinct phosphorylation sites and kinases (Nakamura et al., 1992; Takemura et al., 2002a), e.g., Rho kinase phosphorylating GFAP in cytokinesis (Yasui et al., 1998). Different subpopulations of astrocytes in vivo seem to contain different levels of phosphorylated GFAP, suggesting a role for phosphorylation in the nondividing astroglial cells (Takemura et al., 2002b).

In vivo, IFs are often, if not always, heteropolymeric (Herrmann and Aebi, 2000). Transgenic mice deficient in individual IF proteins were instrumental in determining the partnership in the formation of IF heteropolymers in astrocytes. In nonreactive astrocytes, IFs are formed of GFAP and vimentin, while in reactive astrocytes, nestin can be found as the additional partner in the IF network (Pekny et al., 1998a) (*Table 14-2*). The studies of astrocytes lacking GFAP and/or vimentin revealed that GFAP can form IFs on its own in vimentin-deficient (*Vim*(−/−)) astrocytes, but such filaments form more compact bundles than in wild-type astrocytes (*Figure 14-1a–d*), (*Table 14-3*), suggesting that at least a low level of vimentin is needed for normal IF formation in the astrocytes (Eliasson et al., 1999; Lepekhin et al., 2001; Menet et al., 2001). Studies on mice deficient in GFAP (*GFAP*(−/−)) showed that vimentin does not seem to form IF on its own, or it does so only with a very low efficiency (Pekny et al., 1995; McCall et al., 1996) (*Figure 14-1e–f*). In contrast, the reactive *GFAP*(−/−) astrocytes contain IFs since vimentin can polymerize with nestin, which is expressed in reactive astrocytes (Eliasson et al., 1999). GFAP does not polymerize

**Table 14-2**

**Composition of IFs in nonreactive and reactive astrocytes of wild-type mice and mice deficient in GFAP and/or vimentin**

| Genotype | Composition of IFs | | Reactive astrocytes: IF amount/bundling |
|---|---|---|---|
| | Nonreactive astrocytes | Reactive astrocytes | |
| Wild-type | GFAP, vimentin | GFAP, vimentin, nestin | Normal/normal |
| *GFAP*$^{-/-}$ | No IFs (nonfilamentous vimentin) | Vimentin, nestin | Decreased/normal |
| *Vim*$^{-/-}$ | GFAP | GFAP (nonfilamentous nestin) | Decreased/tight |
| *GFAP*$^{-/-}$ *Vim*$^{-/-}$ | No IFs | No IFs (nonfilamentous nestin) | – |

**Figure 14-1**

**Cytoplasmic details of wild-type astrocytes and astrocytes lacking vimentin (*Vim*(−/−)) or GFAP (*GFAP*(−/−)) in the dorsal funiculus of the cervical spinal cord of healthy adult mice. Compared with wild-type astrocytes (*a*, *c*), in *Vim*(−/−) astrocytes (*b*, *d*), intermediate filaments (IFs) are composed of GFAP but not nestin and form more densely packed bundles with the distance between the adjacent IFs being reduced. The IF bundles were sectioned transversally (*a*, *b*) and longitudinally (*c*, *d*). (Reproduced with permission from Eliasson et al., 1999.) While wild-type astrocytes in the intact central nervous system (CNS) contain abundant IFs (asterisk, *e*), *GFAP*(−/−) astrocytes are devoid of IFs (*f*). (Reproduced with permission from Pekny et al., 1995.)**

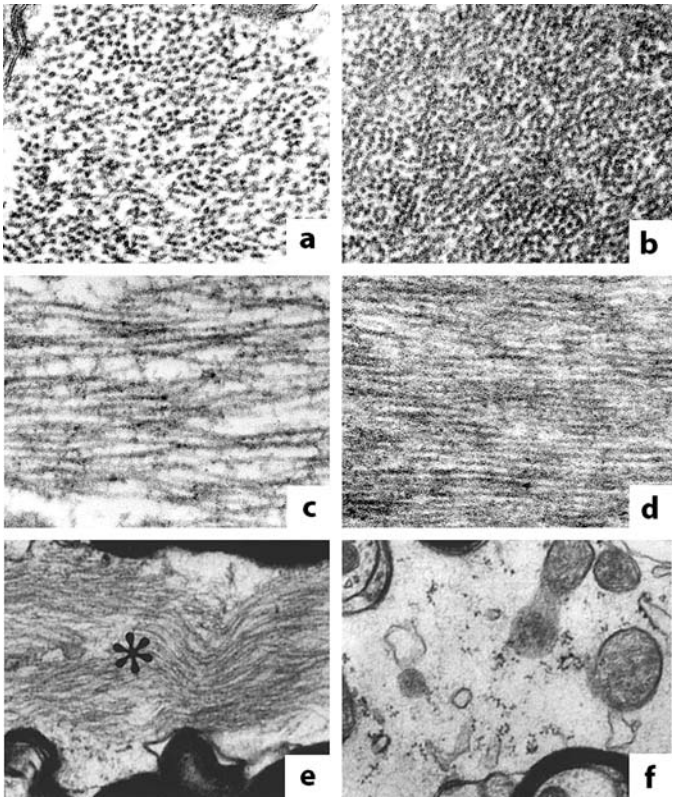


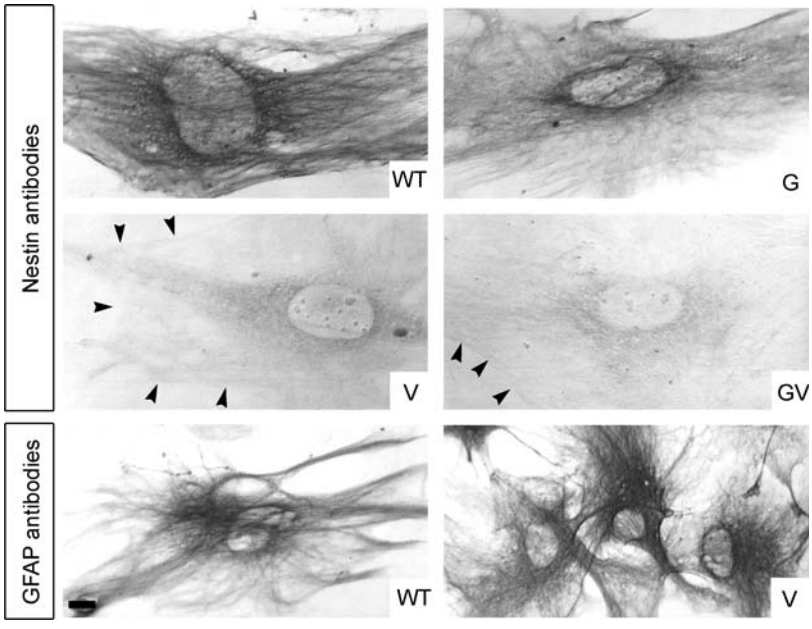
**Table 14-3**

**Quantitative comparison of the density of IFs within IF bundles in wild-type and *Vim*$^{-/-}$ astrocytes in the intact CNS**

| | Mice | Mean ± SEM | Significance |
|---|---|---|---|
| Number of IFs/0.1 μm$^2$ (cross-section) | Wild-type | 215 ± 8 | $p < 0.0001$ |
| | *Vim*$^{-/-}$ | 334 ± 7 | |
| Distance between IFs within a bundle (nm) | Wild-type | 11.8 ± 0.5 | |
| | *Vim*$^{-/-}$ | 4.9 ± 0.3 | $p < 0.0001$ |

**Figure 14-2**

**Reactive astrocytes in vitro from wild-type mice and mice deficient for GFAP and/or vimentin. Reactive astrocytes in a primary culture prepared from wild-type (WT), *GFAP*(−/−) (G), *Vim*(−/−) (V), and *GFAP*(−/−) *Vim*(−/−) (GV) mice. Nestin antibodies facilitate visualization of bundles of intermediate filaments (IFs) in wild-type and *GFAP*(−/−) astrocytes but fail to do so in *Vim*(−/−) or *GFAP*(−/−)*Vim*(−/−) astrocytes, indicating that nestin can neither copolymerize nor coassemble with GFAP. This was also confirmed at a biochemical level (Eliasson et al., 1999). *Vim*(−/−) reactive astrocytes contain IF bundles that can be visualized by antibodies against GFAP, albeit with a reduced distance between individual IFs (see Figure 14-1 and Table 14-3). Bar, 10 μm. (Reproduced with permission from Eliasson et al., 1999.)**




with nestin in reactive *Vim*(−/−) astrocytes and consequently, the IFs contain only GFAP and they exhibit the characteristic tight bundling similar to *Vim*(−/−) nonreactive astrocytes. In reactive astrocytes lacking both GFAP and vimentin (*GFAP*(−/−)*Vim*(−/−)) no IFs are formed, and the nestin protein that is produced stays in a nonfilamentous form (Eliasson et al., 1999) (*Figure 14-2* and *Table 14-2*). Nestin was proposed to facilitate phosphorylation-dependent disassembly of vimentin IFs during mitosis and to play a role in the distribution of IF protein to daughter cells (Chou et al., 2003). The IF protein synemin was detected in some astroglial cell populations that express both GFAP and vimentin, suggesting that in astroglial cells, GFAP and vimentin may be necessary for synemin polymerization (Hirako et al., 2003).

## 4 During Differentiation of Astroglial Cells, Nestin and Vimentin Appear Before GFAP

The first astroglial cells that are derived from neuroectoderm during CNS development are the radial glia. Radial glia are proliferating precursor cells that later on differentiate into neurons and more mature glial cells. The radial processes of these cells span the entire thickness of the neural tube and they function as migratory paths for the immature neurons (reviewed in Gotz et al., 2002). In lower vertebrates the radial glia persist into adulthood (Chanas-Sacre et al., 2000). In mammals, most of radial glia are transformed into astrocytes around the time of birth (Alves et al., 2002; deAzevedo et al., 2003). In some regions of the CNS though, the radial glia persist into adulthood: in the cerebellum as Bergmann glia (Choi and Lapham, 1980) and in the retina as Müller cells (Edwards et al., 1990) (❯ *Figure 14-3a*). During early development, the IFs in the radial glia are composed of nestin (Dahlstrand et al., 1995) and vimentin (Pixley and de Vellis, 1984; Kalman et al., 1998). In primates, the IF network in radial glia also contain GFAP, which is predominantly localized in the main cellular processes (Levitt and Rakic, 1980).

Around the time of birth, the radial glia of the mammalian CNS transform into astrocytes and the expression of vimentin decreases while the expression of GFAP gradually increases (Sancho-Tello et al., 1995). In mice, the amount of GFAP messenger RNA (mRNA) in the brain increases postnatally and reaches the peak at postnatal day 15 when cell proliferation declines (Tardy et al., 1989; Riol et al., 1992). The levels of nestin do not seem to be altered until the transition from radial glia into adult astrocytes is completed and then it is downregulated (Kalman and Ajtai, 2001). The IF protein synemin was reported to be transiently expressed in some immature astrocytes (Sultana et al., 2000).

Thus, the IF network of mature astrocytes is composed of GFAP as the major IF protein and vimentin ranging from very low to intermediate levels depending on the subpopulation of astrocytes (Shaw et al., 1981; Pixley et al., 1984). Mature astrocytes have fine processes extending from the main cellular processes and they give each cell a characteristic bushy appearance (❯ *Figure 14-3b*). The IF network, however, is restricted to the main processes and the soma of astrocytes (Bushong et al., 2002, 2004) (❯ *Figure 14-3d–f*).

It was proposed that GFAP-positive astroglial cells are involved in the baseline neurogenesis in the adult mammalian CNS. Recent data suggest that astrocytes positively control neurogenesis in the two regions of the adult CNS, specifically in the dentate gyrus of the hippocampus and in the subventricular zone, i.e., the only two CNS regions in which new neurons are generated in relatively high numbers even in the adult (Song et al., 2002). Most recently, it was suggested that the majority of neural stem cells in the adult CNS are at some point GFAP-positive, i.e., could be defined as astroglial cells (Doetsch et al., 1999; Laywell et al., 2000; Imura et al., 2003; Morshead et al., 2003). Thus, astroglial cells might be both the cell type that controls adult neurogenesis and the precursors to all neurons that are added in adult life.

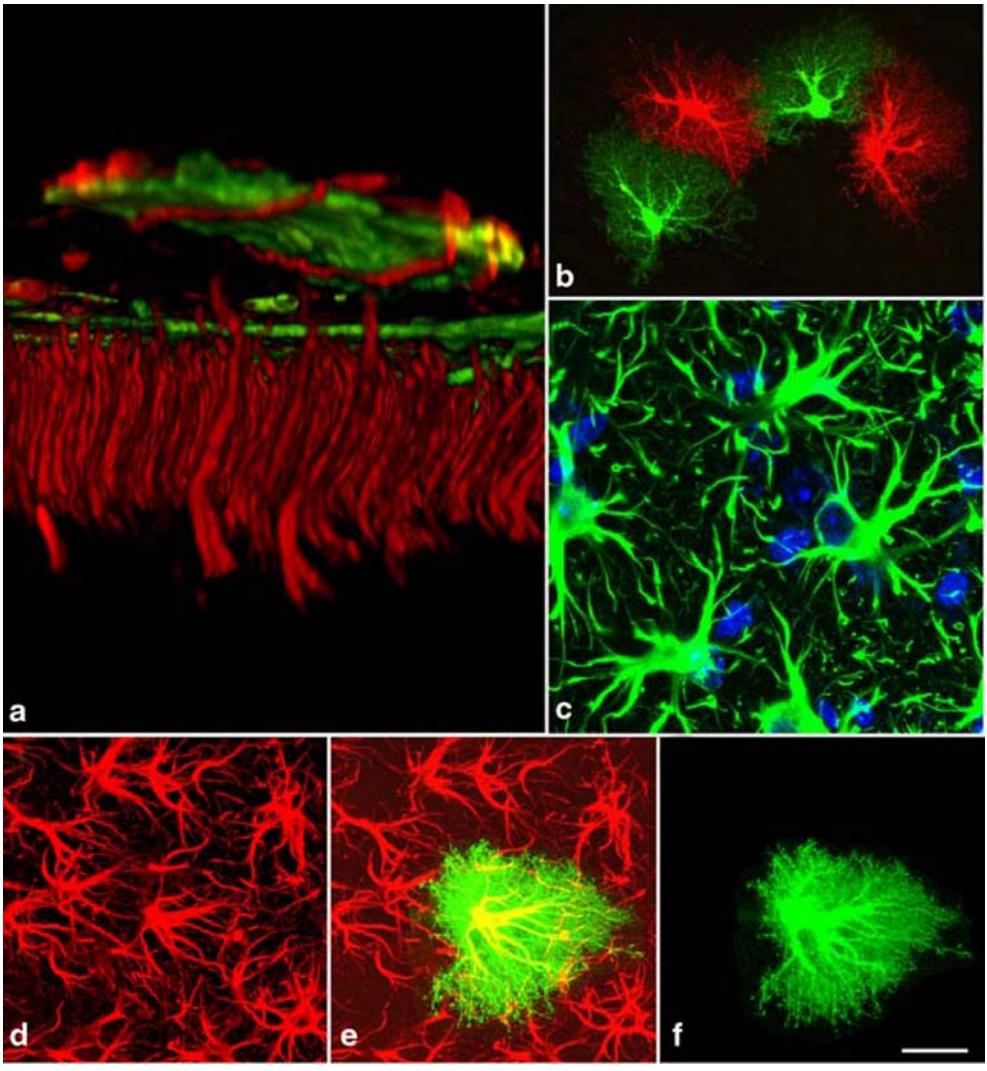
## 5 Mouse Genetic Models to Study the Function of GFAP and Astrocyte IFs

Astrocytes are the most numerous cells in the CNS and they were assumed to be involved in many CNS pathologies, such as trauma, ischemia, or neurodegenerative diseases. In response to any kind of injury in the CNS, astrocytes change their appearance and undergo a characteristic hypertrophy of their cellular processes. This phenomenon is known as reactive gliosis or astrogliosis with its hallmark being upregulation of GFAP and vimentin, reexpression of nestin as well as altered expression profiles of many proteins (Eddleston and Mucke, 1993; Hernandez et al., 2002). Genetic depletion of astrocyte IF proteins in mouse genetic models has shed substantial light on the physiological and pathological function of astrocytes in health and disease (Pekny, 2001). Experiments that subjected the mice with partial or complete depletion of astrocyte IFs to various disease paradigms showed the importance of this part of the cytoskeleton in a number of pathological situations affecting the CNS.

While experiments with mice deficient in GFAP and/or vimentin did not show major CNS phenotypes (Colucci-Guyon et al., 1994; Gomi et al., 1995; Pekny et al., 1995; McCall et al., 1996), they suggested that

**Figure 14-3**

**Visualization of astroglial cell morphology in vivo. *a*, mouse retina with GFAP-positive Müller cells and astrocytes (intermingled with blood vessels in the upper part of the figure). During development of the central nervous system (CNS), radial glia guide neurons into their final destinations. Later on, most radial glia differentiate into various astroglial cell types; however, in the retina and cerebellum, they persist into adulthood as Müller cells and Bergmann glia, respectively. Parallel arrays of Müller cells and a network of astrocytes are both visualized by antibodies against GFAP, vessels are visualized by isolectin. The picture is the courtesy of Lundkvist and Pekny (reproduced from the cover of J Cell Sci, 117:16, 2004); *b*, three-dimensional reconstruction of astrocytes. Astrocytes in the adult mouse hippocampus filled with two different dyes (Alexa 568, gray, and Lucifer yellow, white). The CNS is divided into domains and each of them is accessed by fine cellular processes of an astrocyte. (Courtesy of Wilhelmsson, Bushong, Ellisman, and Pekny.); *c*, astrocytes in the brain cortex visualized by antibodies against GFAP; *d–f*, reactive astrocytes after dye filling and three-dimensional reconstruction. Note the typical bushy appearance of astrocytes with fine cellular processes, which cannot be visualized by antibodies against GFAP (compare the central astrocyte in *d*, *e*, and *f*). Scale bar, 20 μm. (Reproduced with permission from Wilhelmsson et al., 2004.)**



astrocytes influence neuronal physiology in the hippocampus (McCall et al., 1996; Tanaka et al., 2002) and in the cerebellum (Shibuki et al., 1996; Colucci-Guyon et al., 1999). The absence of IF proteins in astroglial cells seems to alter communication between Bergmann glia and Purkinje cells, and this results in impaired eyeblink conditioning and long-term depression in the cerebellum of *GFAP*(−/−) mice (Shibuki et al., 1996) and impaired motor coordination in *Vim*(−/−) mice (Colucci-Guyon et al., 1999). One of the four groups that independently generated *GFAP*(−/−) mice reported white matter pathologies and dysmyelination in their unchallenged *GFAP*(−/−) mice (Liedtke et al., 1996), while the other three groups did not see such changes in their respective *GFAP*(−/−) mice (Gomi et al., 1995; Pekny et al., 1995; McCall et al., 1996). This discrepancy still remains to be solved. Interestingly, the same group reported increased susceptibility of *GFAP*(−/−) mice to experimental autoimmune encephalomyelitis, a model of multiple sclerosis (Liedtke et al., 1998).

## 5.1 GFAP and Blood–Brain Barrier Reconstruction

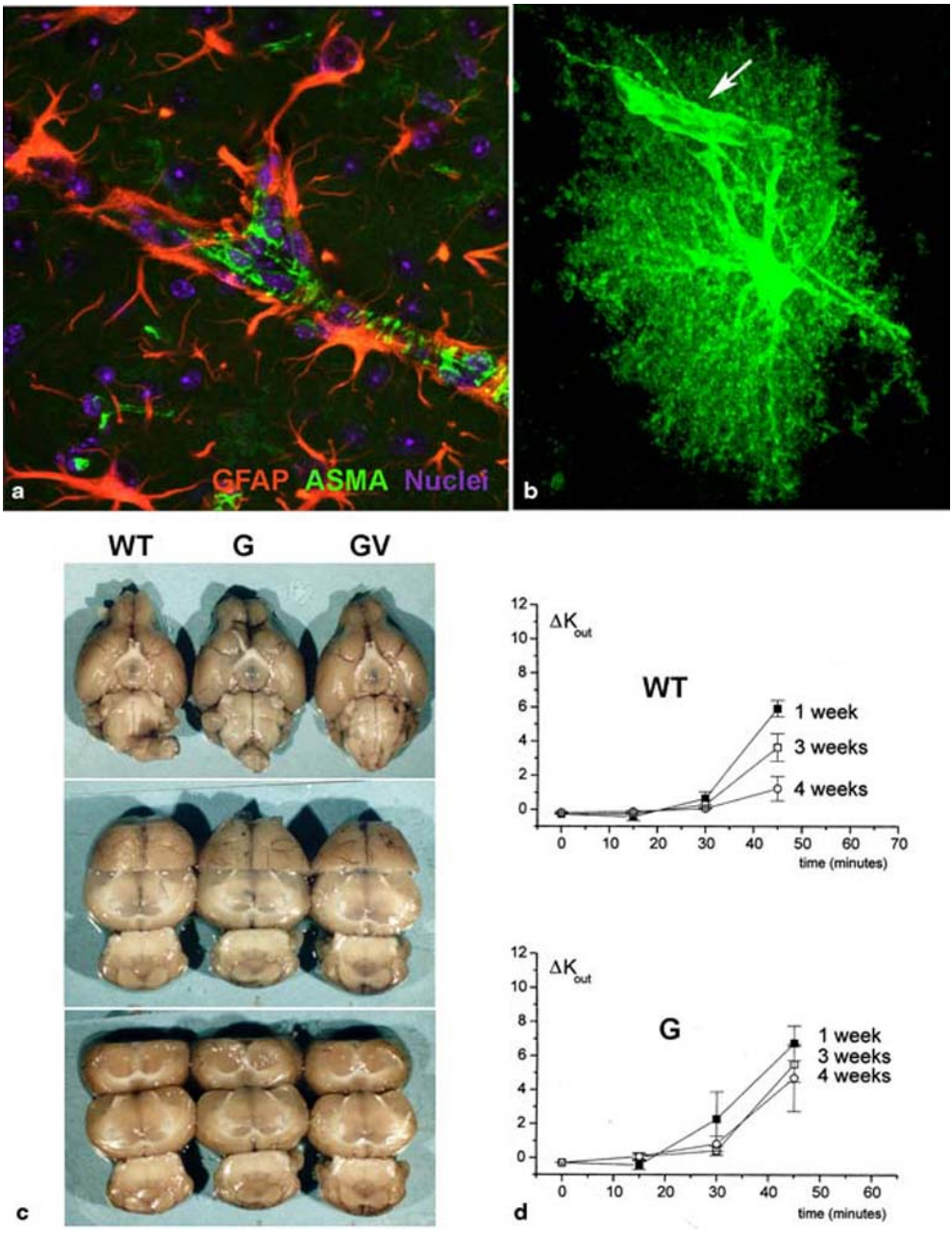
Astrocytes are known to induce blood–brain barrier properties in endothelial cells. These include tight junctions between individual endothelial cells and the presence of active transport mechanisms, the features that limit fluctuations in many biochemical parameters in the brain, spinal cord, or retina (in the latter case, the term blood–retina barrier is often used) (Smith, 2003). Even though the molecular mechanisms behind this induction remain incompletely understood, physical contact or close proximity between astrocytes and endothelial cells seem to be important. In fact, capillaries in the CNS are extensively covered by astrocytic processes that contain abundant IFs (❯ *Figure 14-4a–b*).

In the absence of any injury, the *GFAP*(−/−) mice show normal blood–brain barrier, at least for large molecular complexes, such as albumin–Evans blue (Pekny et al., 1995) or horse radish peroxidase (Shibuki et al., 1996), which readily enter all the other tissues through fenestrations in the capillary wall but not the CNS, in which capillaries lack such fenestrations. These experiments were recently extended to *GFAP*(−/−) *Vim*(−/−) mice, which also showed intact blood–brain barrier for large molecular complexes (❯ *Figure 14-4c*). Reconstruction of blood–brain barrier after CNS trauma or brain ischemia is considered to limit the extent of damage in the CNS. By utilizing the in vitro model of blood–brain barrier, Janigro's and Pekny's groups compared the ability of wild-type and *GFAP*(−/−) reactive astrocytes to induce blood–barrier properties in aortic endothelial cells. *GFAP*(−/−) reactive astrocytes failed to induce a significant barrier for potassium, while the barrier against albumin–Evan blue complexes developed normally (Pekny et al., 1998b) (❯ *Figure 14-4d*).

**Figure 14-4**

**Intermediate filament (IF) proteins and the blood–brain barrier. *a*, astrocytic processes (in gray, visualized by using antibodies against GFAP) make extensive contacts with capillaries throughout the central nervous system (CNS); capillaries are visualized by antibodies against smooth-muscle-specific alpha-actin (ASMA), a marker of pericytes (in white), which are, together with endothelial cells, the main structural components of capillaries (courtesy of Lundkvist and Pekny); *b*, astrocyte processes forming a tube-like structure (arrow) around a capillary are shown after three-dimensional reconstruction of a dye-filled astrocyte in the dentate gyrus of the mouse hippocampus (the capillary itself is invisible, courtesy of Wilhelmsson, Bushong, Ellisman, and Pekny); *c*, Evans blue, following intravenous administration, does not leak out from the vascular system of wild-type (WT), *GFAP*(−/−) (G), and *GFAP*(−/−)*Vim*(−/−) (GV) mice, indicating a functional blood–brain barrier for large molecular complexes in mice deficient for astrocyte IF proteins (*GFAP*(−/−)*Vim*(−/−) mice, Eliasson and Pekny, unpublished data); *d*, the blood–brain barrier in vitro model. In contrast to wild-type astrocytes, which over time induce increasing resistance of endothelial cells to potassium extravasation, *GFAP*(−/−) astrocytes fail to induce blood–brain barrier to intraluminally applied potassium after 1, 3, or 4 weeks of coculturing with endothelial cells. (Reproduced with permission from Pekny et al., 1998b.)**

**Figure 14-4 (overleaf)**



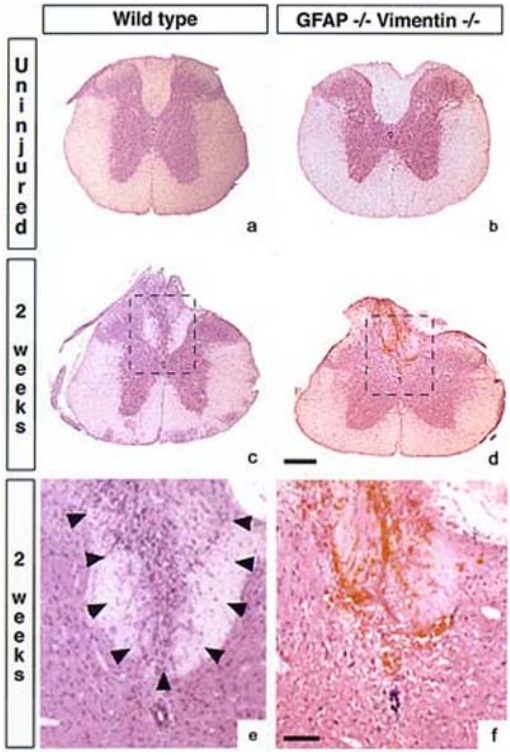

## 5.2 Brain and Spinal Cord Trauma in Mice Deficient in GFAP and/or Vimentin

To assess the role of IF upregulation in reactive astrocytes in CNS injury, several trauma models were applied to mice deficient in GFAP and/or vimentin. Fine needle injury of the brain cortex and transection of the dorsal funiculus in the upper thoracic spinal cord were two of the models used. The responses of

wild-type, *GFAP*(−/−), and *Vim*(−/−) mice were indistinguishable. In *GFAP*(−/−)*Vim*(−/−) mice, however, the posttraumatic glial scarring was looser and less organized, suggesting that upregulation of IFs is an important step in astrocyte activation. These data also imply that reactive astrocytes play a role in posttraumatic healing (Pekny et al., 1999b) (❯ *Figure 14-5*).

**Figure 14-5**

**Glial scarring in the absence of astrocyte intermediate filament (IF) proteins. Wound healing after transection of the dorsal funiculus in the upper thoracic spinal cord takes longer in *GFAP*(−/−)*Vim*(−/−) than in wild-type mice, and the resulting glial scarring is reduced. H&E staining. Bar, 300 μm in *a–d* and 100 μm in *e–f*. (Reproduced with permission from Pekny et al., 1999b.)**




Similarly, extended healing period following CNS injury was reported in mice in which dividing astrocytes had been ablated by GFAP-driven expression of herpes simplex virus thymidine kinase and administration of ganciclovir (Bush et al., 1999; Faulkner et al., 2004).

Another study used hemisections of the lower thoracic spinal cord and reported increased axonal sprouting and better functional recovery in *GFAP*(−/−)*Vim*(−/−) mice than in wild-type controls (Menet et al., 2003). Two groups addressed the role of astrocyte IFs in neurite outgrowth in vitro (Xu et al., 1999; Menet et al., 2000, 2001). One group reported that *GFAP*(−/−)*Vim*(−/−) and *GFAP*(−/−) astrocytes were a better substrate for the outgrowth of neurites in vitro than wild-type astrocytes (Menet et al., 2000, 2001). The other group found comparable neurite outgrowth when neurons were cultured on wild-type and *GFAP* (−/−) astrocytes (Xu et al., 1999). The latter finding is in agreement with the normal axonal sprouting and regeneration assessed after dorsal hemisection of the spinal cord in *GFAP*(−/−) mice (Wang et al., 1997).

Most recently, extensive axonal regeneration was reported in the severed optic nerve of young *GFAP* (−/−) *Vim*(−/−) mice which also carried a transgene overexpressing Bcl2 in neurons (Cho et al., 2005).

## 5.3 Astrocyte IFs and Cell Motility

Lepekhin and coworkers assessed the role of astrocyte IFs in cell motility by comparing the motility of primary cultures of astrocytes from *GFAP*(−/−), *Vim*(−/−), and *GFAP*(−/−)*Vim*(−/−) mice. They showed that the fast-moving subpopulation was depleted partially among *GFAP*(−/−) and *Vim*(−/−) astrocytes and more profoundly among *GFAP*(−/−)*Vim*(−/−) astrocytes (Lepekhin et al., 2001) (*Figure 14-6*). Astrocytes are known to migrate over considerable distances to sites of injury (Johansson et al., 1999) and therefore the slower migration of IF-deficient astrocytes could contribute to the more discrete development of posttraumatic glial scars seen in *GFAP*(−/−)*Vim*(−/−) mice (Pekny et al., 1999b), even though the in vivo relevance of these in vitro data and the molecular mechanisms involved remain to be established. Interestingly, the IFs were also implicated in cell motility in cells other than astrocytes. In vitro studies focusing on the motility of *Vim*(−/−) fibroblasts (Colucci-Guyon et al., 1994) showed reduced resistance to mechanical stress and reduced migration of these cells in the scrape wound assay and in Boyden chambers compared with wild-type fibroblasts (Eckes et al., 1998), even though another study that used monolayer wounding experiments showed comparable mobility of polarized wild-type and *Vim*(−/−) fibroblasts at the edge of the wound (Holwell et al., 1997).

## 5.4 Entorhinal Cortex Lesions Reveal the Yin and Yang of Reactive Gliosis

Entorhinal cortex lesioning interrupts axonal connections (known as the perforant path) between the entorhinal cortex and the projection area in the outer molecular layer of the dentate gyrus of the hippocampus (Turner et al., 1998) where degenerating neurons trigger extensive reactive gliosis. The distance between these two regions allows assessment of astrocyte response, degeneration, and subsequent regeneration in the hippocampus, i.e., the region that is not directly affected by the surgery.

By utilizing this model, we recently showed that reactive astrocytes devoid of IFs (*GFAP*(−/−) *Vim*(−/−)) exhibited only limited hypertrophy of cell processes. Many processes of *GFAP*(−/−) *Vim*(−/−) astrocytes were shorter and less straight than those of wild-type astrocytes, albeit the volume of the CNS tissue reached by a single astrocyte was comparable with that reached in wild-type mice (Wilhelmsson et al., 2004) (*Figure 14-7*). These results, along with in vitro data on the morphology of IF-depleted astrocytes in primary cultures (Lepekhin et al., 2001), show a novel role for IFs in determining astrocyte morphology.

In *GFAP*(−/−)*Vim*(−/−) mice, loss of neuronal synapses in the outer molecular layer of the hippocampal dentate gyrus was prominent 4 days after lesioning (*Figure 14-8a, b, e*). Most interestingly, there was remarkable synaptic regeneration 10 days later (at 14 days after lesions) (*Figure 14-8c–e*). In contrast to wild-type mice, *GFAP*(−/−)*Vim*(−/−) reactive astrocytes did not upregulate the expression of endothelin B receptors, suggesting that the upregulation of this novel marker of reactive astrocytes (Ishikawa et al., 1997; Baba, 1998; Koyama et al., 1999; Peters et al., 2003) is IF-dependent (Wilhelmsson et al., 2004). Thus, the effect of reactive astrocytes after CNS trauma seems to be twofold: reactive astrocytes play a beneficial role in the acute stage after CNS injury; however, later on they act as inhibitors of CNS regeneration. Much less is known about the role of astrocyte IFs and reactive astrocytes in general in neurodegenerative diseases. *GFAP*(−/−) mice showed normal response to prion infection, which leads to neurodegeneration accompanied by massive reactive gliosis (Shibuki et al., 1996; Tatzelt et al., 1996). Thus, it is possible that different pathological insults trigger qualitatively different astrocyte responses.

The studies of IF-null mutants described above provided insights into how reactive astrocytes might influence the clinical outcome of various CNS pathologies. It is feasible that by affecting the abundance or the composition of IFs, it might be possible to control the state of cellular differentiation and thus many cellular functions, which ultimately allow control of complex processes such as the permissiveness of the CNS for regeneration (Pekny et al., 2004; Quinlan and Nilsson, 2004).

**Figure 14-6**

**The impact of GFAP and vimentin on astrocyte motility. Compared with wild type, the migration of *GFAP*(−/−) *Vim*(−/−) reactive astrocytes in vitro is reduced, with the single mutants migrating more slowly than wild-type but faster than *GFAP*(−/−)*Vim*(−/−) astrocytes (*a*). Fast-moving subpopulations of *GFAP*(−/−)*Vim*(−/−) (GV) astrocytes are smaller than in wild type (WT), with *GFAP*(−/−) (G) astrocytes and *Vim*(−/−) (V) astrocytes exhibiting a dose effect (*b*). (Reproduced with permission from Lepekhin et al., 2001.)**

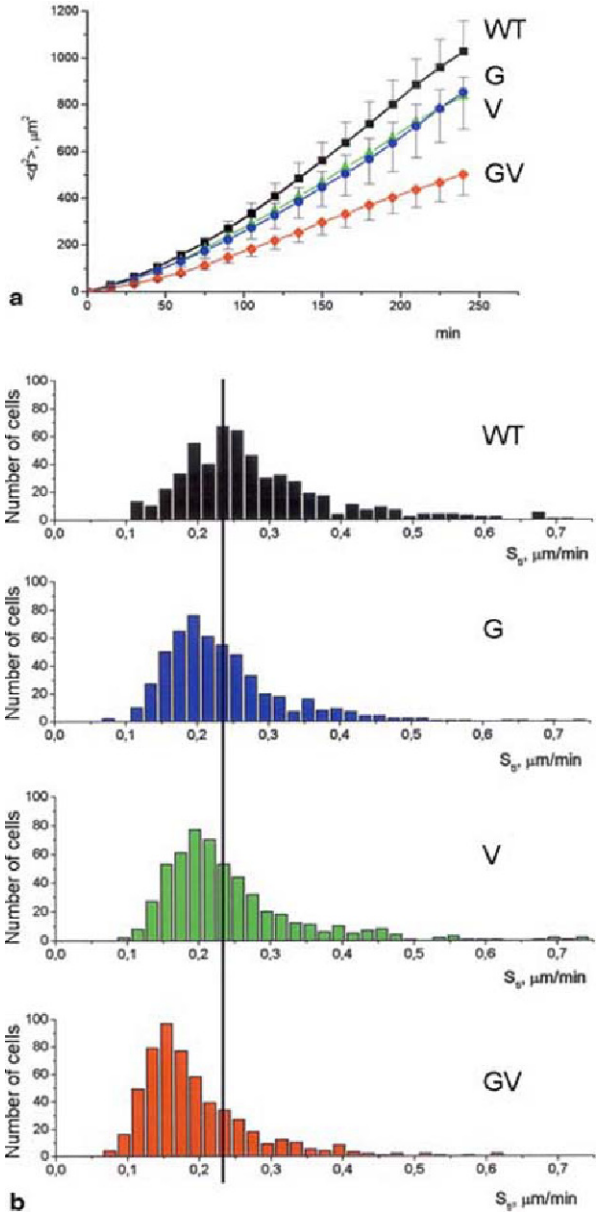
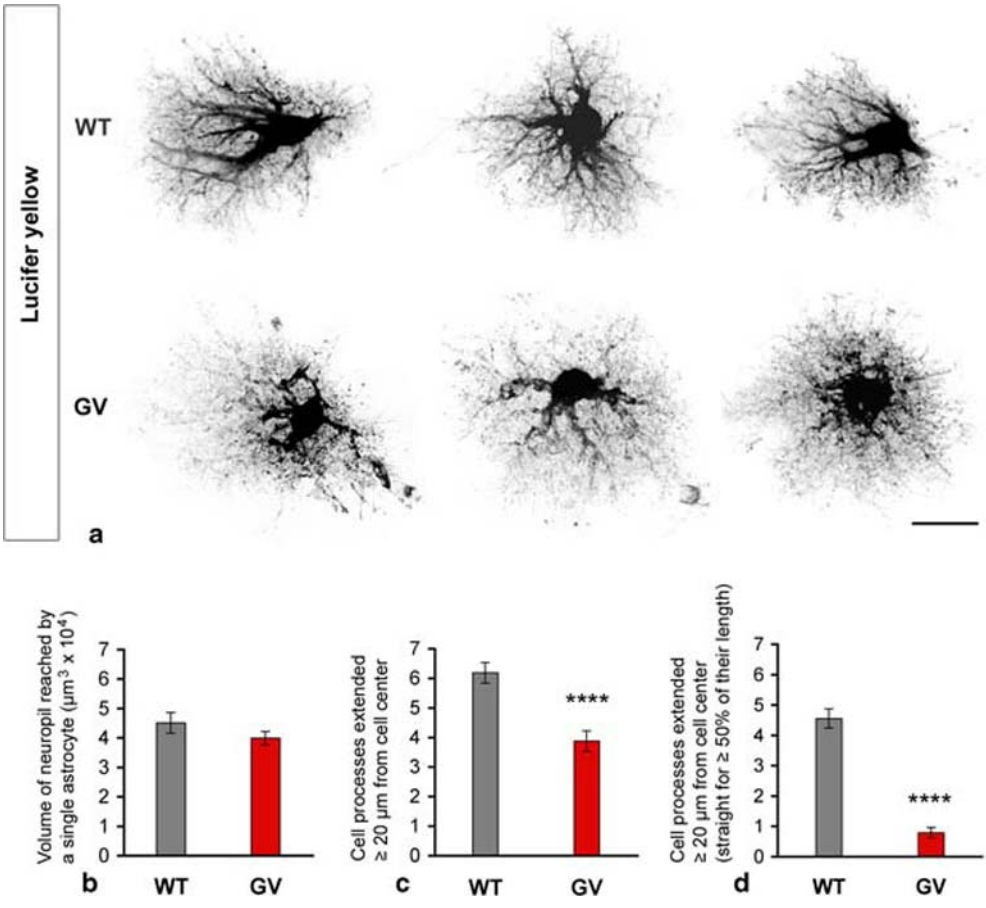


**Figure 14-7**

**The importance of astrocyte intermediate filament (IF) proteins for astrocyte morphology. *GFAP*(−/−)*Vim*(−/−) (GV) reactive astrocytes have fewer long and straight cellular processes than wild type (WT), as shown by the three-dimensional reconstruction of dye-filled reactive astrocytes in the dentate gyrus of the hippocampus after entorhinal cortex lesions (*a*, *c*, *d*). Wild-type and IF-free *GFAP*(−/−)*Vim*(−/−) reactive astrocytes reach comparable volumes of brain tissue (*b*). ****$p<0.0001$. Bar, 20 μm. (Reproduced with permission from Wilhelmsson et al., 2004.)**

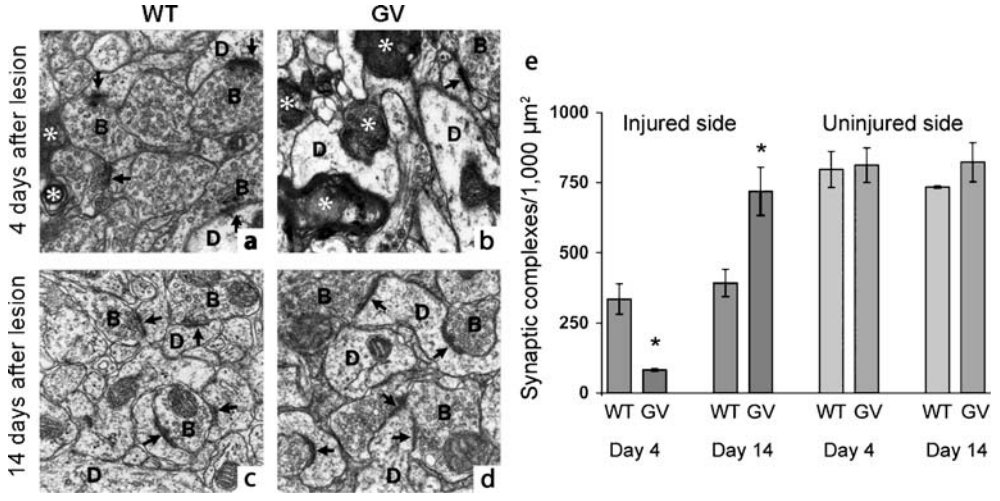



## 5.5 *GFAP*(−/−)*Vim*(−/−) Mice as Recipients of the CNS Transplants

Because of their morphology and abundance in the adult CNS (◉ *Figure 14-3b*), astrocytes have direct physical contact with any cell that moves from one place to another. To assess the impact of astrocyte IFs on the fate of cells migrating from neural transplants, the Chen and Pekny groups transplanted dissociated retinal cells from 0- to 3-week-old donor mice that ubiquitously express enhanced green fluorescent protein (Okabe et al., 1997) into the retinas of adult wild-type and *GFAP*(−/−)*Vim*(−/−) recipients and compared the efficiency of long-term integration of such grafts in the retina (Kinouchi et al., 2003). In wild-type hosts, few transplanted cells migrated from the transplantation site, and few integrated into the retina. In *GFAP*(−/−)*Vim*(−/−) hosts, however, the transplanted cells effectively moved through the retina, differentiated into neurons, integrated into the ganglion cell layer, and some of them even extended neurites about 1 mm

**Figure 14-8**

**The consequences of lesioning of the entorhinal cortex assessed in its projection area in the dentate gyrus of the hippocampus in *GFAP*(−/−)*Vim*(−/−) (GV) and wild-type (WT) mice. At day 4 after lesioning, the synaptic loss and the signs of neurodegeneration were more prominent in *GFAP*(−/−)*Vim*(−/−) than in wild-type mice (*a–b*, *e*). At day 14 after lesioning, the number of synapses in *GFAP*(−/−)*Vim*(−/−), but not wild-type mice, recovered, reaching the levels comparable with the uninjured hemisphere (*c–e*). *Asterisks*, degenerated axons; *arrows*, synaptic complexes; *D*, dendritic profile; *B*, synaptic bouton; **p*<0.05. (Reproduced with permission from Wilhelmsson et al., 2004.)**




into the optic nerve (*Figure 14-9a–d*). The single mutants exhibited a dose effect (*Figure 14-9e–i*). Six months after transplantation, the cells remained alive and well integrated in *GFAP*(−/−)*Vim*(−/−) hosts (Kinouchi et al., 2003).

These results show that the absence of IFs in astroglial cells (astrocytes and Müller cells) of the retina increases the permissiveness of the retinal environment for integration of neural transplants through yet unknown mechanism. The extent to which this reflects increased permissiveness for the migration of transplanted cells remains to be established. However, it is possible to speculate that IF depletion in astroglial cells alters their differentiation state, turning them into cells functionally similar to more immature astrocytes, and thereby also more supportive of CNS regeneration (Emsley et al., 2004; Pekny et al., 2004; Quinlan and Nilsson, 2004). It might be possible that the approaches that would control the expression of IFs and consequently affect cellular differentiation might also be applicable outside the CNS.

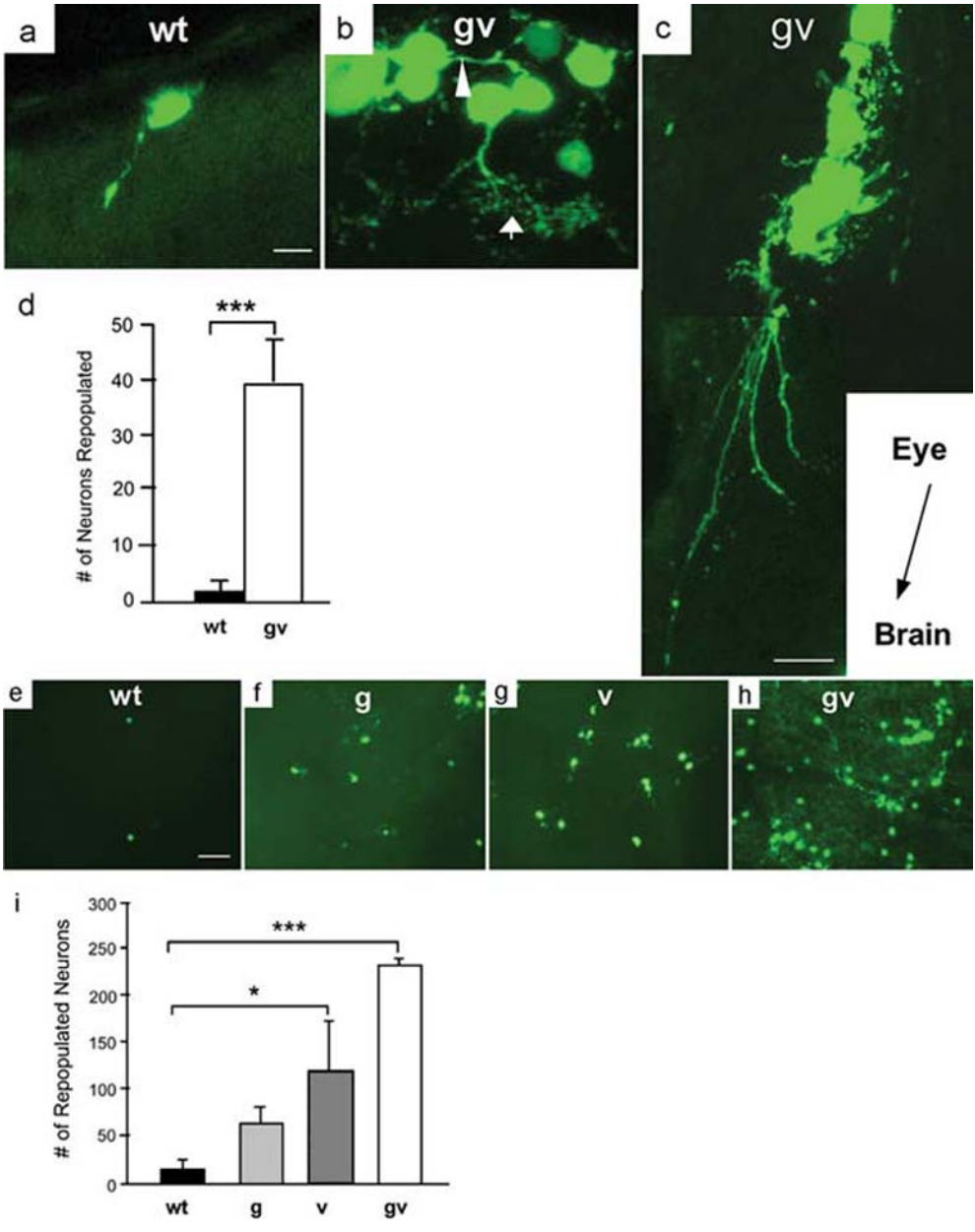
## 5.6 GFAP, Vimentin, and Resistance to Severe Mechanical Stress

While in other tissues, in particular the epidermis, the connection between keratin IFs and resistance to mechanical stress is well established (for review see Fuchs and Cleveland, 1998), the function of astrocyte IFs in maintaining the mechanical integrity of the CNS is unclear. In *GFAP*(−/−) mice, nonreactive astrocytes, which account for the overwhelming majority of astrocytes in a healthy brain, are essentially devoid of IFs (Pekny et al., 1995; McCall et al., 1996). Nevertheless, in three independent studies, *GFAP*(−/−) mice lived normal lives and, if not challenged, had normal CNS morphology (Gomi et al., 1995; Pekny et al., 1995; McCall et al., 1996).

Since the CNS is mechanically well protected, the importance of astrocyte IFs for stabilizing the CNS tissue might become manifest only in situations of severe mechanical stress. Two series of experiments using

**Figure 14-9**

**Integration of retinal transplants in *GFAP*(−/−)*Vim*(−/−) mice. Retinal transplants from mice ubiquitously expressing enhanced green fluorescent protein integrated much better in *GFAP*(−/−)*Vim*(−/−) (GV) than in wild-type (WT) recipients (*a–d*). In *GFAP*(−/−)*Vim*(−/−)recipients, transplanted cells migrated more efficiently from the transplantation site and integrated into the ganglion cell layer (*d*), exhibiting typical morphology of ganglion cells with axon-like process parallel to the retinal surface (arrowhead) and branched dendritic tree-like structures (arrow, *b*). Some of these neurons even extended axons into the optic nerve (*c*). In single mutant recipients (G or V), the transplanted cells spread out more extensively than in wild-type but less efficiently than in *GFAP*(−/−)*Vim*(−/−) recipients (*e–i*). **p*<0.05; ****p*<0.001. Bar, 5 μm in *a–b*, 50 μm in *c*, 100 μm in *e–h*. Data represent mean±SD. (Reproduced with permission from Kinouchi et al., 2003.)**



the head percussion model and the severe mechanical stress applied on the retina, respectively, suggested that this indeed is the case.

In the first of them, *GFAP*(−/−) mice were subjected to head injury from a dropped weight. When placed on a wooden board to prevent head movement at impact, *GFAP*(−/−) mice survived as did wild-type controls. However, when placed on a foam bed that allowed head movement at impact, most of the *GFAP*(−/−) mice, but none of the wild-type controls, died after the injury. The *GFAP*(−/−) mice showed prominent subpial and white matter bleeding in the region of the cervical spinal cord, possibly resulting from a vein rupture (Nawashiro et al., 1998).

Another experimental approach addressed the effect of the absence of IFs in astroglial cells on the mechanical stability of the retina under severe mechanical stress. Being an accessible part of the CNS, retina is well suited for such experiments. In this case, the experiment was performed in mice immediately after death while the retinal tissue was still alive. Application of a severe mechanical stress left the retinas of wild-type controls intact. However, in *GFAP*(−/−)*Vim*(−/−) mice and, to a lesser extent, in *Vim*(−/−)$^{-}$ mice, the inner limiting membrane and adjacent tissue separated from the rest of the retina (❯ *Figure 14-10a–h*). Electron microscopy showed that this retinal "crack" occurred within the end-feet of Müller cells, radial glia-like astroglial cells in the retina that normally contain IFs composed of GFAP and vimentin (Lundkvist et al., 2004). Thus, at least in two specific regions of the CNS, astrocyte IFs seem to be important for resistance to severe mechanical stress, albeit the exact molecular mechanism remains incompletely understood.

## 5.7 GFAP, Vimentin, Osmotic Stress, and CNS Ischemia

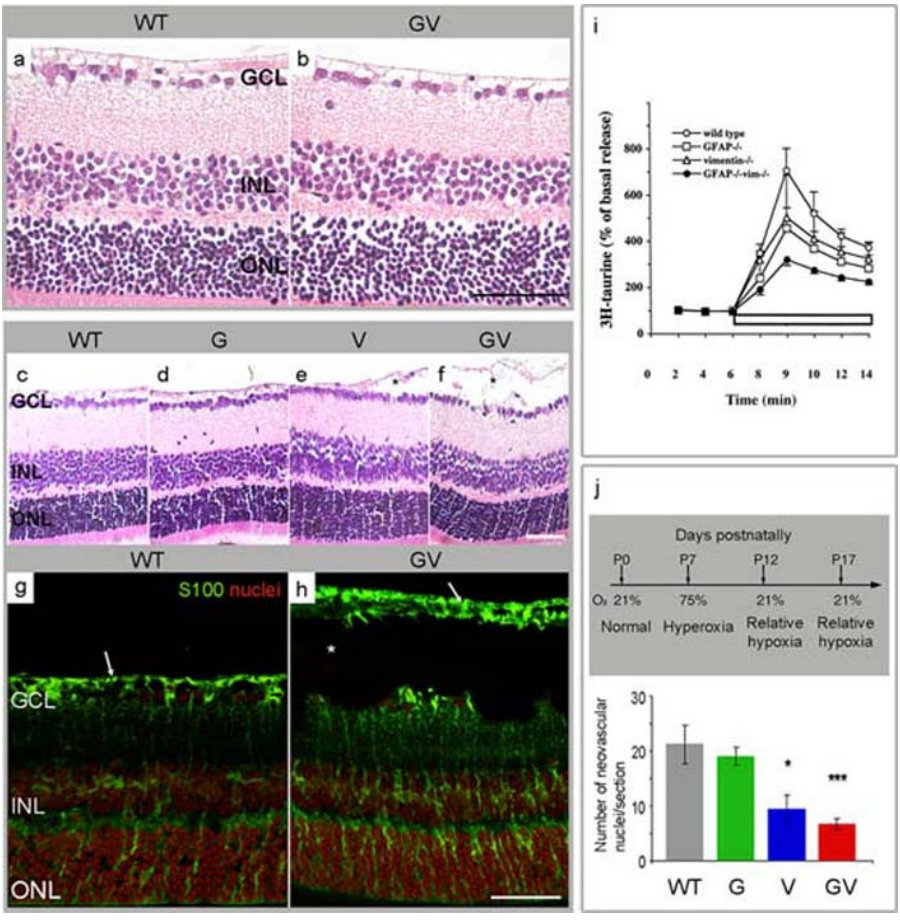
In culture, astrocytes respond to a hypoosmotic environment by transient swelling and within minutes show a tendency to return to their original cell volume (Hoffman, 1991; Kimelberg, 1991). This phenomenon, known as regulatory volume decrease, involves an efflux of osmotically active molecules from astrocytes, such as the amino acid taurine (Pasantes-Morales et al., 1990; Hoffman, 1991; Moran et al., 1994; Vitarella et al., 1994). It was proposed that regulatory volume decrease by astrocytes might be the key mechanism in counteracting the development of brain edema in response to brain ischemia or trauma and that cytoskeleton-linked stretch-activated plasma membrane channels serve as cell-volume sensors (Sanchez-Olea et al., 1991; Cantiello et al., 1993; Moran et al., 1996; Cantiello, 1997).

To address the role of astrocyte IFs in volume regulation, Ding and coworkers subjected primary astrocyte cultures from wild-type, *GFAP*(−/−), *Vim*(−/−), and *GFAP*(−/−)*Vim*(−/−) mice to hypoosmotic stress (corresponding to a 25 mM reduction in NaCl) in perfusion chambers and assessed the efflux of $^{3}$H-taurine. Taurine release was up to 50% lower in *GFAP*(−/−)*Vim*(−/−) than in wild-type astrocytes, but tended to be only slightly decreased in the single mutants (Ding et al., 1998) (❯ *Figure 14-10i*). Anderova and coworkers perfused spinal slices with an isoosmotic solution with an increased concentration of potassium (50 mM) or a hypoosmotic solution with a reduced sodium concentration and found smaller increases in the potassium concentration around astrocytes in slices from *GFAP*(−/−) mice than in those from wild-type controls (Anderova et al., 2001). Thus, genetic ablation of astrocytic IFs seems to diminish the ability of astrocytes to respond to hypoosmotic stress.

Are these findings relevant for brain pathologies, in particular those connected with prominent osmotic stress, such as brain ischemia? Nawashiro and coworkers exposed *GFAP*(−/−) and wild-type mice to brain ischemia induced by middle cerebral artery occlusion for 2 days and reported comparable infarct volumes in the two groups. However, when middle cerebral artery occlusion was combined with transient occlusion of the carotid artery, *GFAP*(−/−) mice had larger infarcts than did controls (Nawashiro et al., 1998). This raises the interesting and unresolved question of whether reactive astrocytes protect ischemically compromised brain tissue around the infarct in stroke patients. In this respect, *GFAP*(−/−) astrocytes in culture showed increased intracellular glutamine levels (Pekny et al., 1999a) and decreased glutamate transport (Hughes et al., 2004), but normal levels of other amino acids and normal glucose and ascorbate uptake (Pekny et al., 1999a). Studies of *GFAP*(−/−)*Vim*(−/−) mice, whose reactive astrocytes are devoid of IFs (Eliasson et al., 1999), in various brain ischemia paradigms should shed more light on this issue.

**Figure 14-10**

**Astrocyte intermediate filaments (IFs), severe mechanical stress, astrocyte volume regulation, and retinal hypoxia. *GFAP*(−/−)*Vim*(−/−) and wild-type retinas are indistinguishable in the absence of a major mechanical challenge (*a–b*). Severe mechanical stress on the retina leads to the complete separation of the inner limiting membrane and adjacent tissue from the rest of the retina (asterisk) in *GFAP*(−/−)*Vim*(−/−) (GV) mice (*f*, *h*) and partial separation (asterisk) in *Vim*(−/−) (V) mice (*e*). The retinas of wild-type (WT; *c*, *g*) or *GFAP*(−/−) (G) mice (*d*) remain intact. *a–f*, H&E staining; *g–h*, visualization of Müller cells and astrocytes by antibodies against S-100. *GCL*, ganglion cell layer; *INL*, inner nuclear layer; *ONL*, outer nuclear layer; *arrow*, the inner limiting membrane. (Reproduced with permission from Lundkvist et al., 2004.); *i*, taurine release following hypoosmostic stress as a measure of the ability of astrocytes to regulate cell volume. In hypoosmotic environment, *GFAP*(−/−)*Vim*(−/−) astrocytes released only about half the amount of $^{3}$H-taurine compared with wild-type ($p<0.01$). The data are presented as mean±SEM. (Reproduced with permission from Ding et al., 1998.); *j*, the oxygen-induced retinopathy model. The number of neovascular nuclei in the vitreous body, a measure of the extent of hypoxia-induced pathological vascularization at postnatal day 17 (P17), is substantially reduced in *GFAP*(−/−)*Vim*(−/−) (GV) retinas and modestly reduced in *Vim*(−/−) (V) retinas. No difference was found between *GFAP*(−/−) (G) and wild-type (WT) retinas. In the absence of hypoxia, blood vessels do not enter the vitreous body, and the normal vascularization of the retina does not depend on the presence of GFAP and vimentin. *$p<0.05$; ***$p<0.005$. (Reproduced with permission from Lundkvist et al., 2004.)**



### 5.8 Decreased Pathological Vascularization in *GFAP*(−/−)*Vim*(−/−) Hypoxic Retinas

In order to assess the pathophysiological implications of the "retinal crack" phenotype found in *GFAP*(−/−) *Vim*(−/−) mice described above, we have turned to a hypoxia model. We exposed *GFAP*(−/−)*Vim*(−/−) mice and single mutants to retinal hypoxia. This leads to oxygen-induced retinopathy, a widely used model of retinopathy of immaturity that also exhibits some features of diabetic retinopathy (Smith et al., 1994). On postnatal day 7, mice are placed into an environment with decreased oxygen concentration, which delays the development of the vascular system. Five days later, the mice are transferred to normooxygenic environment, which leads to massive neovascularization triggered by relative hypoxia (*Figure 14-10j*). The vessels grow from the retina into the vitreous body (as they do in premature babies or patients with diabetes), and their presence there can easily be quantified (Smith et al., 1994). Hypoxia-induced vascularization was decreased substantially in *GFAP*(−/−)*Vim*(−/−) and partially in *Vim*(−/−) mice (*Figure 14-10j*). Thus the absence of IFs in Müller cells of the retina decreases the resistance of their end-feet and consequently of the corresponding layer of the retina to mechanical stress, and it also reduces the extent of ischemia-triggered pathological vascularization (Lundkvist et al., 2004).

## 6 GFAP, Cell Proliferation, and Tumorigenesis

High-grade astrocytomas are the most frequent brain tumors, and they are ranked among the most malignant tumors (Bigner et al., 1998). In many high-grade astrocytomas, the tumor cells lose their GFAP expression, and this often inversely correlates with tumor malignancy (Jacque et al., 1978, 1979; van der Meulen et al., 1978; Velasco et al., 1980; Tascos et al., 1982). Within the same tumor, the cells negative for GFAP were shown to grow faster than the GFAP-positive cells in their vicinity (Hara et al., 1991; Kajiwara et al., 1992).

These data are compatible with a number of in vitro studies. The inhibition of GFAP expression by antisense cDNA in human astrocytoma cell lines results in increased cell proliferation, transformability, and the loss of the tumor cells to extend processes in response to neurons (Weinstein et al., 1991; Rutka et al., 1994). Restoration of GFAP expression reinduces process extension in the tumor cells (Chen and Liem, 1994). GFAP overexpression by previously GFAP-negative astrocytoma cell lines results in a decreased proliferation and cell transformability (Rutka and Smith, 1993; Toda et al., 1994). We reported that primary astrocytes from *GFAP*(−/−) mice grew more quickly in culture and reached higher saturation cell densities than those from wild-type cells (Pekny et al., 1998a). To address a possible role of GFAP in tumor development, astrocytomas were induced in *GFAP*(−/−) and *GFAP*(+/+) mice that had been backcrossed on the *p53*(−/−) genetic background by prenatal exposure to the mutagen ethylnitrosourea (ENU; for the ENU induction of astrocytomas on the *p53*(−/−) background check Oda et al., 1997; Leonard et al., 2001). No difference in tumor incidence, age at tumor detection, tumor size, location, or histology was found between *GFAP*(−/−) and *GFAP*(+/+) mice (Wilhelmsson et al., 2003). This suggests that the loss of GFAP expression does not constitute a step in the development of high-grade astrocytomas. Most likely, it reflects the undifferentiated state of these cells (Wilhelmsson et al., 2003).

## 7 GFAP Mutations Cause Alexander's Disease, a Fatal Neurodegeneration

To study the role of GFAP in astrocyte hypertrophy, Messing, Brenner, and coworkers generated mice overexpressing human GFAP. The astrocytes of these transgenic mice formed complex intracytoplasmic aggregates of GFAP and small stress proteins that were identical to structures known as Rosenthal fibers (Messing et al., 1998). Rosenthal fibers, which accompany chronic reactive astrogliosis, are eosinophilic, elongated structures that when examined ultrastructurally appear as electron-dense, amorphous masses surrounded by and merging with dense bundles of IFs (Messing et al., 1998). Rosenthal fibers are also a hallmark of Alexander's disease, a rare and fatal leukoencephalopathy that most commonly affects infants

and young children, who typically present with feeding problems, paraparesis, seizures, and mental and physical retardation. Juvenile forms of Alexander's disease cause predominantly pseudobulbar and bulbar signs, while adult forms are more variable and often resemble multiple sclerosis. The GFAP-overexpressing mice died at an early age, and although the cause of death remains unknown, these results pointed to an interesting possibility of GFAP as a candidate gene for Alexander's disease.

Subsequent investigations determined that the majority of cases of infantile Alexander's disease, and at least some cases of the late-onset juvenile and adult forms, are due to heterozygous missense mutations in the GFAP gene. The heterozygosity of the mutations suggests that they are dominant. In the majority of cases, the mutations seemed to occur de novo and were not found in either parent (Brenner et al., 2001; Li et al., 2002). However, familial adult cases have been described, raising the interesting issue of reduced penetrance or germline mosaicism (Namekawa et al., 2002; Okamoto et al., 2002). Although these results identify mutated GFAP as at least one of the culprits responsible for a fatal neurological disorder in humans, the mechanism remains unclear. How the mutant GFAP protein causes brain damage and the role of Rosenthal fibers in this process are unknown (Messing and Brenner, 2003a,b). However, Alexander's disease became the first monogenic disease caused by a primary defect in astrocytes and it can be expected that the understanding of its molecular pathogenesis will provide data relevant for other neurodegenerative diseases.

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## References

Alves JA, Barone P, Engelender S, Froes MM, Menezes JR. 2002. Initial stages of radial glia astrocytic transformation in the early postnatal anterior subventricular zone. J Neurobiol 52: 251-265.

Anderova M, Kubinova S, Mazel T, Chvatal A, Eliasson C, et al. 2001. Effect of elevated K(+), hypotonic stress, and cortical spreading depression on astrocyte swelling in GFAP-deficient mice. Glia 35: 189-203.

Angelides KJ, Smith KE, Takeda M. 1989. Assembly and exchange of intermediate filament proteins of neurons: neurofilaments are dynamic structures. J Cell Biol 108: 1495-1506.

Baba A. 1998. Role of endothelin B receptor signals in reactive astrocytes. Life Sci 62: 1711-1715.

Balcarek JM, Cowan NJ. 1985. Structure of the mouse glial fibrillary acidic protein gene: implications for the evolution of the intermediate filament multigene family. Nucleic Acids Res 13: 5527-5543.

Barresi V, Condorelli DF, Giuffrida Stella AM. 1999. GFAP gene methylation in different neural cell types from rat brain. Int J Dev Neurosci 17: 821-828.

Bernal SD, Stahel RA. 1985. Cytoskeleton-associated proteins: their role as cellular integrators in the neoplastic process. Crit Rev Oncol Hematol 3: 191-204.

Bernier L, Colman DR, D'Eustachio P. 1988. Chromosomal locations of genes encoding 2′,3′ cyclic nucleotide 3′-phosphodiesterase and glial fibrillary acidic protein in the mouse. J Neurosci Res 20: 497-504.

Besnard F, Brenner M, Nakatani Y, Chao R, Purohit HJ, et al. 1991. Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. J Biol Chem 266: 18877-18883.

Bigner SH, McLendon RE, Al-dosari N, Rasheed A. 1998. The genetic basis of human cancer. Vogelstein B, Kinsler KW, editors. McGraw-Hill; New York: pp. 661-670.

Brenner M. 1994. Structure and transcriptional regulation of the GFAP gene. Brain Pathol 4: 245-257.

Brenner M, Johnson AB, Boespflug-Tanguy O, Rodriguez D, Goldman JE, et al. 2001. Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. Nat Genet 27: 117-120.

Brenner M, Kisseberth WC, Su Y, Besnard F, Messing A. 1994. GFAP promoter directs astrocyte-specific expression in transgenic mice. J Neurosci 14: 1030-1037.

Brenner M, Messing A. 1996. GFAP transgenic mice. Methods 10: 351-364.

Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, et al. 1999. Leukocyte infiltration, neuronal

degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. Neuron 2: 297-308.

Bushong EA, Martone ME, Ellisman MH. 2004. Maturation of astrocyte morphology and the establishment of astrocyte domains during postnatal hippocampal development. Int J Dev Neurosci 22: 73-86.

Bushong EA, Martone ME, Jones YZ, Ellisman MH. 2002. Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci 22: 183-192.

Cantiello HF. 1997. Role of actin filament organization in cell volume and ion channel regulation. J Exp Zool 279: 425-435.

Cantiello HF, Prat AG, Bonventre JV, Cunningham CC, Hartwig JH, et al. 1993. Actin-binding protein contributes to cell volume regulatory ion channel activation in melanoma cells. J Biol Chem 268: 4596-4599.

Chanas-Sacre G, Rogister B, Moonen G, Leprince P. 2000. Radial glia phenotype: origin, regulation, and transdifferentiation. J Neurosci Res 61: 357-363.

Chen WJ, Liem RK. 1994. Reexpression of glial fibrillary acidic protein rescues the ability of astrocytoma cells to form processes in response to neurons. J Cell Biol 127: 813-823.

Cho KS, Yang L, Lu B, Feng Ma H, Huang X, Pekny M, Chen DF. 2005. Re-establishing the regenerative potential of central nervous system axons in postnatal mice. J Cell Sci 118: 863-872.

Choi BH, Lapham LW. 1980. Evolution of Bergmann glia in developing human fetal cerebellum: a Golgi, electron microscopic and immunofluorescent study. Brain Res 190: 369-383.

Chou YH, Helfand BT, Goldman RD. 2001. New horizons in cytoskeletal dynamics: transport of intermediate filaments along microtubule tracks. Curr Opin Cell Biol 13: 106-109.

Chou YH, Khuon S, Herrmann H, Goldman RD. 2003. Nestin promotes the phosphorylation-dependent disassembly of vimentin intermediate filaments during mitosis. Mol Biol Cell 14: 1468-1478.

Colucci-Guyon E, Gimenez YRM, Maurice T, Babinet C, Privat A. 1999. Cerebellar defect and impaired motor coordination in mice lacking vimentin. Glia 25: 33-43.

Colucci-Guyon E, Portier MM, Dunia I, Paulin D, Pournin S, et al. 1994. Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell 79: 679-694.

Condorelli DF, Dell'Albani P, Conticello SG, Barresi V, Nicoletti VG, et al. 1997. A neural-specific hypomethylated domain in the 5′ flanking region of the glial fibrillary acidic protein gene. Dev Neurosci 19: 446-456.

Condorelli DF, Nicoletti VG, Barresi V, Caruso A, Conticello S, et al. 1994. Tissue-specific DNA methylation patterns of the rat glial fibrillary acidic protein gene. J Neurosci Res 39: 694-707.

Dahlstrand J, Lardelli M, Lendahl U. 1995. Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. Brain Res Dev Brain Res 84: 109-129.

Dahlstrand J, Zimmerman LB, McKay RD, Lendahl U. 1992. Characterization of the human nestin gene reveals a close evolutionary relationship to neurofilaments. J Cell Sci 103 (Pt. 2): 589-597.

deAzevedo LC, Fallet C, Moura-Neto V, Daumas-Duport C, Hedin-Pereira C, et al. 2003. Cortical radial glial cells in human fetuses: depth-correlated transformation into astrocytes. J Neurobiol 55: 288-298.

Ding M, Eliasson C, Betsholtz C, Hamberger A, Pekny M. 1998. Altered taurine release following hypotonic stress in astrocytes from mice deficient for GFAP and vimentin. Brain Res Mol Brain Res 62: 77-81.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97: 703-716.

Eckes B, Dogic D, Colucci-Guyon E, Wang N, Maniotis A, et al. 1998. Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts. J Cell Sci 111 (Pt. 13): 1897-1907.

Eddleston M, Mucke L. 1993. Molecular profile of reactive astrocytes—implications for their role in neurologic disease. Neuroscience 54: 15-36.

Edwards MA, Yamamoto M, Caviness Jr. VS 1990. Organization of radial glia and related cells in the developing murine CNS. An analysis based upon a new monoclonal antibody marker. Neuroscience 36: 121-144.

Eliasson C, Sahlgren C, Berthold CH, Stakeberg J, Celis JE, et al. 1999. Intermediate filament protein partnership in astrocytes. J Biol Chem 274: 23996-24006.

Emsley JG, Arlotta P, Macklis JD. 2004. Star-cross'd neurons: astroglial effects on neural repair in the adult mammalian CNS. Trends Neurosci 27: 238-240.

Eng LF, Ghirnikar RS, Lee YL. 2000. Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000). Neurochem Res 25: 1439-1451.

Eng LF, Vanderhaeghen JJ, Bignami A, Gerstl B. 1971. An acidic protein isolated from fibrous astrocytes. Brain Res 28: 351-354.

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, et al. 2004. Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci 24: 2143-2155.

Ferrari S, Battini R, Kaczmarek L, Rittling S, Calabretta B, et al. 1986. Coding sequence and growth regulation of the human vimentin gene. Mol Cell Biol 6: 3614-3620.

Frisen J, Johansson CB, Torok C, Risling M, Lendahl U. 1995. Rapid, widespread, and longlasting induction of nestin

contributes to the generation of glial scar tissue after CNS injury. J Cell Biol 131: 453-464.

Fuchs E, Cleveland DW. 1998. A structural scaffolding of intermediate filaments in health and disease. Science 279: 514-519.

Goldman RD, Chou YH, Prahlad V, Yoon M. 1999. Intermediate filaments: dynamic processes regulating their assembly, motility, and interactions with other cytoskeletal systems. FASEB J 13 (Suppl. 2): S261-S265.

Gomi H, Yokoyama T, Fujimoto K, Ikeda T, Katoh A, et al. 1995. Mice devoid of the glial fibrillary acidic protein develop normally and are susceptible to scrapie prions. Neuron 14: 29-41.

Gotz M, Hartfuss E, Malatesta P. 2002. Radial glial cells as neuronal precursors: a new perspective on the correlation of morphology and lineage restriction in the developing cerebral cortex of mice. Brain Res Bull 57: 777-788.

Hara A, Sakai N, Yamada H, Niikawa S, Ohno T, et al. 1991. Proliferative assessment of GFAP-positive and GFAP-negative glioma cells by nucleolar organizer region staining. Surg Neurol 36: 190-194.

Helfand BT, Loomis P, Yoon M, Goldman RD. 2003. Rapid transport of neural intermediate filament protein. J Cell Sci 116: 2345-2359.

Hernandez MR, Agapova OA, Yang P, Salvador-Silva M, Ricard CS, et al. 2002. Differential gene expression in astrocytes from human normal and glaucomatous optic nerve head analyzed by cDNA microarray. Glia 38: 45-64.

Herrmann H, Aebi U. 2000. Intermediate filaments and their associates: multi-talented structural elements specifying cytoarchitecture and cytodynamics. Curr Opin Cell Biol 12: 79-90.

Herrmann H, Aebi U. 2004. Intermediate filaments: Molecular structure, assembly mechanism, and integration into functionally distinct intracellular scaffolds. Annu Rev Biochem 73: 749-789.

Herrmann H, Hesse M, Reichenzeller M, Aebi U, Magin TM. 2003. Functional complexity of intermediate filament cytoskeletons: from structure to assembly to gene ablation. Int Rev Cytol 223: 83-175.

Hirako Y, Yamakawa H, Tsujimura Y, Nishizawa Y, Okumura M, et al. 2003. Characterization of mammalian synemin, an intermediate filament protein present in all four classes of muscle cells and some neuroglial cells: co-localization and interaction with type III intermediate filament proteins and keratins. Cell Tissue Res 313: 195-207.

Hockfield S, McKay RD. 1985. Identification of major cell classes in the developing mammalian nervous system. J Neurosci 5: 3310-3328.

Hoffman E. 1991. Volume regulation in cultured cells. Academic Press; New York: pp. 124-180.

Holwell TA, Schweitzer SC, Evans RM. 1997. Tetracycline regulated expression of vimentin in fibroblasts derived from vimentin null mice. J Cell Sci 110: 1947-1956.

Hughes EG, Maguire JL, McMinn MT, Scholz RE, Sutherland ML. 2004. Loss of glial fibrillary acidic protein results in decreased glutamate transport and inhibition of PKA-induced EAAT2 cell surface trafficking. Brain Res Mol Brain Res 124: 114-123.

Imura T, Kornblum HI, Sofroniew MV. 2003. The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. J Neurosci 23: 2824-2832.

Inagaki M, Gonda Y, Nishizawa K, Kitamura S, Sato C, et al. 1990. Phosphorylation sites linked to glial filament disassembly in vitro locate in a non-alpha-helical head domain. J Biol Chem 265: 4722-4729.

Inagaki M, Nakamura Y, Takeda M, Nishimura T, Inagaki N. 1994. Glial fibrillary acidic protein: dynamic property and regulation by phosphorylation. Brain Pathol 4: 239-243.

Ishikawa N, Takemura M, Koyama Y, Shigenaga Y, Okada T, et al. 1997. Endothelins promote the activation of astrocytes in rat neostriatum through ET(B) receptors. Eur J Neurosci 9: 895-901.

Izmailova ES, Wieczorek E, Perkins EB, Zehner ZE. 1999. A GC-box is required for expression of the human vimentin gene. Gene 235: 69-75.

Jacque CM, Kujas M, Poreau A, Raoul M, Collier P, et al. 1979. GFA and S 100 protein levels as an index for malignancy in human gliomas and neurinomas. J Natl Cancer Inst 62: 479-483.

Jacque CM, Vinner C, Kujas M, Raoul M, Racadot J, et al. 1978. Determination of glial fibrillary acidic protein (GFAP) in human brain tumors. J Neurol Sci 35: 147-155.

Johansson CB, Lothian C, Molin M, Okano H, Lendahl U. 2002. Nestin enhancer requirements for expression in normal and injured adult CNS. J Neurosci Res 69: 784-794.

Johansson CB, Momma S, Clarke DL, Risling M, Lendahl U, et al. 1999. Identification of a neural stem cell in the adult mammalian central nervous system. Cell 96: 25-34.

Johnson WB, Ruppe MD, Rockenstein EM, Price J, Sarthy VP, et al. 1995. Indicator expression directed by regulatory sequences of the glial fibrillary acidic protein (GFAP) gene: in vivo comparison of distinct GFAP-lacZ transgenes. Glia 13: 174-184.

Josephson R, Muller T, Pickel J, Okabe S, Reynolds K, et al. 1998. POU transcription factors control expression of CNS stem cell-specific genes. Development 125: 3087-3100.

Kachinsky AM, Dominov JA, Miller JB. 1994. Myogenesis and the intermediate filament protein, nestin. Dev Biol 165: 216-228.

Kajiwara K, Orita T, Nishizaki T, Kamiryo T, Nakayama H, et al. 1992. Glial fibrillary acidic protein (GFAP) expression

and nucleolar organizer regions (NORs) in human gliomas. Brain Res 572: 314-318.

Kalman M, Ajtai BM. 2001. A comparison of intermediate filament markers for presumptive astroglia in the developing rat neocortex: immunostaining against nestin reveals more detail, than GFAP or vimentin. Int J Dev Neurosci 19: 101-108.

Kalman M, Szekely AD, Csillag A. 1998. Distribution of glial fibrillary acidic protein and vimentin-immunopositive elements in the developing chicken brain from hatch to adulthood. Anat Embryol (Berl) 198: 213-235.

Kaneko R, Sueoka N. 1993. Tissue-specific versus cell type-specific expression of the glial fibrillary acidic protein. Proc Natl Acad Sci USA 90: 4698-4702.

Kimelberg HK. 1991. Swelling and volume control in brain astroglial cells. Springer; New York: pp. 81-117.

Kinouchi R, Takeda M, Yang L, Wilhelmsson U, Lundkvist A, et al. 2003. Robust neural integration from retinal transplants in mice deficient in GFAP and vimentin. Nat Neurosci 6: 863-868.

Kosako H, Amano M, Yanagida M, Tanabe K, Nishi Y, et al. 1997. Phosphorylation of glial fibrillary acidic protein at the same sites by cleavage furrow kinase and Rho-associated kinase. J Biol Chem 272: 10333-10336.

Koyama Y, Takemura M, Fujiki K, Ishikawa N, Shigenaga Y, et al. 1999. BQ788, an endothelin ET(B) receptor antagonist, attenuates stab wound injury-induced reactive astrocytes in rat brain. Glia 26: 268-271.

Kryszke MH, Vicart P. 1998. Regulation of the expression of the human vimentin gene: application to cellular immortalization. Pathol Biol (Paris) 46: 39-45.

Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA. 2000. Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. Proc Natl Acad Sci USA 97: 13883-13888.

Lendahl U, Zimmerman LB, McKay RD. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60: 585-595.

Leonard JR, D'Sa C, Klocke BJ, Roth KA. 2001. Neural precursor cell apoptosis and glial tumorigenesis following transplacental ethyl-nitrosourea exposure. Oncogene 20: 8281-8286.

Lepekhin EA, Eliasson C, Berthold CH, Berezin V, Bock E, et al. 2001. Intermediate filaments regulate astrocyte motility. J Neurochem 79: 617-625.

Levitt P, Rakic P. 1980. Immunoperoxidase localization of glial fibrillary acidic protein in radial glial cells and astrocytes of the developing rhesus monkey brain. J Comp Neurol 193: 815-840.

Li R, Messing A, Goldman JE, Brenner M. 2002. GFAP mutations in Alexander disease. Int J Dev Neurosci 20: 259-268.

Liedtke W, Edelmann W, Bieri PL, Chiu FC, Cowan NJ, et al. 1996. GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination. Neuron 17: 607-615.

Liedtke W, Edelmann W, Chiu FC, Kucherlapati R, Raine CS. 1998. Experimental autoimmune encephalomyelitis in mice lacking glial fibrillary acidic protein is characterized by a more severe clinical course and an infiltrative central nervous system lesion. Am J Pathol 152: 251-259.

Lilienbaum A, Paulin D. 1993. Activation of the human vimentin gene by the Tax human T-cell leukemia virus. I. Mechanisms of regulation by the NF-kappa B transcription factor. J Biol Chem 268: 2180-2188.

Lothian C, Lendahl U. 1997. An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. Eur J Neurosci 9: 452-462.

Lothian C, Prakash N, Lendahl U, Wahlstrom GM. 1999. Identification of both general and region-specific embryonic CNS enhancer elements in the nestin promoter. Exp Cell Res 248: 509-519.

Lundkvist A, Reichenbach A, Betsholtz C, Carmeliet P, Wolburg H, et al. 2004. Under stress, the absence of intermediate filaments from Muller cells in the retina has structural and functional consequences. J Cell Sci 117: 3481-3488.

Masood K, Besnard F, Su Y, Brenner M. 1993. Analysis of a segment of the human glial fibrillary acidic protein gene that directs astrocyte-specific transcription. J Neurochem 61: 160-166.

Matsuoka Y, Nishizawa K, Yano T, Shibata M, Ando S, et al. 1992. Two different protein kinases act on a different time schedule as glial filament kinases during mitosis. Embo J 11: 2895-2902.

McCall MA, Gregg RG, Behringer RR, Brenner M, Delaney CL, et al. 1996. Targeted deletion in astrocyte intermediate filament (Gfap) alters neuronal physiology. Proc Natl Acad Sci USA 93: 6361-6366.

Menet V, Gimenez YRM, Chauvet N, Drian MJ, Lannoy J, Colucci-Guyon E, Privat A. 2001. Inactivation of the glial fibrillary acidic protein gene, but not that of vimentin, improves neuronal survival and neurite growth by modifying adhesion molecule expression. J Neurosci 21: 6147-6158.

Menet V, Gimenez YRM, Sandillon F, Privat A. 2000. GFAP null astrocytes are a favorable substrate for neuronal survival and neurite growth. Glia 31: 267-272.

Menet V, Prieto M, Privat A, Gimenez YRM. 2003. Axonal plasticity and functional recovery after spinal cord injury in mice deficient in both glial fibrillary acidic protein and vimentin genes. Proc Natl Acad Sci USA 100: 8999-9004.

Messing A, Brenner M. 2003a. Alexander disease: GFAP mutations unify young and old. Lancet Neurol 2: 75.

Messing A, Brenner M. 2003b. GFAP: functional implications gleaned from studies of genetically engineered mice. Glia 43: 87-90.

Messing A, Head MW, Galles K, Galbreath EJ, Goldman JE, et al. 1998. Fatal encephalopathy with astrocyte inclusions in GFAP transgenic mice. Am J Pathol 152: 391-398.

Miller RK, Vikstrom K, Goldman RD. 1991. Keratin incorporation into intermediate filament networks is a rapid process. J Cell Biol 113: 843-855.

Miura M, Tamura T, Mikoshiba K. 1990. Cell-specific expression of the mouse glial fibrillary acidic protein gene: identification of the cis- and trans-acting promoter elements for astrocyte-specific expression. J Neurochem 55: 1180-1188.

Moran J, Maar T, Pasantes-Morales H. 1994. Cell volume regulation in taurine deficient cultured astrocytes. Adv Exp Med Biol 359: 361-367.

Moran J, Sabanero M, Meza I, Pasantes-Morales H. 1996. Changes of actin cytoskeleton during swelling and regulatory volume decrease in cultured astrocytes. Am J Physiol 271: C1901-C1907.

Morshead CM, Garcia AD, Sofroniew MV, van Der Kooy D. 2003. The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. Eur J Neurosci 18: 76-84.

Moura-Neto V, Kryszke MH, Li Z, Vicart P, Lilienbaum A, et al. 1996. A 28-bp negative element with multiple factor-binding activity controls expression of the vimentin-encoding gene. Gene 168: 261-266.

Nakamura Y, Takeda M, Aimoto S, Hojo H, Takao T, et al. 1992. Assembly regulatory domain of glial fibrillary acidic protein. A single phosphorylation diminishes its assembly-accelerating property. J Biol Chem 267: 23269-23274.

Nakamura Y, Takeda M, Angelides KJ, Tada K, Hariguchi S, et al. 1991. Assembly, disassembly, and exchange of glial fibrillary acidic protein. Glia 4: 101-110.

Nakamura Y, Takeda M, Nishimura T. 1996. Dynamics of bovine glial fibrillary acidic protein phosphorylation. Neurosci Lett 205: 91-94.

Namekawa M, Takiyama Y, Aoki Y, Takayashiki N, Sakoe K, et al. 2002. Identification of GFAP gene mutation in hereditary adult-onset Alexander's disease. Ann Neurol 52: 779-785.

Nawashiro H, Messing A, Azzam N, Brenner M. 1998. Mice lacking GFAP are hypersensitive to traumatic cerebrospinal injury. Neuroreport 9: 1691-1696.

Nielsen AL, Jorgensen AL. 2003. Structural and functional characterization of the zebrafish gene for glial fibrillary acidic protein, GFAP. Gene 310: 123-132.

Nishizawa K, Yano T, Shibata M, Ando S, Saga S, et al. 1991. Specific localization of phosphointermediate filament protein in the constricted area of dividing cells. J Biol Chem 266: 3074-3079.

Oda H, Zhang S, Tsurutani N, Shimizu S, Nakatsuru Y, et al. 1997. Loss of p53 is an early event in induction of brain tumors in mice by transplacental carcinogen exposure. Cancer Res 57: 646-650.

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 1997. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407: 313-319.

Okamoto Y, Mitsuyama H, Jonosono M, Hirata K, Arimura K, et al. 2002. Autosomal dominant palatal myoclonus and spinal cord atrophy. J Neurol Sci 195: 71-76.

Pasantes-Morales H, Moran J, Schousboe A. 1990. Volume-sensitive release of taurine from cultured astrocytes: properties and mechanism. Glia 3: 427-432.

Paulin D, Lilienbaum A, Duprey P, Li Z, Vicart P. 1990. Regulatory elements of the human vimentin gene: activation during proliferation. Reprod Nutr Dev 30: 423-429.

Pekny M. 2001. Astrocytic intermediate filaments: lessons from GFAP and vimentin knock-out mice. Prog Brain Res 132: 23-30.

Pekny M, Eliasson C, Chien CL, Kindblom LG, Liem R, et al. 1998a. GFAP-deficient astrocytes are capable of stellation in vitro when cocultured with neurons and exhibit a reduced amount of intermediate filaments and an increased cell saturation density. Exp Cell Res 239: 332-343.

Pekny M, Eliasson C, Siushansian R, Ding M, Dixon SJ, et al. 1999a. The impact of genetic removal of GFAP and/or vimentin on glutamine levels and transport of glucose and ascorbate in astrocytes. Neurochem Res 24: 1357-1362.

Pekny M, Johansson CB, Eliasson C, Stakeberg J, Wallen A, et al. 1999b. Abnormal reaction to central nervous system injury in mice lacking glial fibrillary acidic protein and vimentin. J Cell Biol 145: 503-514.

Pekny M, Leveen P, Pekna M, Eliasson C, Berthold CH, et al. 1995. Mice lacking glial fibrillary acidic protein display astrocytes devoid of intermediate filaments but develop and reproduce normally. EMBO J 14: 1590-1598.

Pekny M, Pekna M, Wilhelmsson U, Chen DF. 2004. Response to Quinlan and Nilsson: astroglia sitting at the controls? Trends Neurosci 27: 243-244.

Pekny M, Stanness KA, Eliasson C, Betsholtz C, Janigro D. 1998b. Impaired induction of blood–brain barrier properties in aortic endothelial cells by astrocytes from GFAP-deficient mice. Glia 22: 390-400.

Peters CM, Rogers SD, Pomonis JD, Egnazyck GF, Keyser CP, et al. 2003. Endothelin receptor expression in the normal and injured spinal cord: potential involvement in injury-induced ischemia and gliosis. Exp Neurol 180: 1-13.

Pieper FR, Van de Klundert FA, Raats JM, Henderik JB, Schaart G, et al. 1992. Regulation of vimentin expression in cultured epithelial cells. Eur J Biochem 210: 509-519.

Pieper FR, Slobbe RL, Ramaekers FC, Cuypers HT, Bloemendal H. 1987. Upstream regions of the hamster desmin and vimentin genes regulate expression during in vitro myogenesis. Embo J 6: 3611-3618.

Pixley SK, Kobayashi Y, de Vellis J. 1984. A monoclonal antibody against vimentin: characterization. Brain Res 317: 185-199.

Pixley SK, de Vellis J. 1984. Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. Brain Res 317: 201-209.

Prahlad V, Yoon M, Moir RD, Vale R D, Goldman RD. 1998. Rapid movements of vimentin on microtubule tracks: kinesin-dependent assembly of intermediate filament networks. J Cell Biol 143: 159-170.

Quinlan R, Nilsson M. 2004. Reloading the retina by modifying the glial matrix. Trends Neurosci 27: 241-242.

Reeves SA, Helman LJ, Allison A, Israel MA. 1989. Molecular cloning and primary structure of human glial fibrillary acidic protein. Proc Natl Acad Sci USA 86: 5178-5182.

Renner W, Franke WW, Schmid E, Geisler N, Weber K, et al. 1981. Reconstitution of intermediate-sized filaments from denatured monomeric vimentin. J Mol Biol 149: 285-306.

Riol H, Fages C, Tardy M. 1992. Transcriptional regulation of glial fibrillary acidic protein (GFAP)-mRNA expression during postnatal development of mouse brain. J Neurosci Res 32: 79-85.

Rueger DC, Huston JS, Dahl D, Bignami A. 1979. Formation of 100 A filaments from purified glial fibrillary acidic protein in vitro. J Mol Biol 135: 53-68.

Rutka JT, Hubbard SL, Fukuyama K, Matsuzawa K, Dirks PB, et al. 1994. Effects of antisense glial fibrillary acidic protein complementary DNA on the growth, invasion, and adhesion of human astrocytoma cells. Cancer Res 54: 3267-3272.

Rutka JT, Smith SL. 1993. Transfection of human astrocytoma cells with glial fibrillary acidic protein complementary DNA: analysis of expression, proliferation, and tumorigenicity. Cancer Res 53: 3624-3631.

Sanchez-Olea R, Moran J, Schousboe A, Pasantes-Morales H. 1991. Hyposmolarity-activated fluxes of taurine in astrocytes are mediated by diffusion. Neurosci Lett 130: 233-236.

Sancho-Tello M, Valles S, Montoliu C, Renau-Piqueras J, Guerri C. 1995. Developmental pattern of GFAP and vimentin gene expression in rat brain and in radial glial cultures. Glia 15: 157-166.

Sarid J. 1991. Identification of a cis-acting positive regulatory element of the glial fibrillary acidic protein gene. J Neurosci Res 28: 217-228.

Sarkar S, Cowan NJ. 1991. Regulation of expression of glial filament acidic protein. J Cell Sci Suppl 15: 97-102.

Sejersen T, Lendahl U. 1993. Transient expression of the intermediate filament nestin during skeletal muscle development. J Cell Sci 106(Pt. 4): 1291-1300.

Shaw G, Osborn M, Weber K. 1981. An immunofluorescence microscopical study of the neurofilament triplet proteins, vimentin and glial fibrillary acidic protein within the adult rat brain. Eur J Cell Biol 26: 68-82.

Shibuki K, Gomi H, Chen L, Bao S, Kim JJ, et al. 1996. Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. Neuron 16: 587-599.

Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, et al. 1994. Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci 35: 101-111.

Smith QR. 2003. A review of blood–brain barrier transport techniques. Methods Mol Med 89: 193-208.

Sommers CL, Skerker JM, Chrysogelos SA, Bosseler M, Gelmann EP. 1994. Regulation of vimentin gene transcription in human breast cancer cell lines. Cell Growth Differ 5: 839-846.

Song H, Stevens CF, Gage FH. 2002. Astroglia induce neurogenesis from adult neural stem cells. Nature 417: 39-44.

Song MR, Ghosh A. 2004. FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat Neurosci 7: 229-235.

Steinert PM, Parry DA. 1985. Intermediate filaments: conformity and diversity of expression and structure. Annu Rev Cell Biol 1: 41-65.

Steinert PM, Roop DR. 1988. Molecular and cellular biology of intermediate filaments. Annu Rev Biochem 57: 593-625.

Su M, Hu H, Lee Y, d'Azzo A, Messing A, et al. 2004. Expression specificity of GFAP transgenes. Neurochem Res 29: 2075-2093.

Sugawara K, Kurihara H, Negishi M, Saito N, Nakazato Y, et al. 2002. Nestin as a marker for proliferative endothelium in gliomas. Lab Invest 82: 345-351.

Sultana S, Sernett SW, Bellin RM, Robson RM, Skalli O. 2000. Intermediate filament protein synemin is transiently expressed in a subset of astrocytes during development. Glia 30: 143-153.

Takemura M, Gomi H, Colucci-Guyon E, Itohara S. 2002a. Protective role of phosphorylation in turnover of glial fibrillary acidic protein in mice. J Neurosci 22: 6972-6979.

Takemura M, Nishiyama H, Itohara S. 2002b. Distribution of phosphorylated glial fibrillary acidic protein in the mouse central nervous system. Genes Cells 7: 295-307.

Takizawa T, Nakashima K, Namihira M, Ochiai W, Uemura A, et al. 2001. DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 1: 749-758.

Tanaka H, Katoh A, Oguro K, Shimazaki K, Gomi H, et al. 2002. Disturbance of hippocampal long-term potentiation after transient ischemia in GFAP deficient mice. J Neurosci Res 67: 11-20.

Tardy M, Fages C, Riol H, Le Prince G, Rataboul P, et al. 1989. Developmental expression of the glial fibrillary acidic protein mRNA in the central nervous system and in cultured astrocytes. J Neurochem 52: 162-167.

Tascos NA, Parr J, Gonatas NK. 1982. Immunocytochemical study of the glial fibrillary acidic protein in human neoplasms of the central nervous system. Hum Pathol 13: 454-458.

Tatzelt J, Maeda N, Pekny M, Yang SL, Betsholtz C, et al. 1996. Scrapie in mice deficient in apolipoprotein E or glial fibrillary acidic protein. Neurology 47: 449-453.

Teter B, Osterburg HH, Anderson CP, Finch CE. 1994. Methylation of the rat glial fibrillary acidic protein gene shows tissue-specific domains. J Neurosci Res 39: 680-693.

Teter B, Rozovsky I, Krohn K, Anderson C, Osterburg H, et al. 1996. Methylation of the glial fibrillary acidic protein gene shows novel biphasic changes during brain development. Glia 17: 195-205.

Toda M, Miura M, Asou H, Toya S, Uyemura K. 1994. Cell growth suppression of astrocytoma C6 cells by glial fibrillary acidic protein cDNA transfection. J Neurochem 63: 1975-1978.

Tsujimura K, Tanaka J, Ando S, Matsuoka Y, Kusubata M, et al. 1994. Identification of phosphorylation sites on glial fibrillary acidic protein for cdc2 kinase and Ca(2+)-calmodulin-dependent protein kinase II. J Biochem (Tokyo) 116: 426-434.

Turner DA, Buhl EH, Hailer NP, Nitsch R. 1998. Morphological features of the entorhinal-hippocampal connection. Prog Neurobiol 55: 537-562.

van de Klundert FA, van Eldik GJ, Pieper FR, Jansen HJ, Bloemendal H. 1992. Identification of two silencers flanking an AP-1 enhancer in the vimentin promoter. Gene 122: 337-343.

van der Meulen JD, Houthoff HJ, Ebels EJ. 1978. Glial fibrillary acidic protein in human gliomas. Neuropathol Appl Neurobiol 4: 177-190.

Velasco ME, Dahl D, Roessmann U, Gambetti P. 1980. Immunohistochemical localization of glial fibrillary acidic protein in human glial neoplasms. Cancer 45: 484-494.

Vikstrom KL, Lim SS, Goldman RD, Borisy GG. 1992. Steady state dynamics of intermediate filament networks. J Cell Biol 118: 121-129.

Vitarella D, DiRisio DJ, Kimelberg HK, Aschner M. 1994. Potassium and taurine release are highly correlated with regulatory volume decrease in neonatal primary rat astrocyte cultures. J Neurochem 63: 1143-1149.

Wang X, Messing A, David S. 1997. Axonal and nonneuronal cell responses to spinal cord injury in mice lacking glial fibrillary acidic protein. Exp Neurol 148: 568-576.

Weinstein DE, Shelanski ML, Liem RK. 1991. Suppression by antisense mRNA demonstrates a requirement for the glial fibrillary acidic protein in the formation of stable astrocytic processes in response to neurons. J Cell Biol 112: 1205-1213.

Wieczorek E, Lin Z, Perkins EB, Law DJ, Merchant JL, et al. 2000. The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1. J Biol Chem 275: 12879-12888.

Wiegers W, Honer B, Traub P. 1991. Microinjection of intermediate filament proteins into living cells with and without preexisting intermediate filament network. Cell Biol Int Rep 15: 287-296.

Wilhelmsson U, Eliasson C, Bjerkvig R, Pekny M. 2003. Loss of GFAP expression in high-grade astrocytomas does not contribute to tumor development or progression. Oncogene 22: 3407-3411.

Wilhelmsson U, Li L, Pekna M, Berthold CH, Blom S, et al. 2004. Absence of glial fibrillary acidic protein and vimentin prevents hypertrophy of astrocytic processes and improves post-traumatic regeneration. J Neurosci 24: 5016-5021.

Xu K, Malouf AT, Messing A, Silver J. 1999. Glial fibrillary acidic protein is necessary for mature astrocytes to react to beta-amyloid. Glia 25: 390-403.

Yasui Y, Amano M, Nagata K, Inagaki N, Nakamura H, et al. 1998. Roles of Rho-associated kinase in cytokinesis; mutations in Rho-associated kinase phosphorylation sites impair cytokinetic segregation of glial filaments. J Cell Biol 143: 1249-1258.

Yaworsky PJ, Kappen C. 1999. Heterogeneity of neural progenitor cells revealed by enhancers in the nestin gene. Dev Biol 205: 309-321.

Yoon M, Moir RD, Prahlad V, Goldman RD. 1998. Motile properties of vimentin intermediate filament networks in living cells. J Cell Biol 143: 147-157.

Yu AC, Lee YL, Fu WY, Eng LF. 1995. Gene expression in astrocytes during and after ischemia. Prog Brain Res 105: 245-253.

Zhang X, Diab IH, Zehner ZE. 2003. ZBP-89 represses vimentin gene transcription by interacting with the transcriptional activator, Sp1. Nucleic Acids Res 31: 2900-2914.

Zimmerman L, Parr B, Lendahl U, Cunningham M, McKay R, et al. 1994. Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. Neuron 12: 11-24.

# 15 GAP-43 in Neural Development and Plasticity

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**Abstract:** The growth-associated protein-43 (GAP-43) is known to participate in mechanisms of synaptic plasticity during neuronal development, nerve regeneration, and learning and memory. Despite the large number of studies using GAP-43 as a marker of neural plasticity, the precise molecular function of GAP-43 in these processes remains to be elucidated. The goal of this chapter is to highlight some of the roles of GAP-43 in brain function by focusing on a few well-documented findings ranging from the control of gene expression to its role in membrane–cytoskeleton dynamics and cell signaling. GAP-43 expression is controlled at the transcriptional and posttranscriptional levels, allowing for more flexibility and diversity in the amounts of GAP-43 protein present in different neuronal populations. Once translated, this protein becomes phosphorylated and displays distinct interactions with different signaling molecules. These interactions participate in modulating growth cone function during neuronal development and synaptic release during adult synaptic plasticity. Finally, the levels and distribution GAP-43 are altered both during normal brain processes like learning and memory and in disease states such as schizophrenia, temporal lobe epilepsy, and Alzheimer's disease. These studies support the notion that tightly regulated control of GAP-43 expression is important for maintaining normal brain function.

**List of Abbreviations:** AA, arachidonic acid; bHLH, basic helix–loop–helix; CaM, calmodulin; CaMKII, calcium/calmodulin-dependent kinase II; CFC, contextual fear conditioning; CNS, central nervous system; DRG, dorsal root ganglion; ELAV, embryonic lethal abnormal vision; f-actin, filamentous actin; GAP, growth-associated protein; GAP-43, growth-associated protein-43; kb, kilobase; LTP, long-term potentiation; mRNA, messenger ribonucleic acid; N-CAM, neuronal cell adhesion molecule; NMDA, *N*-methyl-D-aspartate; PC12, pheochromocytoma cell line; PFC, prefrontal cortex; $PI(4,5)P_2$, phosphatidyl inositol-4,5-bis-phosphate; PKC, protein kinase C; PNS, peripheral nervous system; 3′ UTR, 3′ untranslated region; (−/−), homozygous null; (+/−), heterozygous

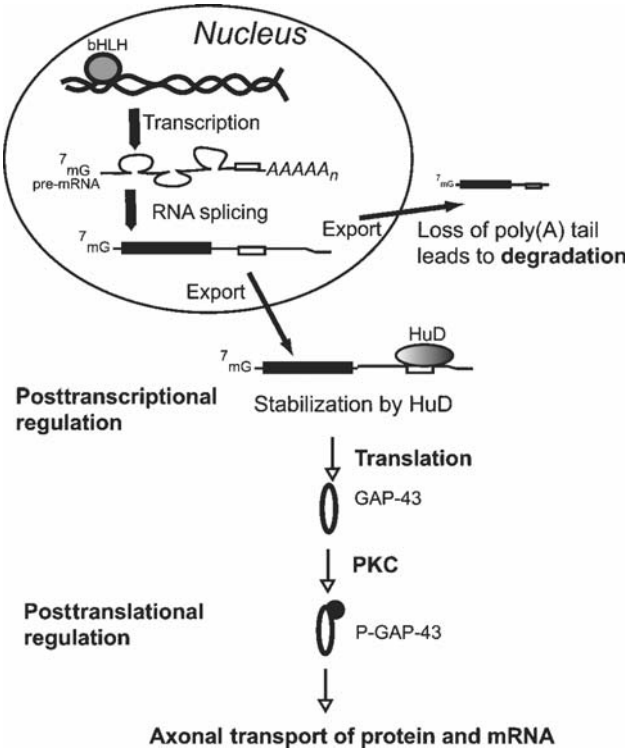
## 1 GAP-43: from Gene to Protein

### 1.1 Mechanisms of Gene Regulation

The neuronal growth-associated protein-43 (GAP-43) was independently identified as a protein associated with axonal outgrowth during development and regeneration (Benowitz et al., 1981; Skene and Willard, 1981a, b), a protein regulated by $Ca^{2+}$ and several peptides (Ehrlich and Routtenberg, 1974; Zwiers et al., 1978), and a major substrate of protein kinase C (PKC) (Aloyo et al., 1983; Akers and Routtenberg, 1985). GAP-43 is encoded in a 50-kb gene that is found on chromosome 3 in humans (Kosik et al., 1988). The resulting messenger RNA (mRNA) is 1.5 kb and is generated from three widely spaced exons (Grabczyk et al., 1990). GAP-43 expression is regulated through both transcriptional and posttranscriptional mechanisms (*Figure 15-1*). Sequences in the promoter region of the GAP-43 gene regulate its expression in the nervous system (Nedivi et al., 1992; Eggen et al., 1994; Reinhard et al., 1994; Weber and Skene, 1997, 1998). Although two promoters were described for the gene, 95% of the transcripts are derived from the second promoter that lies closer to the transcription start site (Eggen et al., 1994). GAP-43 transcription is directed by the activity of basic helix–loop–helix (bHLH) proteins that, depending on the protein–protein interactions, can act as transcriptional activators or repressors (Chiaramello et al., 1996; Kinney et al., 1996). Among these, the NeuroD/MATH family member Nex1 was shown to mediate increases in GAP-43 expression during neuronal differentiation of PC12 cells (Uittenbogaard et al., 2003). In transgenic mice, elements in the promoter and part of the first intron were sufficient to direct the neural-specific expression of a reporter construct in a similar temporal and spatial pattern as endogenous GAP-43 (Vanselow et al., 1994; Namgung et al., 1997). GAP-43 gene expression is also regulated through posttranscriptional mechanisms as shown in cell culture (Federoff et al., 1988; Perrone-Bizzozero et al., 1993; Anderson et al., 2000; Mobarak et al., 2000) and in living animals (Perrone-Bizzozero et al., 1991; Cantallops and Routtenberg, 1999; Namgung and Routtenberg, 2000). In cultured PC12 cells, GAP-43 expression is controlled by changes in the stability of the GAP-43 mRNA, which depends on the activation of PKC

**Figure 15-1**

**Control of GAP-43 gene expression. GAP-43 transcription is regulated by transcription factors from the basic helix–loop–helix (bHLH) family. GAP-43 primary transcript is processed in the nucleus and exported to the cytoplasm where it is stabilized by HuD. In the absence of HuD, GAP-43 messenger RNA (mRNA) is quickly degraded. GAP-43 mRNA is translated in the soma and the protein is phosphorylated by protein kinase C (PKC) and transported to the nerve terminal. In developing neurons, the GAP-43 mRNA is also transported to the growth cone**




and is independent of translation (Perrone-Bizzozero et al., 1993). Furthermore, several RNA-binding proteins were shown to bind instability-conferring elements in the 3′ untranslated region (3′ UTR) of this mRNA (Kohn et al., 1996; Chung et al., 1997; Irwin et al., 1997, 2002; DeFranco et al., 1998). One of these, the neuronal-specific RNA-binding protein HuD, is one of the Hu proteins identified for the association with paraneoplastic neurological syndromes (Szabo et al., 1991). This protein is highly conserved in evolution and related to ELAV (embryonic lethal abnormal vision), an RNA-binding protein identified in *Drosophila*, where the gene is required for normal development and maintenance of the nervous system (Campos et al., 1985). HuD was shown to increase the stability of the GAP-43 mRNA both in cell culture and in vivo, leading to increase GAP-43 protein expression and process outgrowth (Anderson et al., 2000; Mobarak et al., 2000; Tanner et al., 2004a; Bolognani et al., 2006). HuD binds to a highly conserved U-rich element in the 3′ UTR (Kohn et al., 1996; Chung et al., 1997; Tsai et al., 1997) and stabilizes mRNAs that contain a long poly(A) tail (Beckel-Mitchener et al., 2002), suggesting that only translationally competent GAP-43 mRNA is stabilized by HuD.

GAP-43 translation occurs primarily in the soma and the protein is transported by fast axonal transport to the nerve terminal (*Figure 15-1*). In developing neurons, GAP-43 mRNA is also transported to growth

cones where it colocalizes with both HuD and ribosomes, suggesting that it may be used for local protein synthesis (Smith et al., 2004).

### 1.2 Structural Domains in GAP-43

The GAP-43 mRNA is translated into a different-sized protein ranging from 194 to 238 amino acids depending on the species. This protein contains an unusually high proportion of acidic amino acids and only few hydrophobic residues (reviewed in Benowitz and Routtenberg, 1997). The N terminus is highly conserved between vertebrate species and the first ten amino acids contain two cysteines that direct the protein to the nerve terminal membrane via protein palmitoylation mechanisms (Skene and Virag, 1989; Zuber et al., 1989; Liu et al., 1991; Palacios et al., 1994). GAP-43 also contains a highly conserved calmodulin (CaM)-binding domain, also known as the isoleucine–glutamine (IQ) domain for the presence of these amino acids (Alexander et al., 1987; Chapman et al., 1991). This site is adjacent to the serine residue at position 41, which is the phosphorylation site of PKC in the rat protein (Coggins and Zwiers, 1989). Unlike other CaM-binding proteins, GAP-43 binds this protein at low concentrations of calcium. When calcium levels rise in the cell or when GAP-43 is phosphorylated by PKC the affinity of this protein for CaM decreases dramatically (Alexander et al., 1987). Because of these properties, it was postulated that GAP-43 acts as a "calmodulin sponge," bringing CaM down to the growth cone or nerve terminal and then releasing it upon depolarization or PKC activation (Skene, 1990; see ❯ *Figure 15-2*).

## 2 GAP-43 Function: from Growth Cones to Mature Synapses

### 2.1 GAP-43 and Growth Cone Motility

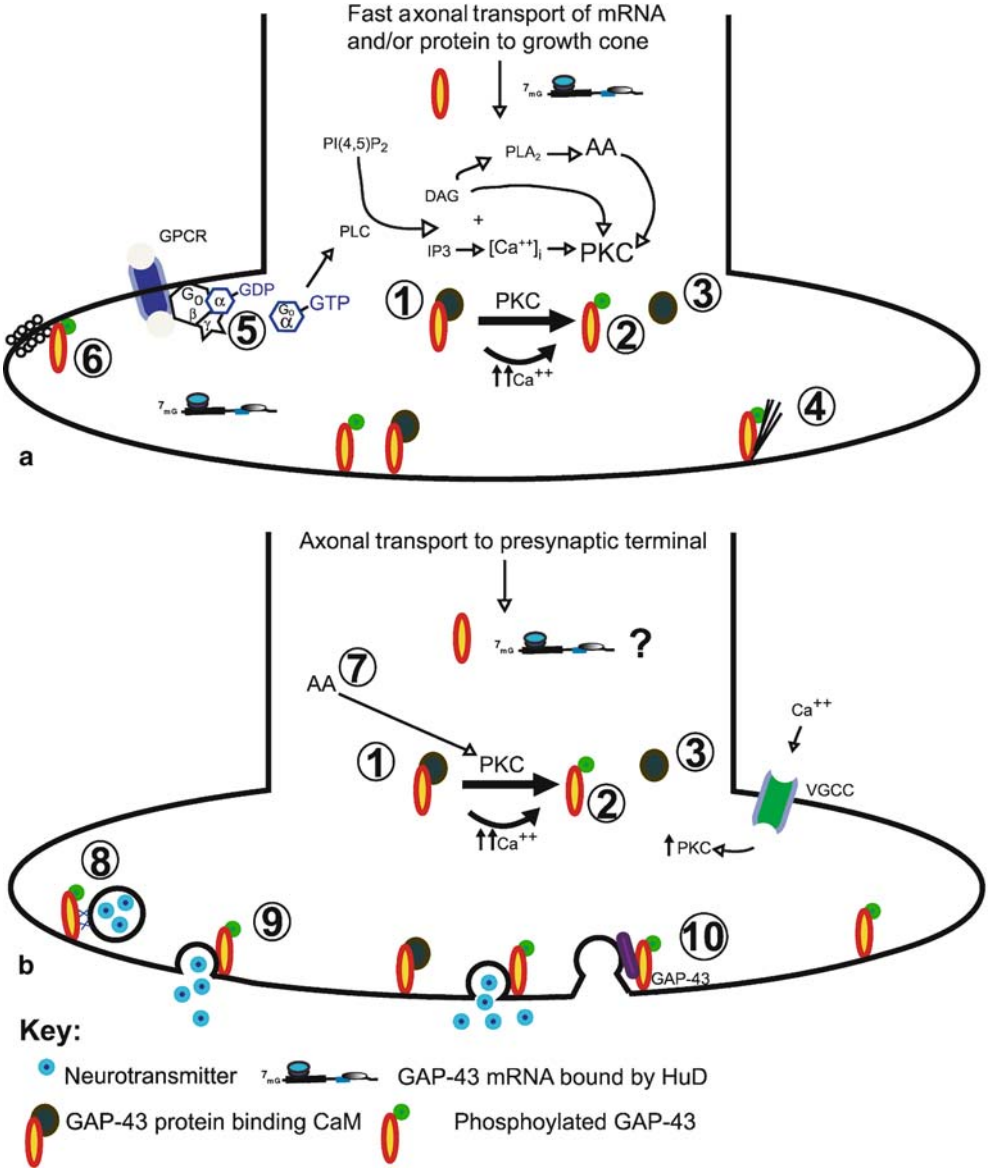
GAP-43 binds filamentous actin (f-actin) directly and its phosphorylation by PKC stabilizes actin filaments. Conversely, binding of unphosphorylated GAP-43 to f-actin inhibits filament phosphorylation (He et al., 1997). In growth cones, phosphorylated GAP-43 is found in regions where growth cones are advancing while regions that are retracting contain the unphosphorylated form (Dent and Meiri, 1998). Neurite outgrowth stimulated by neural cell adhesion molecule (N-CAM) is dependent on GAP-43, and IgCAMs can directly stimulate GAP-43 phosphorylation via a phospholipase C (PLC)-mediated signaling cascade (Hille, 1992; Meiri et al., 1998; see ❯ *Figure 15-2a*). Phosphorylated GAP-43 also activates Go α subunits (Strittmatter and Fishman, 1991), which enhance neurite outgrowth (Strittmatter et al., 1994). GAP-43 also interacts with PI(4,5)P2 in specific plasmalemmal microdomains (rafts), which cluster pools of signaling molecules controlling reorganization of the actin cytoskeleton (Laux et al., 2000). Finally, the association of GAP-43 with NCAM in multiple raft populations is required to stimulate neurite outgrowth (He and Meiri, 2002; Niethammer et al., 2002).

### 2.2 GAP-43 and Synaptic Release

In the mature synapse, GAP-43 performs similar functions in membrane dynamics and cytoskeletal reorganization but the precise mechanism by which GAP-43 performs these functions is less understood (❯ *Figure 15-2b*). Phosphorylated GAP-43 is involved in exocytosis and neurotransmitter release (Dekker et al., 1989; Ivins et al., 1993; Hens et al., 1998). Although GAP-43 is not part of the basic vesicular release machinery, this protein was shown to bind to the synaptic core complex and synaptotagmin in a $Ca^{2+}$-dependent manner (Haruta et al., 1997). Phosphorylated GAP-43 is also involved in vesicle recycling, as shown by its interactions with rabaptin-5, an effector of the guanine nucleotide triphosphatase Rab5 (Neve et al., 1998). GAP-43 interacts with PI(4,5)$P_2$ in the mature synapse, as described above (Laux et al., 2000), potentiates cytoskeletal reorganization in membrane rafts (Aarts et al., 1999), and binds to the presynaptic form of brain spectrin (Riederer and Routtenberg, 1999). Both *N*-methyl-D-aspartate (NMDA) receptor

**Figure 15-2**

**Functions of GAP-43 in growth cones and mature synapses. (a) GAP-43 has a high affinity for calmodulin (CaM) and is thought to be a CaM sponge (1; see text for specific references) but is readily phosphorylated by protein kinase C (PKC) (2). PKC phosphorylation displaces CaM, which is an activator of CaMKII (3). Phosphorylated GAP-43 has a number of roles in membrane–cytoskeletal dynamics and cell signaling, such as mediating the polymerization of f-actin (4), activating the Gα subunit of $G_o$ (5), and clustering $PIP_2$ in plasmalemmal microdomains (6). (b) In the mature synapse, GAP-43 performs a number of the same functions. In addition, the phosphorylation of GAP-43 is thought to be activated by the retrograde messenger arachidonic acid (AA; 7). Phosphorylated GAP-43 is involved in neurotransmitter exocytosis through binding the synaptic core complex and synaptotagmin (8), and its required presence for normal transmitter release (9). Phospho-GAP-43 also regulates vesicle endocytosis via rabaptin-5 (10). Finally, PKC-induced phosphorylation of GAP-43 facilitates long-term potentiation (LTP) and contextual fear conditioning (CFC), a learning and memory paradigm**



activation and arachidonic acid (AA) have been identified as stimulating PKC and thereby inducing the phosphorylation of GAP-43 in the maintenance phase of long-term potentiation (LTP) (Luo and Weinstein, 1993; McNamara and Routtenberg, 1995), further implicating GAP-43 in neurotransmitter release and in synaptic strengthening.

## 3 The Role of GAP-43 in Nervous System Development

### 3.1 Expression of GAP-43 in Developing Neurons

GAP-43 is first expressed in multipotent neuroectodermal precursors before they start differentiating into neurons (Esdar et al., 1999; Mani et al., 2001; Shen et al., 2004). In cultured neurons and PC12 cells, GAP-43 expression is low before process outgrowth but as cells differentiate and extend, the neurite levels of this protein increase severalfold (Perrone-Bizzozero et al., 1986; Meiri et al., 1988; Goslin and Banker, 1989; Van Hooff et al., 1989).

The expression pattern of GAP-43 shifts throughout the course of development. Initially, GAP-43 is expressed in virtually all neuronal populations, and protein expression sharply declines after birth (Benowitz and Perrone-Bizzozero, 1991). For instance, GAP-43 protein levels in the neocortex drop about tenfold between neonatal and mature rat brains (Skene et al., 1986). GAP-43 expression proceeds in a caudal to rostral manner and follows a similar pattern in both the peripheral nervous system (PNS) and the central nervous system (CNS) although CNS expression persists in some neurons throughout life (De la Monte et al., 1989; Dani et al., 1991; Meberg and Routtenberg, 1991). In the rat neocortex and hippocampal formation, GAP-43 expression becomes maximal during the first 2 postnatal weeks, which corresponds to axon-terminal branching and synapse formation. During this period, dentate granule cells express GAP-43 in an outside-in pattern corresponding to their time course of maturation. This pattern of expression is controlled at the posttranscriptional level and requires NMDA receptor activation (Catallops and Routtenberg, 1999). In the striate cortex of the cat, the highest levels of GAP-43 coincide with the critical period for activity-dependent plasticity in synaptic organization (Dani et al., 1991). Furthermore, GAP-43 mRNA levels are higher in dark-reared kittens than in light-reared controls but upon exposure to light, the mRNA falls to near-normal levels within 12 h (Neve and Bear, 1989). Altogether, these findings suggest that a developmental drop in GAP-43 gene expression is involved in the irreversible transformation of soft-wired connections to hard-wired circuits in the brain.

### 3.2 Involvement of GAP-43 in Neural Circuitry

The significance of GAP-43 in the establishment of neural circuitry during development and in adulthood was further demonstrated in GAP-43 knockout mice. The absence of GAP-43 in null mice (−/−) results in a lethal phenotype as the great majority of the animals die within the first postnatal week (Strittmatter et al., 1995). Furthermore, these animals exhibited abnormal axonal pathfinding as demonstrated by the inability of optic nerve axons to cross the chiasm (Strittmatter et al., 1995). In addition, GAP-43(−/−) show malformed retinogeniculate and retinotectal connections as well as abnormal hypothalamic projections (Zhu and Julien, 1999). The most severe defects in GAP-43-null mice are seen in the telencephalon where synaptic organization is severely disrupted. Pathfinding deficits in thalamocortical axons result in the absence of somatotopic maps in the cortex (Maier et al., 1999). In the rat, the barrel fields of the somatosensory cortex, which represent the facial vibrissae, show peak GAP-43 immunostaining at postnatal days 4 and 5 and almost no staining at postnatal day 8; however, in GAP-43(−/−) mice, barrel fields are not formed, while in GAP-43(+/−) mice the barrel fields are abnormally enlarged (Maier et al., 1999; McIlvain et al., 2003). In addition, midbrain serotonergic neurons fail to innervate the cortex (Donovan et al., 2002) and none of the telencephalic commissures (anterior commissure, corpus callosum, and hippocampal commissure) form in GAP-43(−/−) mice (Shen et al., 2002). An opposite phenotype was observed in

transgenic mice overexpressing this protein. These animals display excessive axonal sprouting and increased propensity to spontaneous seizures (Aigner et al., 1995; Holtmaat et al., 1995).

## 4 Function of GAP-43 in the Mature Nervous System

### 4.1 Patterns of GAP-43 Expression in the Adult Brain

After the critical period, GAP-43 expression decreases in the visual system and most motor/sensory pathways; however, some regions of the adult neocortex express constitutive high levels of GAP-43 mRNA and protein. It has been suggested that the distribution pattern of GAP-43 allows structural and functional changes in some neurons for information storage (Benowitz et al., 1988, 1989; Neve et al., 1988). In limbic and associative brain regions where mRNA and protein are abundant, GAP-43 has been shown to influence the release of monoamines (Dekker et al., 1989; Ivins et al., 1993) and glutamate (Hens et al., 1998) and the establishment of synaptic plasticity mechanisms (Akers and Routtenberg, 1985; Routtenberg et al., 2000). There may be a correlation between the neurons that possess GAP-43 mRNA and their neurotransmitters. Neurons that contain biogenic amines, such as substantia nigra pars compacta (dopamine), the locus coeruleus (norepinephrine), and dorsal raphe (serotonin) possess high levels of GAP-43 whereas cholinergic neurons in the basal forebrain and medial habenula express little or no mRNA (Meberg and Routtenberg, 1991). In vivo, GAP-43 is primarily localized to glutamatergic neurons with little or no expression in GABAergic interneurons (Benowitz and Routtenberg, 1997). While GAP-43 is not found in mature glia, its expression by glial progenitors appears to be critical in early brain development. For example, failure of GAP-43(−/−) callosal axons to cross the midline is due in part to aberrant differentiation of the glia wedge (Shen et al., 2004).

### 4.2 Role of GAP-43 in Synaptic Plasticity

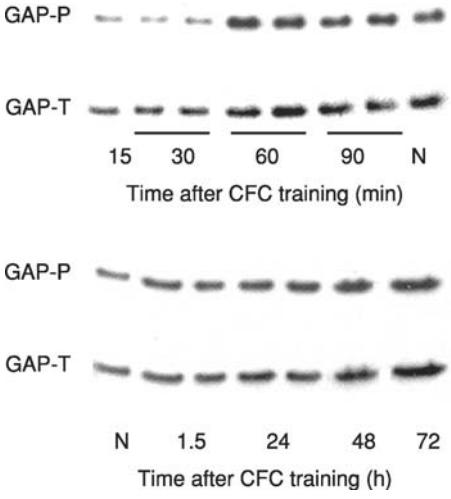
Multiple lines of evidence confirm that GAP-43 plays an important role in synaptic plasticity mechanisms underlying learning and memory. PKC phosphorylation of GAP-43 changes as a function of LTP (Lovinger et al., 1986; Linden et al., 1988; Ramakers et al., 1999a), long-term depression (Ramakers et al., 1999b), contextual fear conditioning (CFC) (Young et al., 2002), and fetal alcohol exposure (Tanner et al., 2004b). Using a CFC task, we have recently shown that GAP-43 phosphorylation and levels of expression change during the establishment of both short- and long-term memory (Young et al., 2002; see ❯ *Figure 15-3*). After rats are trained using a CFC paradigm, the levels of GAP-43 phosphorylation decrease within 15–30 min of training, are back to normal levels after 60 min, and increase after 90 min. Both GAP-43 phosphorylation and GAP-43 protein levels remain elevated in the hippocampus of CFC-trained animals for at least 72 h. There is a correlation between GAP-43 phosphorylation, gene expression, and learning and alterations in GAP-43 protein level and phosphorylation in transgenic mice result in corresponding changes in synaptic plasticity. Specifically, increased expression and phosphorylation of GAP-43 in transgenic mice was found to increase both spatial learning in animals overexpressing normal GAP-43 (Routtenberg et al., 2000) and LTP in mice overexpressing a constitutively phosphorylated form of this protein (Routtenberg et al., 2000; Hulo et al., 2002). Furthermore, animals with impaired learning have altered GAP-43 expression and phosphorylation in the hippocampus (Di Luca et al., 1993; Young et al., 2000; Tanner et al., 2004b).

### 4.3 GAP-43 and Nerve Regeneration

Expression of genes encoding GAP-43 and other growth-associated proteins (GAPs), are normally upregulated following PNS injury and lesions of selective regions of the adult rat brain such as the hippocampus. Also, GAP-43 expression is known to increase after injury of the optic nerve of lower vertebrates, which

**Figure 15-3**

**Contextual fear conditioning (CFC) alters GAP-43 phosphorylation and protein levels in the hippocampus. Rats were trained using a single-trial CFC and hippocampi were dissected at different time points after training. The levels of phosphorylated GAP-43 (GAP-P) (A) and total GAP-43 protein (GAP-T) (B) were measured by Western blotting. Values in naive (untrained) rats (N) are shown for comparison. Reproduced from Young et al., 2000 with permission from John Wiley and Sons**




leads to successful regeneration (Skene and Willard, 1981a, b; Benowitz and Lewis, 1983). In contrast, in mammals most regions of the adult CNS, including the optic nerve and spinal cord, fail to regenerate. In these neurons, there is a transient increase in the expression of GAP-43 and other GAPs but the levels of mRNA and protein decline shortly after axonal damage (Teztlaff et al., 1991; Doster et al., 1991). It is now apparent that multiple positive and negative factors, both intrinsic and extrinsic to the injured neurons, influence the regenerative capacity of CNS nerves. With regard to the intrinsic factors, overexpression of GAP-43 was sufficient to sustain regeneration of adult Purkinje cells (Gianola and Rossi, 2004) but not of thalamic cortical connections (Mason et al., 2000). Also, a combination of GAP-43 and CAP-23, another GAP, was shown to foster regeneration of mature dorsal root ganglion (DRG) neurons into the spinal cord (Bomze et al., 2001). Still, successful regeneration of spinal tracts poses a tremendous challenge, requiring the coordinated actions of a multiplicity of factors from neurotrophins to blockage of NogoA and other growth inhibitory factors (for reviews see Plunet et al. (2002), Filbin (2003), and Schwab (2004)).

## 5 GAP-43 in Disease States

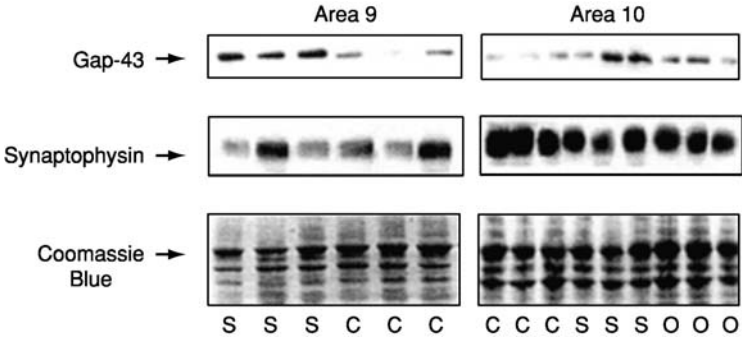
### 5.1 GAP-43 and Schizophrenia

Abnormal GAP-43 expression has also been associated with neurodevelopmental disorders such as schizophrenia (Perrone-Bizzozero et al., 1996; Eastwood and Harrison, 1998), fetal alcohol exposure (Perrone-Bizzozero et al., 1998; Tanner et al., 2004b), and temporal lobe epilepsy (Proper et al., 2000), suggesting a role of this protein in the pathophysiology of these disorders.

As shown in *Figure 15-4*, GAP-43 levels are increased in Brodmann's areas 9 and 10 in the prefrontal cortex (PFC) of patients with schizophrenia relative to matched controls. A similar increase was observed in the visual association cortex (area 20) but not in the primary visual cortex (area 17) (Perrone-Bizzozero et al., 1996). Furthermore, we found an inverse correlation between GAP-43 protein levels and the levels of synaptic vesicle protein synaptophysin in the PFC of patients with schizophrenia. While the pathophysiological

**Figure 15-4**

**Increased GAP-43 levels in the prefrontal cortex (PFC) of patients with schizophrenia. Synaptic protein levels were measured in Brodmann's areas 9 and 10 from patients with schizophrenia (*S*), age-, sex-, and postmortem interval-matched normal controls (*C*) and subjects with other psychiatric conditions receiving similar medication (*O*). Panels show representative Western blots of GAP-43 and synaptophysin in PFC samples. Coomassie blue staining was used to verify that equal amounts of protein were loaded in each lane. *Arrow* (bottom panel) indicates the migration of GAP-43. Reproduced from Perrone-Bizzozero et al., 1996 with permission of the Proceedings of the National Academy of Sciences, USA**




consequences of altered GAP-43/synaptophysin ratios in these brain regions remain to be elucidated, one possible explanation is that, given the association of GAP-43 with neuronal plasticity, synapses in associative areas of the brain may be more plastic or less mature in patients with schizophrenia than in age-matched control subjects.

In contrast to PFC, GAP-43 levels in the hippocampus were found to be decreased in patients with schizophrenia (Eastwood and Harrison, 1998; Perrone-Bizzozero et al., 1999). These changes were more prominent in the hilus region of the dentate gyrus (Chambers et al., 2005). As patients with schizophrenia perform poorly on several PFC- and hippocampal-dependent tasks (Heckers et al., 1998; Bertolino et al., 2000; Kuperberg and Heckers, 2000), the observed changes in GAP-43 levels may be related to cognitive deficits seen in these patients.

## 5.2 GAP-43 and Other Neuropsychiatric Disorders

There are other neuropsychiatric conditions with selective alterations in GAP-43 expression in the hippocampus, such as Alzheimer's disease and temporal lobe epilepsy (Masliah et al., 1991; Proper et al., 2000; Rekart et al., 2004). In contrast to patients with schizophrenia, Alzheimer's disease patients have increased deposition of GAP-43 protein surrounding plaques (Masliah et al., 1991) and significant increases along the stratum lacunosum moleculare (Rekart et al., 2004). GAP-43 protein levels were also found to increase in animal models and human brain tissues in response to traumatic (Hulsebosch et al., 1998) or ischemic brain injury (Ng et al., 1988), suggesting a role for GAP-43 in the repair of mature CNS neurons.

Patients with temporal lobe epilepsy have decreased levels of GAP-43 in the hilus but increased levels in the inner molecular layer of the dentate gyrus (Proper et al., 2000). Likewise, in the rat hippocampal formation, seizures induced through electrical stimulation (Meberg et al., 1993) or kainic acid injections (Bendotti et al., 1994; McNamara and Routtenberg, 1995; Cantallops and Routtenberg, 1996) show similar increases in GAP-43 mRNA expression. Kainic acid-induced seizures also elicit mossy fiber sprouting in the inner molecular layer of the dentate gyrus, the effects of which can be greatly reduced with treatment of the noncompetitive NMDA receptor inhibitor MK-801 (Bendotti et al., 1994; McNamara and Routtenberg, 1995; Cantallops and Routtenberg, 1996; Bendotti et al., 1997). In mouse models the picture is less clear. Kainate administration to mice produces seizures without induction of GAP-43 mRNA or mossy fiber

sprouting (McNamara and Routtenberg, 1995; Schauwecker and Steward, 1997; Schauwecker et al., 2000). It has been demonstrated that C57BL/6 mice are not sensitive to excitotoxic cell death induced by kainate (Schauwecker and Steward, 1997). This insensitivity is thought to underlie the fact that mice that do not show an induction of GAP-43 mRNA or mossy fiber sprouting (McNamara and Routtenberg, 1995; Schauwecker et al., 2000). Despite a lack of GAP-43 induction, transcription of the GAP-43 promoter remains high in adult dentate granule cells suggesting that the failure in induction is due to a posttranscriptional mechanism (Namgung and Routtenberg, 2000). Supporting this idea, expression of the RNA-binding protein HuD in dentate granule cells was found to rescue GAP-43 mRNA and protein expression in transgenic mice (Tanner et al., 2004a; Bolognani et al., 2006). While ongoing work is examining the impact of GAP-43 reexpression in these cells, it is interesting to note that GAP-43 is normally expressed at high levels in human and monkey dentate granule cells, suggesting that synaptic plasticity in these cells increased with evolution.

## 6 Concluding Remarks

The work reviewed in the previous sections highlights the functional significance of GAP-43 in the initial establishment and remodeling of neural connections. In growth cones, GAP-43 is involved in membrane–cytoskeletal dynamics and signaling mechanisms. While most neurons suppress the expression of GAP-43 after the critical period, expression of this protein persists in highly plastic brain regions such as the association cortices and the hippocampus. The significance of this protein in the mature nervous system is highlighted not only by the association of GAP-43 with normal brain functions such as learning and memory but also by its alterations in neuropsychiatric conditions affecting these regions. While abnormally high levels of GAP-43 are associated with aberrant or compensatory sprouting, failure to express GAP-43 leads to abnormal pathfinding or abortive regeneration. These conditions underscore the need for tight regulation of GAP-43 expression in neurons. Consistent with this idea, neurons control GAP-43 expression at multiple levels from transcription, mRNA stability, transport, and localized synthesis. This combination of regulatory mechanisms provides not only for distinct patterns of expression in different neuronal populations but also for additional safeguards against unregulated expression.

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## References

Aarts LH, Verkade P, Schrama LH, Oestreicher AB, Gispen WH, et al. 1999. Local accumulations of B-50/GAP-43 evoke excessive bleb formation in PC12 cells. Mol Neurobiol 20: 17-28.

Aigner L, Arber S, Kapfhammer JP, Laux T, Schneider C, et al. 1995. Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. Cell 83: 269-278.

Akers RF, Routtenberg A. 1985. Protein kinase C phosphorylates a 47 Mr protein (F1) directly related to synaptic plasticity. Brain Res 334: 147-151.

Alexander KA, Cimler BM, Meier KE, Storm DR. 1987. Regulation of calmodulin binding to P-57. A neurospecific calmodulin binding protein. J Biol Chem 262: 6108-6113.

Aloyo VJ, Zwiers H, Gispen WH. 1983. Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 protein kinase. J Neurochem 41: 649-653.

Anderson KD, Morin MA, Beckel-Mitchener AC, Mobarak CD, Neve RL, et al. 2000. Overexpression of HuD, but not of its truncated form HuD I+II, promotes GAP-43 gene expression and neurite outgrowth in PC12 cells in the

absence of nerve growth factor. J Neurochem 75: 1103-1114.

Beckel-Mitchener AC, Miera A, Keller R, Perrone-Bizzozero NI. 2002. Poly(A) tail length dependent stabilization of GAP-43 mRNA by the RNA binding protein HuD. J Biol Chem 277: 27996-28002.

Bendotti C, Baldessari S, Pende M, Southgate T, Guglielmetti F, et al. 1997. Relationship between GAP-43 expression in the dentate gyrus and synaptic reorganization of hippocampal mossy fibres in rats treated with kainic acid. Eur J Neurosci 9: 93-101.

Bendotti C, Pende M, Samanin R. 1994. Expression of GAP-43 in the granule cells of rat hippocampus after seizure-induced sprouting of mossy fibres: in situ hybridization and immunocytochemical studies. Eur J Neurosci 6: 509-515.

Benowitz LI, Apostolides PJ, Perrone-Bizzozero NI, Finklestein SP, Zwiers H. 1988. Anatomical distribution of the growth-associated protein GAP-43/B-50 in the adult rat brain. J Neurosci 8: 339-352.

Benowitz LI, Lewis ER. 1983. Increased transport of 44,000- to 49,000-dalton acidic proteins during regeneration of the goldfish optic nerve: a two-dimensional gel analysis. J Neurosci 3: 2153-2163.

Benowitz LI, Perrone-Bizzozero NI. 1991. The expression of GAP-43 in relation to neuronal growth and plasticity: when, where, how, and why? Prog Brain Res 89: 69-87.

Benowitz LI, Perrone-Bizzozero NI, Finklestein SP, Bird ED. 1989. Localization of the growth-associated phosphoprotein GAP-43 (B-50, F1) in the human cerebral cortex. J Neurosci 9: 990-995.

Benowitz LI, Routtenberg A. 1997. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci 20: 84-91.

Benowitz LI, Shashoua VE, Yoon MG. 1981. Specific changes in rapidly transported proteins during regeneration of the goldfish optic nerve. J Neurosci 1: 300-307.

Bertolino A, Esposito G, Callicott JH, Mattay VS, Van Horn JD, et al. 2000. Specific relationship between prefrontal neuronal *N*-acetylaspartate and activation of the working memory cortical network in schizophrenia. Am J Psychiatry 157: 26-33.

Bolognani F, Tanner DC, Merhege M, Deschenes-Furry J, Jasmin B, Perrone-Bizzozero NI. 2006. In vivo post-transcriptional regulation of GAP-43 mRNA by overexpression of the RNA-binding protein HuD. J Neurochem, in press.

Bomze HM, Bulsara KR, Iskandar BJ, Caroni P, Skene JH. 2001. Spinal axon regeneration evoked by replacing two growth cone proteins in adult neurons. Nat Neurosci 4: 38-43.

Campos AR, Grossman D, White K. 1985. Mutant alleles at the locus elav in *Drosophila melanogaster* lead to nervous system defects. A developmental-genetic analysis. J Neurogenet 2: 197-218.

Cantallops I, Routtenberg A. 1996. Rapid induction by kainic acid of both axonal growth and F1/GAP-43 protein in the adult rat hippocampal granule cells. J Comp Neurol 366: 303-319.

Cantallops I, Routtenberg A. 1999. Activity-dependent regulation of axonal growth: posttranscriptional control of the GAP-43 gene by the NMDA receptor in developing hippocampus. J Neurobiol 41: 208-220.

Chambers JS, Thomas D, Saland L, Neve RL, Perrone-Bizzozero NI. 2005. Growth-associated protein 43 (GAP-43) and synaptophysin alterations in the dentate gyrus of patients with schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 29: 283-290.

Chapman ER, Au D, Alexander KA, Nicolson TA, Storm DR. 1991. Characterization of the calmodulin binding domain of neuromodulin. Functional significance of serine 41 and phenylalanine 42. J Biol Chem 266: 207-213.

Chiaramello A, Neuman T, Peavy DR, Zuber MX. 1996. The GAP-43 gene is a direct downstream target of the basic helix–loop–helix transcription factors. J Biol Chem 271: 22035-22043.

Chung S, Eckrich M, Perrone-Bizzozero NI, Kohn DT, Furneaux H. 1997. The Elav-like proteins bind to a conserved regulatory element in the 3′-untranslated region of GAP-43 mRNA. J Biol Chem 272: 6593-6598.

Coggins PJ, Zwiers H. 1989. Evidence for a single protein kinase C-mediated phosphorylation site in rat brain protein B-50. J Neurochem 53: 1895-1901.

Dani JW, Armstrong DM, Benowitz LI. 1991. Mapping the development of the rat brain by GAP-43 immunocytochemistry. Neuroscience 40: 277-287.

De la Monte SM, Federoff HJ, Ng SC, Grabczyk E, Fishman MC. 1989. GAP-43 gene expression during development: persistence in a distinctive set of neurons in the mature central nervous system. Brain Res Dev Brain Res 46: 161-168.

De Franco C, Chicurel ME, Potter H. 1998. A general RNA-binding protein complex that includes the cytoskeleton-associated protein MAP 1A. Mol Biol Cell 9: 1695-1708.

Dekker LV, De Graan PN, Oestreicher AB, Versteeg DH, Gispen WH. 1989. Inhibition of noradrenaline release by antibodies to B-50 (GAP-43). Nature 342: 74-76.

Dent EW, Meiri KF. 1998. Distribution of phosphorylated GAP-43 (neuromodulin) in growth cones directly reflects growth cone behavior. J Neurobiol 35: 287-299.

Di Luca M, Merazzi F, De Graan PN, Cimino M, Balduini W, et al. 1993. Selective alteration in B-50/GAP-43 phosphorylation in brain areas of animals characterized by cognitive impairment. Brain Res 607: 329-332.

Donovan SL, Mamounas LA, Andrews AM, Blue ME, McCasland JS. 2002. GAP-43 is critical for normal

development of the serotonergic innervation in forebrain. J Neurosci 22: 3543-3552.

Doster SK, Lozano AM, Aguayo AJ, Willard MB. 1991. Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. Neuron 6: 635-647.

Eastwood SL, Harrison PJ. 1998. Hippocampal and cortical growth-associated protein-43 messenger RNA in schizophrenia. Neuroscience 86: 437-448.

Eggen BJ, Nielander HB, Rensen-de Leeuw MG, Schotman P, Gispen WH, et al. 1994. Identification of two promoter regions in the rat B-50/GAP-43 gene. Brain Res Mol Brain Res 23: 221-234.

Ehrlich YH, Routtenberg A. 1974. Cyclic AMP regulates phosphorylation of three protein components of rat cerebral cortex membranes for thirty minutes. FEBS Lett 45: 237-243.

Esdar C, Oehrlein SA, Reinhardt S, Maelicke A, Herget T. 1999. The protein kinase C (PKC) substrate GAP-43 is already expressed in neural precursor cells, colocalizes with PKCeta and binds calmodulin. Eur J Neurosci 11: 503-516.

Federoff HJ, Grabczyk E, Fishman MC. 1988. Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids. J Biol Chem 263: 19290-19295.

Filbin MT. 2003. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4: 703-713.

Gianola S, Rossi F. 2004. GAP-43 overexpression in adult mouse Purkinje cells overrides myelin-derived inhibition of neurite growth. Eur J Neurosci 19: 819-830.

Goslin K, Banker G. 1989. Experimental observations on the development of polarity by hippocampal neurons in culture. J Cell Biol 108: 1507-1516.

Grabczyk E, Zuber MX, Federoff HJ, Ng SC, Pack A, et al. 1990. Cloning and characterization of the rat gene encoding GAP-43. Eur J Neurosci 2: 822-827.

Haruta T, Takami N, Ohmura M, Misumi Y, Ikehara Y. 1997. Ca2+-dependent interaction of the growth-associated protein GAP-43 with the synaptic core complex. Biochem J 325 (Pt 2): 455-463.

He Q, Dent EW, Meiri KF. 1997. Modulation of actin filament behavior by GAP-43 (neuromodulin) is dependent on the phosphorylation status of serine 41, the protein kinase C site. J Neurosci 17: 3515-3524.

He Q, Meiri KF. 2002. Isolation and characterization of detergent-resistant microdomains responsive to NCAM-mediated signaling from growth cones. Mol Cell Neurosci 19: 18-31.

Heckers S, Rauch SL, Goff D, Savage CR, Schacter DL, et al. 1998. Impaired recruitment of the hippocampus during conscious recollection in schizophrenia. Nat Neurosci 1: 318-323.

Hens JJ, Ghijsen WE, Weller U, Spierenburg HA, Boomsma F, et al. 1998. Anti-B-50 (GAP-43) antibodies decrease exocytosis of glutamate in permeated synaptosomes. Eur J Pharmacol 363: 229-240.

Hille B. 1992. G protein-coupled mechanisms and nervous signaling. Neuron 9: 187-195.

Holtmaat AJ, Dijkhuizen PA, Oestreicher AB, Romijn HJ, Van der Lugt NM, et al. 1995. Directed expression of the growth-associated protein B-50/GAP-43 to olfactory neurons in transgenic mice results in changes in axon morphology and extraglomerular fiber growth. J Neurosci 15: 7953-7965.

Hulo S, Alberi S, Laux T, Muller D, Caroni P. 2002. A point mutant of GAP-43 induces enhanced short-term and long-term hippocampal plasticity. Eur J Neurosci 15: 1976-1982.

Hulsebosch CE, De Witt DS, Jenkins LW, Prough DS. 1998. Traumatic brain injury in rats results in increased expression of Gap-43 that correlates with behavioral recovery. Neurosci Lett 255: 83-86.

Irwin N, Baekelandt V, Goritchenko L, Benowitz L. 1997. Identification of two proteins that bind to a pyrimidine-rich sequence in the 3′-untranslated region of GAP-43 mRNA. Nucleic Acids Res 25: 1281-1288.

Irwin N, Chao S, Goritchenko L, Horiuchi A, Greengard P, et al. 2002. Nerve growth factor controls GAP-43 mRNA stability via the phosphoprotein ARPP-19. Proc Natl Acad Sci USA 99: 12427-12431.

Ivins KJ, Neve KA, Feller DJ, Fidel SA, Neve RL. 1993. Antisense GAP-43 inhibits the evoked release of dopamine from PC12 cells. J Neurochem 60: 626-633.

Kinney WR, McNamara RK, Valcourt E, Routtenberg A. 1996. Prolonged alteration in E-box binding after a single systemic kainate injection: potential relation to F1/GAP-43 gene expression. Brain Res Mol Brain Res 38: 25-36.

Kohn DT, Tsai KC, Cansino VV, Neve RL, Perrone-Bizzozero NI. 1996. Role of highly conserved pyrimidine-rich sequences in the 3′ untranslated region of the GAP-43 mRNA in mRNA stability and RNA–protein interactions. Brain Res Mol Brain Res 36: 240-250.

Kosik KS, Orecchio LD, Bruns GA, Benowitz LI, Mac Donald GP, et al. 1988. Human GAP-43: its deduced amino acid sequence and chromosomal localization in mouse and human. Neuron 1: 127-132.

Kuperberg G, Heckers S. 2000. Schizophrenia and cognitive function. Curr Opin Neurobiol 10: 205-210.

Laux T, Fukami K, Thelen M, Golub T, Frey D, et al. 2000. GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J Cell Biol 149: 1455-1472.

Linden DJ, Wong KL, Sheu FS, Routtenberg A. 1988. NMDA receptor blockade prevents the increase in protein kinase C

substrate (protein F1) phosphorylation produced by long-term potentiation. Brain Res 458: 142-146.

Liu YC, Chapman ER, Storm DR. 1991. Targeting of neuromodulin (GAP-43) fusion proteins to growth cones in cultured rat embryonic neurons. Neuron 6: 411-420.

Lovinger DM, Colley PA, Akers RF, Nelson RB, Routtenberg A. 1986. Direct relation of long-term synaptic potentiation to phosphorylation of membrane protein F1, a substrate for membrane protein kinase C. Brain Res 399: 205-211.

Luo JH, Weinstein IB. 1993. Calcium-dependent activation of protein kinase C. The role of the C2 domain in divalent cation selectivity. J Biol Chem 268: 23580-23584.

Maier DL, Mani S, Donovan SL, Soppet D, Tessarollo L, et al. 1999. Disrupted cortical map and absence of cortical barrels in growth-associated protein (GAP)-43 knockout mice. Proc Natl Acad Sci USA 96: 9397-9402.

Mani S, Shen Y, Schaefer J, Meiri KF. 2001. Failure to express GAP-43 during neurogenesis affects cell cycle regulation and differentiation of neural precursors and stimulates apoptosis of neurons. Mol Cell Neurosci 17: 54-66.

Masliah E, Fagan AM, Terry RD, De Teresa R, Mallory M, et al. 1991. Reactive synaptogenesis assessed by synaptophysin immunoreactivity is associated with GAP-43 in the dentate gyrus of the adult rat. Exp Neurol 113: 131-142.

Mason MR, Campbell G, Caroni P, Anderson PN, Lieberman AR. 2000. Overexpression of GAP-43 in thalamic projection neurons of transgenic mice does not enable them to regenerate axons through peripheral nerve grafts. Exp Neurol 165: 143-152.

McIlvain VA, Robertson DR, Maimone MM, McCasland JS. 2003. Abnormal thalamocortical pathfinding and terminal arbors lead to enlarged barrels in neonatal GAP-43 heterozygous mice. J Comp Neurol. 462: 252-264.

McNamara RK, Routtenberg A. 1995. NMDA receptor blockade prevents kainate induction of protein F1/GAP-43 mRNA in hippocampal granule cells and subsequent mossy fiber sprouting in the rat. Brain Res Mol Brain Res 33: 22-28.

Meberg PJ, Gall CM, Routtenberg A. 1993. Induction of F1/GAP-43 gene expression in hippocampal granule cells after seizures. Brain Res Mol Brain Res 17: 295-299.

Meberg PJ, Routtenberg A. 1991. Selective expression of protein F1/(GAP-43) mRNA in pyramidal but not granule cells of the hippocampus. Neuroscience 45: 721-733.

Meiri KF, Saffell JL, Walsh FS, Doherty P. 1998. Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. J Neurosci 18: 10429-10437.

Meiri KF, Willard M, Johnson MI. 1988. Distribution and phosphorylation of the growth-associated protein GAP-43 in regenerating sympathetic neurons in culture. J Neurosci 8: 2571-2581.

Mobarak CD, Anderson KD, Morin M, Beckel-Mitchener AC, Rogers SL, et al. 2000. The RNA-binding protein HuD is required for GAP-43 mRNA stability, GAP-43 gene expression, and PKC-dependent neurite outgrowth in PC12 cells. Mol Biol Cell 11: 3191-3203.

Namgung U, Matsuyama S, Routtenberg A. 1997. Long-term potentiation activates the GAP-43 promoter: selective participation of hippocampal mossy cells. Proc Natl Acad Sci USA 94: 11675-11680.

Namgung U, Routtenberg A. 2000. Transcriptional and post-transcriptional regulation of a brain growth protein: regional differentiation and regeneration induction of GAP-43. Eur J Neurosci 12: 3124-3136.

Nedivi E, Basi GS, Akey IV, Skene JH. 1992. A neural-specific GAP-43 core promoter located between unusual DNA elements that interact to regulate its activity. J Neurosci 12: 691-704.

Neve RL, Bear MF. 1989. Visual experience regulates gene expression in the developing striate cortex. Proc Natl Acad Sci USA 86: 4781-4784.

Neve RL, Coopersmith R, McPhie DL, Santeufemio C, Pratt KG, et al. 1998. The neuronal growth-associated protein GAP-43 interacts with rabaptin-5 and participates in endocytosis. J Neurosci 18: 7757-7767.

Neve RL, Finch EA, Bird ED, Benowitz LI. 1988. Growth-associated protein GAP-43 is expressed selectively in associative regions of the adult human brain. Proc Natl Acad Sci USA 85: 3638-3642.

Ng SC, de la Monte SM, Conboy GL, Karns LR, Fishman MC. 1988. Cloning of human GAP-43: growth association and ischemic resurgence. Neuron 1: 133-139.

Niethammer P, Delling M, Sytnyk V, Dityatev A, Fukami K, et al. 2002. Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neuritogenesis. J Cell Biol 157: 521-532.

Palacios G, Mengod G, Sarasa M, Baudier J, Palacios JM. 1994. De novo synthesis of GAP-43: in situ hybridization histochemistry and light and electron microscopy immunocytochemical studies in regenerating motor neurons of cranial nerve nuclei in the rat brain. Brain Res Mol Brain Res 24: 107-117.

Perrone-Bizzozero NI, Cansino VV, Kohn DT. 1993. Posttranscriptional regulation of GAP-43 gene expression in PC12 cells through protein kinase C-dependent stabilization of the mRNA. J Cell Biol 120: 1263-1270.

Perrone-Bizzozero NI, Finklestein SP, Benowitz LI. 1986. Synthesis of a growth-associated protein by embryonic rat cerebrocortical neurons in vitro. J Neurosci 6: 3721-3730.

Perrone-Bizzozero NI, Isaacson TV, Keidan GM, Eriqat C, Meiri KF, et al. 1998. Prenatal ethanol exposure decreases GAP-43 phosphorylation and protein kinase C activity in

the hippocampus of adult rat offspring. J Neurochem 71: 2104-2111.

Perrone-Bizzozero NI, Neve RL, Irwin N, Lewis S, Fischer I, et al. 1991. Post-transcriptional regulation of GAP-43 mRNA levels during neuronal differentiation and nerve regeneration. Mol Cell Neurosci 2: 402-409.

Perrone-Bizzozero NI, Sower AC, Bird ED, Benowitz LI, Ivins KJ, et al. 1996. Levels of the growth-associated protein GAP-43 are selectively increased in association cortices in schizophrenia. Proc Natl Acad Sci USA 93: 14182-14187.

Perrone-Bizzozero NI, Thomas D, Harji F, Saland L, Hassinger L, et al. 1999. Molecular and cytoarchitectural alterations in the hippocampus of patients with schizophrenia revealed by immunostaining against HuD, GAP-43, and other neuronal markers. Schizophr Res 36: 83.

Plunet W, Kwon BK, Tetzlaff W. 2002. Promoting axonal regeneration in the central nervous system by enhancing the cell body response to axotomy. J Neurosci Res 68: 1-6.

Proper EA, Oestreicher AB, Jansen GH, Veelen CW, van Rijen PC, et al. 2000. Immunohistochemical characterization of mossy fibre sprouting in the hippocampus of patients with pharmaco-resistant temporal lobe epilepsy. Brain 123 (Pt 1): 19-30.

Ramakers GM, Gerendasy DD, de Graan PN. 1999a. Substrate phosphorylation in the protein kinase C gamma knockout mouse. J Biol Chem 274: 1873-1874.

Ramakers GM, McNamara RK, Lenox RH, De Graan PN. 1999b. Differential changes in the phosphorylation of the protein kinase C substrates myristoylated alanine-rich C kinase substrate and growth-associated protein-43/B-50 following Schaffer collateral long-term potentiation and long-term depression. J Neurochem 73: 2175-2183.

Reinhard E, Nedivi E, Wegner J, Skene JH, Westerfield M. 1994. Neural selective activation and temporal regulation of a mammalian GAP-43 promoter in zebrafish. Development 120: 1767-1775.

Rekart JL, Quinn B, Mesulam MM, Routtenberg A. 2004. Subfield-specific increase in brain growth protein in postmortem hippocampus of Alzheimer's patients. Neuroscience 126: 579-584.

Riederer BM, Routtenberg A. 1999. Can GAP-43 interact with brain spectrin? Brain Res Mol Brain Res 71: 345-348.

Routtenberg A, Cantallops I, Zaffuto S, Serrano P, Namgung U. 2000. Enhanced learning after genetic overexpression of a brain growth protein. Proc Natl Acad Sci USA 97: 7657-7662.

Schauwecker PE, Ramirez JJ, Steward O. 2000. Genetic dissection of the signals that induce synaptic reorganization. Exp Neurol 161: 139-152.

Schauwecker PE, Steward O. 1997. Genetic determinants of susceptibility to excitotoxic cell death: implications for gene targeting approaches. Proc Natl Acad Sci USA 94: 4103-4108.

Schwab ME. 2004. Nogo axon regeneration. Curr Opin Neurobiol 14: 118-124.

Shen Y, Mani S, Donovan SL, Schwob JE, Meiri KF. 2002. Growth-associated protein-43 is required for commissural axon guidance in the developing vertebrate nervous system. J Neurosci 22: 239-247.

Shen Y, Mani S, Meiri KF. 2004. Failure to express GAP-43 leads to disruption of a multipotent precursor and inhibits astrocyte differentiation. Mol Cell Neurosci 26: 390-405.

Skene JH. 1990. GAP-43 as a 'calmodulin sponge' and some implications for calcium signalling in axon terminals. Neurosci Res Suppl 13: S112-S125.

Skene JH, Jacobson RD, Snipes GJ, McGuire CB, Norden JJ, et al. 1986. A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. Science 233: 783-786.

Skene JH, Virag I. 1989. Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. J Cell Biol 108: 613-624.

Skene JH, Willard M. 1981a. Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. J Cell Biol 89: 96-103.

Skene JH, Willard M. 1981b. Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. J Cell Biol 89: 86-95.

Smith CL, Afroz R, Bassell GJ, Furneaux HM, Perrone-Bizzozero NI, et al. 2004. GAP-43 mRNA in growth cones is associated with HuD and ribosomes. J Neurobiol 61: 222-235.

Strittmatter SM, Fankhauser C, Huang PL, Mashimo H, Fishman MC. 1995. Neuronal pathfinding is abnormal in mice lacking the neuronal growth cone protein GAP-43. Cell 80: 445-452.

Strittmatter SM, Fishman MC. 1991. The neuronal growth cone as a specialized transduction system. Bioessays 13: 127-134.

Strittmatter SM, Fishman MC, Zhu XP. 1994. Activated mutants of the alpha subunit of G(o) promote an increased number of neurites per cell. J Neurosci 14: 2327-2338.

Szabo A, Dalmau J, Manley G, Rosenfeld M, Wong E, et al. 1991. HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal. Cell 67 (2): 325-333.

Tanner DC, Bolognani F, Perrone-Bizzozero NI. 2004a. Rescue of GAP-43 mRNA in dentate granule cells of adult transgenic mice. J Neurochem (Conference Proceedings) v. 88: 38.

Tanner DC, Githinji AW, Young EA, Meiri KF, Savage DD, et al. 2004b. Fetal alcohol exposure alters GAP-43 phosphorylation and protein kinase C responses to contextual

fear conditioning in the hippocampus of adult rat offspring. Alcohol Clin Exp Res 28: 113-122.

Tetzlaff W, Alexander SW, Miller FD, Bisby MA. 1991. Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. J Neurosci 11: 2528-2544.

Tsai KC, Cansino VV, Kohn DT, Neve RL, Perrone-Bizzozero NI. 1997. Post-transcriptional regulation of the GAP-43 gene by specific sequences in the 3′ untranslated region of the mRNA. J Neurosci 17: 1950-1958.

Uittenbogaard M, Martinka DL, Chiaramello A. 2003. The basic helix–loop–helix differentiation factor Nex1/MATH-2 functions as a key activator of the GAP-43 gene. J Neurochem 84: 678-688.

Van Hooff CO, Holthuis JC, Oestreicher AB, Boonstra J, De Graan PN, et al. 1989. Nerve growth factor-induced changes in the intracellular localization of the protein kinase C substrate B-50 in pheochromocytoma PC12 cells. J Cell Biol 108: 1115-1125.

Vanselow J, Grabczyk E, Ping J, Baetscher M, Teng S, et al. 1994. GAP-43 transgenic mice: dispersed genomic sequences confer a GAP-43-like expression pattern during development and regeneration. J Neurosci 14: 499-510.

Weber JR, Skene JH. 1997. Identification of a novel repressive element that contributes to neuron-specific gene expression. J Neurosci 17: 7583-7593.

Weber JR, Skene JH. 1998. The activity of a highly promiscuous AP-1 element can be confined to neurons by a tissue-selective repressive element. J Neurosci 18: 5264-5274.

Young E, Cesena T, Meiri KF, Perrone-Bizzozero NI. 2002. Changes in protein kinase C (PKC) activity, isozyme translocation, and GAP-43 phosphorylation in the rat hippocampal formation after a single-trial contextual fear conditioning paradigm. Hippocampus 12: 457-464.

Young EA, Owen EH, Meiri KF, Wehner JM. 2000. Alterations in hippocampal GAP-43 phosphorylation and protein level following contextual fear conditioning. Brain Res 860: 95-103.

Zhu Q, Julien JP. 1999. A key role for GAP-43 in the retinotectal topographic organization. Exp Neurol 155: 228-242.

Zuber MX, Strittmatter SM, Fishman MC. 1989. A membrane-targeting signal in the amino terminus of the neuronal protein GAP-43. Nature 341: 345-348.

Zwiers H, Wiegant VM, Schotman P, Gispen WH. 1978. ACTH-induced inhibition of endogenous rat brain protein phosphorylation in vitro: structure activity. Neurochem Res 3: 455-463.

# 16 Protein Attractants and Repellants in Axonal Guidance

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**Abstract:** The function of the nervous system depends on the complex network of neurons that forms during embryonic development. The precision and complexity of nervous system wiring are achieved through the action of a large number of axon guidance molecules that specify the directional information for axonal growth cones to navigate within the developing embryos. This chapter covers the basic information about major classes of axonal attractants and repellents important for nervous system wiring. Midline axon pathfinding and retinotopic mapping are described as examples to illustrate how these molecules function during nervous system development. Finally, some of the axonal inhibitors that prevent axonal regeneration in adult central nervous system (CNS) are discussed.

**List of Abbreviations:** BMP-7, bone morphogenetic protein-7; CALEB, chicken acidic leucine-rich EGF-like-domain-containing brain protein; CDs, cadherin domains; CSPG, chondroitin sulfate proteoglycans; DCC, deleted in colorectal cancer; ECMs, extracellular matrix molecules; EGF, epidermal growth factor; FGFR, fibroblast growth factor receptor; Fn, fibronectin; GAG, glycosaminoglycan; GDF-7, growth/differentiation factor 7; GPI, glycosylphosphatidylinositol; HSPG, heparin sulfate proteoglycans; Ig-CAMs, immunoglobulin superfamily cell adhesion molecules; *KAL1*, Kallmann's syndrome gene 1; LRR, leucine-rich repeats; MAG, myelin-associated glycoprotein; MAM, meprin/A5-protein/PTPmu; NgR, nogo receptor; OMgp, oligodendrocyte–myelin glycoprotein; PDZ, PSD-95, discs-large, Z0-1; RGD, arginine–glycine–aspartate; Shh, sonic hedgehog; TGF-β, transforming growth factor-β

## 1 Introduction

A key step in building the functional nervous system is to establish the precise neuronal connections during the development of the nervous system (Tessier-Lavigne and Goodman, 1996). To make connections with their synaptic partners, neurons send out axons over complicated embryonic and fetal structures through a process called axon guidance. There are over 100 billion neurons in our nervous system, and each neuron makes multiple connections with other targets. Nervous system wiring is a very complex process, which is controlled by a genetic program encoding a large number of axon guidance molecules (Tessier-Lavigne and Goodman, 1996; Dickson, 2002).

In recent years, studies utilizing biochemical and genetic approaches led to the discovery of a large collection of axon guidance molecules (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). The structure and function of axon guidance molecules and their signaling receptors are highly conserved throughout the animal kingdom, from nematodes to mammals. Axon guidance molecules can be categorized into four classes (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). They are long-range attractants, long-range repellents, contact-mediated attractants, and contact-mediated repellents. Each class is composed of multiple protein families. Many axon guidance proteins are bifunctional and can either be attractive or repulsive. For the convenience of discussion, we will group axon guidance cues into diffusible and nondiffusible cues to avoid repetitive mentioning of the same molecules in the attractant and repellent categories.

This chapter covers proteins that have been demonstrated to have axon guidance functions, conveying directional information. Proteins that influence the growth of axons, such as neurotrophins, are covered in previous chapters. Molecules that have growth-inhibiting effects in adult central nervous system (CNS), such as Nogo, myelin-associated glycoprotein (MAG), oligodendrocyte–myelin glycoprotein (OMgp), have important implications in nervous system regeneration and are discussed, although their functions in embryonic development and directional growth are not addressed in this chapter. The list of axon guidance molecules will likely grow in the future, which will be reflected by periodical updating of this chapter. Receptors mediating these guidance molecules will be mentioned, but their signaling mechanisms will not be discussed in detail.

## 2 Axon Guidance Molecules in Development

### 2.1 Diffusible Cues

#### 2.1.1 Netrins

Netrins are a family of secreted proteins of ~600 amino acids with regions homologous to domains VI and V of laminin chains. The vertebrate and invertebrate netrins are most closely related to laminin B2 chain, and domain V contains three epidermal growth factor (EGF) repeats (Kennedy et al., 1994; Serafini et al., 1994). The C-terminal domain, sometimes referred to as "netrin-like domain", is highly basic and contains an RGD (arginine–glycine–aspartate) motif, a recognition sequence for integrins. The root of the word netrin, "netr," is derived from Sanskrit, meaning "one who guides" (Serafini et al., 1994).

Each vertebrate genome has several members of the *netrin* family. Two netrin genes, *netrin 1* and *netrin 3* were first found in mice (Wang et al., 1999a). A remote relative, *netrin 4*, was later found in mammals (Yin et al., 2000). Two netrins, *netrin 1* and *netrin 2*, were found in the chick. Only one *netrin* homolog, *unc*-6, is present in *Caenorhabditis elegans*. Drosophila has two netrin genes, *netrin A* and *netrin B*. Two molecules remotely related to netrins, define a family of glycosylphosphatidylinositol (GPI)-linked proteins called laminets (Nakashiba et al., 2000; Yin et al., 2002). The laminets are not diffusible and are structurally more related to laminins than to netrins.

The netrin family cues are bifunctional (either attractive or repulsive), regulating the growth of a variety of axons. Attraction is mediated by a transmembrane receptor, DCC (deleted in colorectal cancer) (Keino-Masu et al., 1996; Fazeli et al., 1997; Stein et al., 2001). The signaling mechanisms are highly conserved from *C. elegans* to vertebrates (Chan et al., 1996; Kolodziej et al., 1996. Repulsion is mediated by the DCC and Unc5 receptor complexes (Hedgecock et al., 1990; Hong et al., 1999; Keleman and Dickson, 2001).

#### 2.1.2 Secreted Semaphorins

Secreted semaphorins are highly diffusible glycoproteins. They are characterized by a ~500-amino-acid semaphorin domain that contains ~15 conserved cysteine residues and many other blocks of conserved residues and no obvious repeats. C-terminal to the semaphorin domain are a single C2 type immunoglobulin-like (Ig) domain and a 70–120 amino acid C-terminal domain. The vertebrate secreted semaphorins contain a stretch of highly basic amino acids in this C-terminal region. The name "semaphorin" is derived from "semaphore," which means to send visual signals using signaling apparatus like flags, lights, or mechanically moving arms, as ones used on a railroad.

There are six vertebrate members in class 3 semaphorin (diffusible vertebrate semaphorins) family and one diffusible Drosophila semaphorin, D-semaII, which belongs to class 2 semaphorin (diffusible invertebrate semaphorins) family (Semaphorin Nomenclature Committee, 1999).

The function of secreted semaphorins is primarily repulsive, mediated by the neuropilin–plexin complexes (Kolodkin et al., 1993; He and Tessier-Lavigne, 1997; Winberg et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999; Chen et al., 2000a; Giger et al., 2000; Cheng et al., 2001; Grunwald and Klein, 2002; Swiercz et al., 2002). The structure and function of semaphorins and the plexins are also highly conserved. Some secreted semaphorins have been reported to be attractive (Bagnard et al., 1998; de Castro et al., 1999). It is currently unclear which receptor mediates attraction by secreted semaphorins.

#### 2.1.3 Slits

Slits are a family of extracellular matrix molecules (ECMs) with four tandem arrays of leucine-rich repeats (LRR) (flanked by conserved amino- and carboxy-terminal sequences), a long stretch of EGF repeats, an agrin–laminin–perlecan–slit (ALPS) conserved spacer motif, and a cysteine knot (a dimerization motif

found in several secreted growth factors) (Rothberg et al., 1990; Brose et al., 1999; Kidd et al., 1999; Brose and Tessier-Lavigne, 2000). On the basis of vertebrate studies, the slits were found to be proteolytically cleaved into two fragments (Slit-N and Slit-C; 120–130 kDa and 55–60 kDa, respectively). Slit-C undergoes further cleavage or degradation, and Slit-N has potent repulsive activity (Brose et al., 1999).

Three Slit genes are found in vertebrates, *Slits 1–3*, whereas only one is found in Drosophila and in *C. elegans*. Vertebrate slit proteins differ from the invertebrate slits with one extra leucine-rich repeat in the third leucine-rich array and two more EGF repeats (Brose et al., 1999).

Slits are repulsive axon guidance cues, and the repulsion is mediated by their receptors, robos, members of immunoglobulin superfamily cell adhesion molecules (Ig-CAMs) (Brose and Tessier-Lavigne, 2000). The repulsive function of the slits and their signaling pathways are conserved from worms, flies to mammals (Zallen et al., 1998; Kidd et al., 1999; Hao et al., 2001; Plump et al., 2002; Long et al., 2004). The slits have been shown to stimulate axonal branching in vitro (Wang et al., 1999b).

### 2.1.4 TGF-β Family Proteins

Transforming growth factor-β (TGF-β) family proteins are secreted growth factors. TGF-β superfamily is a large one, with more than 30 members and having multiple functions (Miyazono et al., 2001; Nusse, 2003). Bone morphogenetic protein-7 (BMP-7) and growth/differentiation factor 7 (GDF-7) have been implicated in axonal repulsion (Augsburger et al., 1999). A *C. elegans* TGF-β family member, Unc129, has been implicated in motor axon guidance (Colavita et al., 1998). TGF-β proteins were the first morphogens implicated in axon guidance.

TGF-β family members are mediated by type I and II receptors. A type I receptor has been implicated in mediating axon guidance (Liu et al., 2003).

### 2.1.5 Sonic Hedgehog (Shh)

Sonic hedgehog is a secreted protein and belongs to the hedgehog family, which are highly conserved signaling proteins (Nusse, 2003). The Shh protein is first made as a precursor, consisting of a C-terminal protease domain (26–27 kDa) and an N-terminal signaling unit (19 kDa). The C-terminal protease of Shh cleaves the precursor in an autocatalytic manner to release the active signaling domain, HhNp. Sonic hedgehog protein has two lipid modifications. The N-terminal becomes modified by the fatty acid palmitate, on a conserved cysteine residue that is exposed at the very N-terminal end of the protein after its signal sequence has been removed. During this cleavage, the C terminus of HhNp becomes covalently modified by a cholesterol molecule. Sonic hedgehog protein binds to a membrane receptor Patched and in doing so derepresses the inhibition on the Smoothened by Patched, allowing activation of signaling downstream of Smoothened, a seven transmembrane domain protein.

Sonic hedgehog has been shown to be an axonal attractant mediated by Smoothened (Charron et al., 2003) and a repellent for retinal ganglion cell axons (Trousse et al., 2001). The receptor mediating repulsion has not been identified.

### 2.1.6 Wnts

The Wnt proteins are a large family of secreted, cysteine-rich proteins, which are both lipid modified and glycosylated (Nusse, 2003). The first conserved cysteine, which is essential for Wnt function, is palmitoylated, making Wnts very hydrophobic and hard to purify. Glycosylation takes place on conserved N-linked glycosylation sites. Wnts were the first guidance cues to be implicated in axon guidance along the rostral–caudal axis of the neuraxis (Lyuksyutova et al., 2003).

In mammals, 19 members of the Wnt family of proteins have been reported. So far multiple Wnts have been shown to have the ability to regulate CNS axon growth (Lyuksyutova et al., 2003).

Wnts are key players in several aspects of development, including axis formation, patterning, cell fate determination, proliferation, tissue polarity, morphogenesis, cell motility, and synaptogenesis (Wodarz and Nusse, 1998; Adler and Lee, 2001; Zorn, 2001; Tada et al., 2002; Keller, 2002). Wnt functions are mediated by frizzled family of receptors. More recently, evidence began to emerge that Wnt proteins may also regulate axon development (Lucas and Salinas, 1997; Salinas, 1999; Krylova et al., 2002; Lyuksyutova et al., 2003; Yoshikawa et al., 2003). A member of the frizzled family proteins, the cell surface receptors of Wnts, has also been shown to be involved in axon growth in vivo (Wang et al., 2002c). Attractive function of Wnts is mediated by frizzled-3 (Lyuksyutova et al., 2003). A receptor tyrosine kinase like protein, Ryk, mediates repulsion by Wnts (Yoshikawa et al., 2003).

## 2.2 Nondiffusible Cues

### 2.2.1 Immunoglobulin Superfamily Cell Adhesion Molecules (Ig-CAMs)

Immunoglobulin superfamily cell adhesion molecules are either transmembrane proteins or GPI-linked cell surface proteins, which contain one or multiple immunoglobulin repeats (Ig repeats) (Brummendorf and Rathjen, 1993, 1995). The Ig superfamily also includes secreted members, which have similar domain arrangements except that they are not tethered to cell membranes (Rougon and Hobert, 2003). Some secreted Ig superfamily molecules do regulate axon guidance, but they belong to the class of diffusible axon guidance molecules. The Ig repeats are approximately 100-amino-acids long, with several β-strands distributed in two β-sheets forming a sandwich stabilized by a conserved disulfide bond (Williams and Barclay, 1988; Halaby et al., 1999). Many Ig superfamily axon guidance molecules include fibronectin (Fn) type-III repeats following the Ig repeats. Sometimes, the Ig domains are followed by other protein interaction domains, such as the MAM (meprin/A5-protein/PTPmu) domain (Litwack et al., 2004). The Ig domains are sometimes present in the classes of axon guidance molecules that are usually not referred to as Ig-CAMs, as seen in several classes of semaphorin family (1999).

Ig superfamily includes a large number of members. The number of Ig repeats and Fn repeats and their combinations can vary. Some members contain large numbers of splice variants (Schmucker et al., 2000).

Cell adhesion molecules of the Ig superfamily can act as both ligands and receptors to mediate contact-mediated attraction or repulsion. They have been shown to play important roles in many axonal pathfinding events (Tessier-Lavigne and Goodman, 1996). They can act as receptors to mediate axon repulsion as in the case of F11/contactin (Pesheva et al., 1993). Some Ig superfamily molecules have signaling capacity, with protein tyrosine kinase or protein tyrosine phosphatase domains, and can act as receptors through homophilic or heterophilic interactions (Tessier-Lavigne and Goodman, 1996).

### 2.2.2 Cadherins

Classical cadherins are single transmembrane domain glycoproteins, containing five cadherin domains (CDs), which mediate calcium-dependent homophilic interaction (Ranscht, 2000). Calcium binds to the interface of neighboring CDs and stabilizes the conformation. Homophilic interactions among same types of cadherins are generally stronger than heterophilic interactions among different types of cadherins. Cadherins also mediate heterophilic interactions with integrins and fibroblast growth factor receptor (FGFR). A conserved intracellular domain of cadherins binds to α- and β-catenins.

The cadherins family is large, with about 80 members in vertebrates. In addition to the classical N-, E-, and R-cadherins, some cadherins have different structural organization. The T-cadherin has the same ectodomain structure but is tethered to the membrane by GPI linkage. Many new members belong to protocadherin family as their ectodomains contain more than five CDs, and the cytoplasmic domains are divergent from the classical cadherins.

N- and R-cadherins have been shown to promote growth and pathfinding of retinal ganglion cell axons. Homophilic interaction of T-cadherin triggers repulsive effects on motor axon projection (Ranscht, 2000).

### 2.2.3 Extracellular Matrix Molecules (ECMs)

Several classes of extracellular matrix molecules play important roles in axon growth, although it is not clear whether they play a role in axon guidance or simply growth regulation. These molecules include laminin, fibronectin, vitronectin, collagen, tenascin, thrombospondin families, and proteoglycans such as chondroitin sulfate proteoglycans (CSPGs) and heparin sulfate proteoglycans (HSPG). Some extracellular matrix molecules, such as reelin and anosmin (Kallmann's syndrome gene 1 (*KAL1*) product), are probably not as abundant as the other ECM molecules but have been shown to regulate axon growth. The slits are sometimes referred to as extracellular matrix molecules as well. Some of the ECMs, such as CSPGs and tenascins, have been implicated to be the major inhibitory cues in glial scars that prevent axon regeneration in adult CNS. The receptors that mediate growth control of axons are integrins, Ig superfamily, and proteoglycans (Tessier-Lavigne and Goodman, 1996). Many of the receptors for ECM molecules have not been identified.

### 2.2.4 Ephrins

The Ephrins are a large family of contact-mediated repellents, mediated by the ephrin (Eph) receptors, which are receptor tyrosine kinases and are among the more recently identified contact-mediated cues (Grunwald and Klein, 2002). There are two major classes, the A-class and B-class. The A-class ephrins are tethered to the cell membrane by GPI linkage. The B-class ephrins are transmembrane proteins. All ephrins contain unique conserved, extracellular receptor-binding domain with homology to photocyanins and plant nodulins (Himanen and Nikolov, 2003). B-class ephrins possess a short but highly conserved cytoplasmic domain and a C-terminal PSD-95, discs-large, Z0-1 (PDZ)-binding motif involved in protein interactions in reverse signaling. There are five A class ephrins and three B class ephrins in mammals.

Ephrins play important roles in guiding axon wiring, particularly in the formation of topographic maps and midline crossing by repulsive as well as attractive mechanisms (Cheng and Flanagan, 1994; Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996; Feldheim et al., 1998, 2000; Flanagan and Vanderhaeghen, 1998; Frisen et al., 1998; Hindges et al., 2002; Kullander and Klein, 2002; Mann et al., 2002; Pittman and Chien, 2002; Williams et al., 2003). Bidirectional signaling has been demonstrated in B ephrins as well as A ephrins (Davy and Robbins, 2000). There are 14 Eph receptors, 8 EphAs and 6 EphBs. A-class ephrins bind to EphA promiscuously and B-class ephrins bind to EphB promiscuously. There are two exceptions to that rule: B ephrins bind to EphA4 and ephrinA5 binds to EphB2 (Himanen et al., 2004).

### 2.2.5 Membrane-Bound Semaphorins

Membrane-bound semaphorins contain the conserved ~500-amino-acid sema domain in the extracellular region but are bound to the cell membrane by one transmembrane domain (Classes 1, 4, 5, and 6) or by GPI linkage (Class 7) (Semaphorin Nomenclature Committee, 1999).

The first semaphorin discovered was a transmembrane semaphorin that regulates axon fasciculation (Kolodkin et al., 1992, 1993) Both repulsive and attractive functions have been shown in membrane-bound semaphorins. Plexins have been implicated in mediating repulsive response/growth cone collapse (Swiercz et al., 2002), whereas integrins were implicated in attractive response (Pasterkamp et al., 2003). Bidirectional signaling has also been suggested in a transmembrane semaphorin with intracellular domain (sema4D) (Elhabazi et al., 2003). Much is still to be learned about membrane-bound semaphorins, which comprise the majority of the semaphorin family of axon guidance molecules.

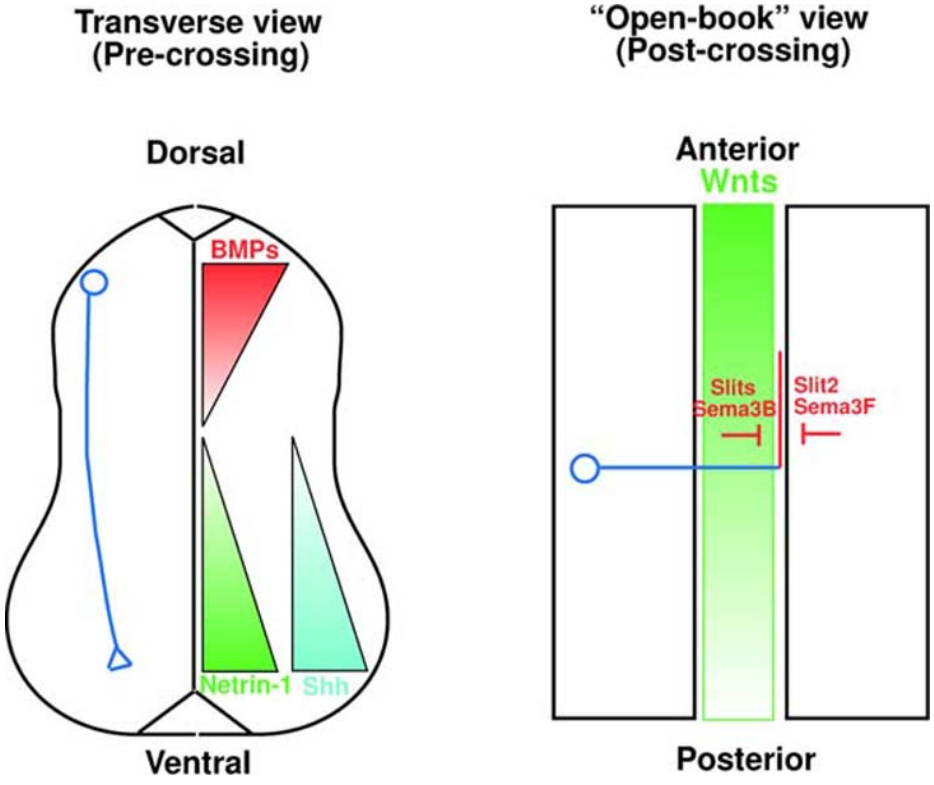
## 3 Axon Guidance at the Midline of Central Nervous System

The midline is an important source of guidance information and therefore a key organizer of nervous system wiring. Along the anterior–posterior (rostral–caudal) axis, the ventral midline plays an essential role

in guiding many classes of axons. At the forebrain, corpus callosum, which connects the left and right cerebral cortices, and optic nerves, which connect the eyes to forebrain and midbrain targets, have been extensively studied. In the spinal cord, commissural neurons have been a model system for axon guidance studies and the floor plate is one of the best-known intermediate targets for axon wiring (*Figure 16-1*).

**Figure 16-1**

**Multiple guidance cues control the pathfinding of spinal cord commissural axons. In a transverse view, precrossing commissural axons are shown projecting to the ventral midline in response to the midline chemoattractants netrin-1 and sonic hedgehog. Chemorepellants, such as the bone morphogenetic proteins (BMPs), also play a role in directing the ventral growth. After midline crossing as shown in "open-book" view, postcrossing commissural axons turn anteriorly. Postcrossing commissural axons lose responsiveness to chemoattractants, netrin-1 and sonic Hedgehog, but gain responsiveness to chemorepellents such as slits and semaphorins, which turn their trajectory from the dorsal–ventral axis to the anterior–posterior axis. Commissural axons also gain responsiveness to Wnt proteins, which direct them to turn anteriorly to project to the brain**




Axon trajectories are often very long and complex. To establish such complex connections is not a trivial task. The trajectory is often broken down into smaller segments so that pathfinding is much simplified at a given segment. At the end of these segments, growth cones usually pause and change responsiveness to guidance cues and then move on to the next segment, often in a new direction. The floor plate at the ventral midline is one such intermediate target.

Commissural neuron cell bodies are located in the dorsal half of the spinal cord and send axons toward the ventral midline, a process guided by netrin-1, a diffusible attractant (Kennedy et al., 1994; Serafini et al.,

1994, 1996), mediated by its receptor DCC (Keino-Masu et al., 1996; Fazeli et al., 1997), a member of Ig-CAM. Sonic hedgehog, a morphogen, which patterns the nervous system during earlier development, collaborates with netrin-1 in guiding commissural axons to grow to the ventral midline (Charron et al., 2003). While commissural axons are projecting ventrally, they are also repelled by BMP-7, a TGF-β family member, emanating from the roof plate (Augsburger et al., 1999). Therefore, precrossing commissural axon growth cones respond to netrin-1, Shh, and BMP-7 simultaneously.

After reaching the ventral midline, commissural axons cross to the contralateral side and turn rostrally immediately after reaching the contralateral border of the floor plate. During midline crossing, commissural axons lose responsiveness to netrin-1 (Shirasaki et al., 1998). Interestingly, before reaching the midline, commissural axons do not respond to slits and secreted semaphorins; once they cross the midline, postcrossing commissural axons gain responsiveness to both slits and a subset of secreted semaphorins, sema3B and sema3F (Zou et al., 2000) (*Figure 16-1*). The repulsion by these chemorepellents from the midline and ventral spinal cord turns commissural axons from the dorsal–ventral axis into their longitudinal trajectory.

Recent work showed that commissural axons ignore Wnt proteins before midline crossing but become attracted by Wnt proteins after midline crossing (Lyuksyutova et al., 2003). An anterior–posterior gradient of Wnt function is required for normal anterior turning of commissural axons after midline crossing. Therefore, commissural axon growth cones respond sequentially first to netrin-1, BMPs, and Shh, and then to slits, secreted semaphorins, and the Wnt proteins.

## 4 Axon Target Selection in Retinotopic Mapping

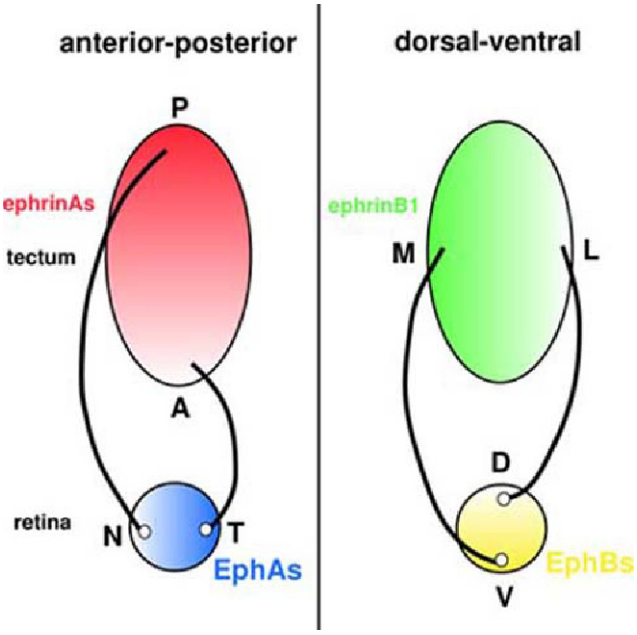
Topographic mapping of axonal connection is a fundamental feature of nervous system wiring. In several sensory systems, such as visual, auditory, and somatosensory systems, the spatial order of the sensory receptors is precisely mapped to brain targets. This is achieved largely by patterning axonal connections between the sensory receptors and their targets in a topographically organized manner. In the visual system, retinotectal projections are organized along the anterior–posterior and dorsal–ventral axes. Temporal axons project to anterior tectum (superior colliculus in rodents), and nasal axons project to posterior tectum (superior colliculus in rodents). Ventral retinal axons terminate in medial (dorsal) tectum, and dorsal retinal axons terminate at lateral (ventral) tectum (*Figure 16-2*).

Studies have implicated A-class ephrins in retinal ganglion cell axon target selection along the anterior–posterior axis via a repulsive mechanism through EphA (Feldheim et al., 1998, 2000; Flanagan and Vanderhaeghen, 1998; Frisen et al., 1998) and that an attractive interaction involving ephrinB–EphB controls dorsal–ventral patterning of axon branch termination (Hindges et al., 2002; Mann et al., 2002). Interestingly, in *ephB2-/ephB3-* double mutant, interstitial branches always shifted laterally, suggesting the presence of another activity apposing the attractive ephrinB1–EphB interaction, which directs axon branches medially (Hindges et al., 2002).

## 5 Inhibitors of Axon Regeneration in Adult Central Nervous System

During adult life, axons in the CNS lose regenerative capability so that injuries to axons in the spinal cord and brain are permanent, causing long-term functional loss. The CNS contains inhibitory molecules that are either present or induced after injury that only affect the growth of adult CNS axons. The adult CNS axons can grow if they are present with peripheral nervous system (PNS) tissues, such as sciatic nerves (Richardson et al., 1980), suggesting that adult CNS axons will regenerate if the inhibitors in the CNS are blocked. Therefore, much effort has been dedicated to the identification of these inhibitory factors and how they act to prevent axon regeneration. In general, two sources of inhibitors are present in the CNS: the myelin-derived inhibitors and the glial-scar-derived inhibitors. The myelin sheath, which wraps around adult axons, contains a number of potent inhibitors that prevent axon growth. Upon injury, glial

**Figure 16-2**
**Retinotopic mapping in vertebrate visual system. Type-A ephrins (*top left*) form an anterior–posterior low-to-high gradient in the tectal (superior colliculus in rodents) membrane. Their receptors (EphAs) are expressed in a high-to-low gradient along the temporal nasal axis in the retina. As ephrinAs repel retinal ganglion cell axons, temporal axons preferably project to anterior tectum (superior colliculus in rodents) and nasal axons project to the posterior tectum (superior colliculus in rodents), forming a topographic map. EphrinB1 (*top right*), in a medial to lateral decreasing gradient, attracts RGC axons via EphB receptors, which are expressed in a ventral to dorsal decreasing gradient in the retina. Therefore, the ventral axons preferably target to medial tectum (superior colliculus in rodents) and dorsal axons target to lateral tectum (superior colliculus in rodents), forming the topographic map along the medial lateral axis**




scars form to fill the injured CNS tissue, which creates a physical barrier and also contains inhibitory molecules.

## 5.1 Myelin-Derived Inhibitors

### 5.1.1 Nogo

Nogo proteins have potent inhibitory activity and are made up of three isoforms (Nogo-A, Nogo-B, and Nogo-C) (Chen et al., 2000b; GrandPre et al., 2000; Prinjha et al., 2000). The Nogo proteins belong to the reticulon family. The three isoforms are generated in the oligodendrocytes through alternative splicing (Nogo-A and Nogo-B) and alternative promoters (Nogo-C). The C-terminal domain (188 amino acids) is common to all isoforms and contains a 66-amino-acid subdomain, which is present on the cell surface. The Nogo-66 domain binds to Nogo receptor, NgR, which together with a coreceptor P75, causes axon growth inhibition (Fournier et al., 2001; Wang et al., 2002a; Wong et al., 2002). Nogo-A has a long N-terminal region, which can inhibit axon regeneration, although the membrane topology of Nogo-A N-terminal region is still not clear (whether it is exposed extracellularly). An antibody against the N-terminal region of Nogo-A, IN-1, promotes regeneration in vivo.

### 5.1.2 MAG

Myelin-associated glycoprotein (MAG) is a member of Ig-superfamily molecules with five C2-type Ig repeats (Salzer et al., 1987), and inhibits adult CNS axon growth (McKerracher et al., 1994; Mukhopadhyay et al., 1994). MAG is found in the myelin sheaths of both oligodendrocytes and Schwann cells (Filbin, 1995). Interestingly, MAG can promote neurite extension in neonatal and postnatal day 1 DRG neurons. MAG shares a common receptor complex with Nogo-66, i.e., NgR-P75 (Domeniconi et al., 2002).

### 5.1.3 OMgp

The oligodendrocyte–myelin glycoprotein (OMgp) is a GPI-anchored protein expressed in both neurons and oligodendrocytes in the CNS (Vourc'h and Andres, 2004). It is a 120 kDa, highly glycosylated protein. The polypeptide backbone is 46 kDa. The 440-amino-acid peptide sequence contains several domains: a 32-amino-acid cysteine-rich region (with four cysteines, likely forming two disulfide bonds), a 197-amino-acid serine–threonine rich (S/TR) domain with putative N- and O-glycosylation sites, and a 172-amino-acid LRR domain (eight tandem LRRs). OMgp inhibits axon growth and shares a common receptor complex with Nogo and MAG, i.e., NgR-P75 (Wang et al., 2002a, b).

## 5.2 Glial-Scar-Derived Inhibitors

Following injury, a strong inhibitory scar tissue forms, which prevents CNS axons to grow across. Even PNS axons cannot grow across this inhibitory environment. Scar tissue contains astrocytes, precursors of oligodendrocytes, microglia, and meningeal cells. Two major classes of extracellular matrix molecules are known to contribute to axon growth inhibition:CSPGs and tenascins.

### 5.2.1 CSPGs

CSPGs are a class of proteoglycans with a protein core and long, unbranched polysaccharides (glycosaminoglycans or GAGs) containing chondroitin sulfate (CS) disaccharide unit repeats (Properzi et al., 2003). The core proteins are hyalectans (brevican, neurocan, versican, aggrecan), NG2, phosphacan, appican, decorin, biglycan, and neuroglycan. The GAGs can be formed from six different CS disaccharide units, each of which carries one or two sulfate groups. The GAGs are generally thought to be mainly responsible for the inhibitory activity, although in some cases domains in the protein core are also implicated and may appear more important (Chen et al., 2002; Ughrin et al., 2003). Multiple CSPG core proteins are upregulated in the glial scar and are highly expressed during development. The relative contributions of individual CSPGs in axon growth inhibition are not yet determined.

### 5.2.2 Tenascins

The tenascins are a family of extracellular molecules that form dimers, trimers, and tetramers (Faissner, 1997). The amino-termini of tenascins contain several (3 to 17.5) cysteine-rich EGF-like repeats, followed by several (usually 8) fibronectin type III repeats and a carboxyl fibronectin-b- and fibronectin-g-like domain. Between the fifth and sixth FNIII domains are various numbers of alternatively spliced FNIII domains. There are four known tenascins: C, R, X, and Y. Tenascin-X and -Y are not expressed in the nervous system. Tenascin-C is expressed mainly in astroctyes and tenascin-R is expressed in oligodendrocytes and their precursors as well as Schwann cells. Tenascins play important roles in axon growth and guidance in development. Identified receptors for tenascin-C are F11/contactin and CALEB (chicken acidic leucine-rich EGF-like-domain-containing brain protein). During injury, both tenascin-C and -R are induced and have inhibitory effects on regenerating axons.

## References


Adler PN, Lee H. 2001. Frizzled signaling and cell–cell interactions in planar polarity. Curr Opin Cell Biol 13: 635-640.

Augsburger A, Schuchardt A, Hoskins S, Dodd J, Butler S. 1999. BMPs as mediators of roof plate repulsion of commissural neurons. Neuron 24: 127-141.

Bagnard D, Lohrum M, Uziel D, Puschel AW, Bolz J. 1998. Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. Development 125: 5043-5053.

Brose K, Bland KS, Wang KH, Arnott D, Henzel W, et al. 1999. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell 96: 795-806.

Brose K, Tessier-Lavigne M. 2000. Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Curr Opin Neurobiol 10: 95-102.

Brummendorf T, Rathjen FG. 1993. Axonal glycoproteins with immunoglobulin- and fibronectin type III-related domains in vertebrates: structural features, binding activities, and signal transduction. J Neurochem 61: 1207-1219.

Brummendorf T, Rathjen FG. 1995. Cell adhesion molecules 1: immunoglobulin superfamily. Protein Profile 2: 963-1108.

Chan SS, Zheng H, Su MW, Wilk R, Killeen MT, et al. 1996. UNC-40, a *C. elegans* homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. Cell 87: 187-195.

Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M. 2003. The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. Cell 113: 11-23.

Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, et al. 2000a. Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron 25: 43-56.

Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, et al. 2000b. Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403: 434-439.

Chen ZJ, Ughrin Y, Levine JM. 2002. Inhibition of axon growth by oligodendrocyte precursor cells. Mol Cell Neurosci 20: 125-139.

Cheng HJ, Bagri A, Yaron A, Stein E, Pleasure SJ, et al. 2001. Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. Neuron 32: 249-263.

Cheng HJ, Flanagan JG. 1994. Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. Cell 79: 157-168.

Cheng HJ, Nakamoto M, Bergemann AD, Flanagan JG. 1995. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell 82: 371-381.

Colavita A, Krishna S, Zheng H, Padgett RW, Culotti JG. 1998. Pioneer axon guidance by UNC-129, a *C. elegans* TGF-beta. Science 281: 706-709.

Davy A, Robbins SM. 2000. Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. EMBO J 19: 5396-5405.

de Castro F, Hu L, Drabkin H, Sotelo C, Chedotal A. 1999. Chemoattraction and chemorepulsion of olfactory bulb axons by different secreted semaphorins. J Neurosci 19: 4428-4436.

Dickson BJ. 2002. Molecular mechanisms of axon guidance. Science 298: 1959-1964.

Domeniconi M, Cao Z, Spencer T, Sivasankaran R, Wang K, et al. 2002. Myelin-associated glycoprotein interacts with the Nogo-66 receptor to inhibit neurite outgrowth. Neuron 35: 283-290.

Drescher U, Kremoser C, Handwerker C, Loschinger J, Noda M, et al. 1995. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell 82: 359-370.

Elhabazi A, Marie-Cardine A, Chabbert-de Ponnat I, Bensussan A, Boumsell L. 2003. Structure and function of the immune semaphorin CD100/SEMA4D. Crit Rev Immunol 23: 65-81.

Faissner A. 1997. The tenascin gene family in axon growth and guidance. Cell Tissue Res 290: 331-341.

Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, et al. 1997. Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature 386: 796-804.

Feldheim DA, Kim YI, Bergemann AD, Frisen J, Barbacid M, et al. 2000. Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron 25: 563-574.

Feldheim DA, Vanderhaeghen P, Hansen MJ, Frisen J, Lu Q, et al. 1998. Topographic guidance labels in a sensory projection to the forebrain. Neuron 21: 1303-1313.

Filbin MT. 1995. Myelin-associated glycoprotein: a role in myelination and in the inhibition of axonal regeneration? Curr Opin Neurobiol 5: 588-595.

Flanagan JG, Vanderhaeghen P. 1998. The ephrins and Eph receptors in neural development. Annu Rev Neurosci 21: 309-345.

Fournier AE, Grand Pre T, Strittmatter SM. 2001. Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409: 341-346.

Frisen J, Yates PA, McLaughlin T, Friedman GC, O'Leary DD, et al. 1998. Ephrin-A5 (AL-1/RAGS) is essential for proper

retinal axon guidance and topographic mapping in the mammalian visual system. Neuron 20: 235-243.

Giger RJ, Cloutier JF, Sahay A, Prinjha RK, Levengood DV, et al. 2000. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron 26: 29-41.

Grand Pre T, Nakamura F, Vartanian T, Strittmatter SM. 2000. Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403: 439-444.

Grunwald IC, Klein R. 2002. Axon guidance: receptor complexes and signaling mechanisms. Curr Opin Neurobiol 12: 250-259.

Halaby DM, Poupon A, Mornon J. 1999. The immunoglobulin fold family: sequence analysis and 3D structure comparisons. Protein Eng 12: 563-571.

Hao JC, Yu TW, Fujisawa K, Culotti JG, Gengyo-Ando K, et al. 2001. *C. elegans* slit acts in midline, dorsal-ventral, and anterior-posterior guidance via the SAX-3/Robo receptor. Neuron 32: 25-38.

He Z, Tessier-Lavigne M. 1997. Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90: 739-751.

Hedgecock EM, Culotti JG, Hall DH. 1990. The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in *C. elegans*. Neuron 4: 61-85.

Himanen JP, Chumley MJ, Lackmann M, Li C, Barton WA, et al. 2004. Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. Nat Neurosci 7: 501-509.

Himanen JP, Nikolov DB. 2003. Eph receptors and ephrins. Int J Biochem Cell Biol 35: 130-134.

Hindges R, McLaughlin T, Genoud N, Henkemeyer M, O'Leary DD. 2002. EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. Neuron 35: 475-487.

Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, et al. 1999. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 97: 927-941.

Keino-Masu K, Masu M, Hinck L, Leonardo ED, Chan SS, et al. 1996. Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell 87: 175-185.

Keleman K, Dickson BJ. 2001. Short- and long-range repulsion by the Drosophila Unc5 netrin receptor. Neuron 32: 605-617.

Keller R. 2002. Shaping the vertebrate body plan by polarized embryonic cell movements. Science 298: 1950-1954.

Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. 1994. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell 78: 425-435.

Kidd T, Bland KS, Goodman CS. 1999. Slit is the midline repellent for the robo receptor in Drosophila. Cell 96: 785-794.

Kolodkin AL, Matthes DJ, Goodman CS. 1993. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 75: 1389-1399.

Kolodkin AL, Matthes DJ, O'Connor TP, Patel NH, Admon A, et al. 1992. Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron 9: 831-845.

Kolodziej PA, Timpe LC, Mitchell KJ, Fried SR, Goodman CS, et al. 1996. Frazzled encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell 87: 197-204.

Krylova O, Herreros J, Cleverley KE, Ehler E, Henriquez JP, et al. 2002. WNT-3, expressed by motoneurons, regulates terminal arborization of neurotrophin-3-responsive spinal sensory neurons. Neuron 35: 1043-1056.

Kullander K, Klein R. 2002. Mechanisms and functions of Eph and ephrin signalling. Nat Rev Mol Cell Biol 3: 475-486.

Litwack ED, Babey R, Buser R, Gesemann M, O'Leary DD. 2004. Identification and characterization of two novel brain-derived immunoglobulin superfamily members with a unique structural organization. Mol Cell Neurosci 25: 263-274.

Liu J, Wilson S, Reh T. 2003. BMP receptor 1b is required for axon guidance and cell survival in the developing retina. Dev Biol 256: 34-48.

Long H, Sabatier C, Ma L, Plump A, Yuan W, et al. 2004. Conserved roles for Slit and Robo proteins in midline commissural axon guidance. Neuron 42: 213-223.

Lucas FR, Salinas PC. 1997. WNT-7a induces axonal remodeling and increases synapsin I levels in cerebellar neurons. Dev Biol 192: 31-44.

Lyuksyutova AI, Lu CC, Milanesio N, King LA, Guo N, et al. 2003. Anterior–posterior guidance of commissural axons by Wnt-frizzled signaling. Science 302: 1984-1988.

Mann F, Ray S, Harris W, Holt C. 2002. Topographic mapping in dorsoventral axis of the *Xenopus* retinotectal system depends on signaling through ephrin-B ligands. Neuron 35: 461-473.

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, et al. 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13: 805-811.

Miyazono K, Kusanagi K, Inoue H. 2001. Divergence and convergence of TGF-beta/BMP signaling. J Cell Physiol 187: 265-276.

Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT. 1994. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13: 757-767.

Nakamoto M, Cheng HJ, Friedman GC, McLaughlin T, Hansen MJ, et al. 1996. Topographically specific effects of ELF-1 on retinal axon guidance in vitro and retinal axon mapping in vivo. Cell 86: 755-766.

Nakashiba T, Ikeda T, Nishimura S, Tashiro K, Honjo T, et al. 2000. Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins. J Neurosci 20: 6540-6550.

Nusse R. 2003. Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. Development 130: 5297-5305.

Pasterkamp RJ, Peschon JJ, Spriggs MK, Kolodkin AL. 2003. Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. Nature 424: 398-405.

Pesheva P, Gennarini G, Goridis C, Schachner M. 1993. The F3/11 cell adhesion molecule mediates the repulsion of neurons by the extracellular matrix glycoprotein J1-160/180. Neuron 10: 69-82.

Pittman A, Chien CB. 2002. Understanding dorsoventral topography: backwards and forwards. Neuron 35: 409-411.

Plump AS, Erskine L, Sabatier C, Brose K, Epstein CJ, et al. 2002. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron 33: 219-232.

Prinjha R, Moore SE, Vinson M, Blake S, Morrow R, et al. 2000. Inhibitor of neurite outgrowth in humans. Nature 403: 383-384.

Properzi F, Asher RA, Fawcett JW. 2003. Chondroitin sulphate proteoglycans in the central nervous system: changes and synthesis after injury. Biochem Soc Trans 31: 335-336.

Ranscht B. 2000. Cadherins: molecular codes for axon guidance and synapse formation. Int J Dev Neurosci 18: 643-651.

Richardson PM, McGuinness UM, Aguayo AJ. 1980. Axons from CNS neurons regenerate into PNS grafts. Nature 284: 264-265.

Rothberg JM, Jacobs JR, Goodman CS, Artavanis-Tsakonas S. 1990. Slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. Genes Dev 4: 2169-2187.

Rougon G, Hobert O. 2003. New insights into the diversity and function of neuronal immunoglobulin superfamily molecules. Annu Rev Neurosci 207-238.

Salinas PC. 1999. Wnt factors in axonal remodelling and synaptogenesis. Biochem Soc Symp 65: 101-109.

Salzer JL, Holmes WP, Colman DR. 1987. The amino acid sequences of the myelin-associated glycoproteins: homology to the immunoglobulin gene superfamily. J Cell Biol 104: 957-965.

Schmucker D, Clemens JC, Shu H, Worby CA, Xiao J, et al. 2000. Drosophila Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. Cell 101: 671-684.

Semaphorin Nomenclature Committee. 1999. Unified nomenclature for the semaphorins/collapsins. Cell 97: 551-552.

Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, et al. 1996. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87: 1001-1014.

Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM, et al. 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. Cell 78: 409-424.

Shirasaki R, Katsumata R, Murakami F. 1998. Change in chemoattractant responsiveness of developing axons at an intermediate target. Science 279: 105-107.

Stein E, Zou Y, Poo M, Tessier-Lavigne M. 2001. Binding of DCC by netrin-1 to mediate axon guidance independent of adenosine A2B receptor activation. Science 291: 1976-1982.

Swiercz JM, Kuner R, Behrens J, Offermanns S. 2002. Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. Neuron 35: 51-63.

Tada M, Concha ML, Heisenberg CP. 2002. Non-canonical Wnt signalling and regulation of gastrulation movements. Semin Cell Dev Biol 13: 251-260.

Takahashi T, Fournier A, Nakamura F, Wang LH, Murakami Y, et al. 1999. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell 99: 59-69.

Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, et al. 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99: 71-80.

Tessier-Lavigne M, Goodman CS. 1996. The molecular biology of axon guidance. Science 274: 1123-1133.

Trousse F, Marti E, Gruss P, Torres M, Bovolenta P. 2001. Control of retinal ganglion cell axon growth: a new role for Sonic hedgehog. Development 128: 3927-3936.

Ughrin YM, Chen ZJ, Levine JM. 2003. Multiple regions of the NG2 proteoglycan inhibit neurite growth and induce growth cone collapse. J Neurosci 23: 175-186.

Vourc'h P, Andres C. 2004. Oligodendrocyte myelin glycoprotein (OMgp): evolution, structure, and function. Brain Res Rev 45: 115-124.

Wang KH, Brose K, Arnott D, Kidd T, Goodman CS, et al. 1999b. Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. Cell 96: 771-784.

Wang H, Copeland NG, Gilbert DJ, Jenkins NA, Tessier-Lavigne M. 1999a. Netrin-3, a mouse homolog of human

NTN2L, is highly expressed in sensory ganglia and shows differential binding to netrin receptors. J Neurosci 19: 4938-4947.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z. 2002a. P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG, and OMgp. Nature 420: 74-78.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, et al. 2002b. Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417: 941-944.

Wang Y, Thekdi N, Smallwood PM, Macke JP, Nathans J. 2002c. Frizzled-3 is required for the development of major fiber tracts in the rostral CNS. J Neurosci 22: 8563-8573.

Williams AF, Barclay AN. 1988. The immunoglobulin superfamily—domains for cell surface recognition. Annu Rev Immunol 6: 381-405.

Williams SE, Mann F, Erskine L, Sakurai T, Wei S, et al. 2003. Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. Neuron 39: 919-935.

Winberg ML, Noordermeer JN, Tamagnone L, Comoglio PM, Spriggs MK, et al. 1998. Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell 95: 903-916.

Wodarz A, Nusse R. 1998. Mechanisms of Wnt signaling in development. Annu Rev Cell Dev Biol 14: 59-88.

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, et al. 2002. A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 5: 1302-1308.

Yin Y, Miner JH, Sanes JR. 2002. Laminets: laminin- and netrin-related genes expressed in distinct neuronal subsets. Mol Cell Neurosci 19: 344-358.

Yin Y, Sanes JR, Miner JH. 2000. Identification and expression of mouse netrin-4. Mech Dev 96: 115-119.

Yoshikawa S, McKinnon RD, Kokel M, Thomas JB. 2003. Wnt-mediated axon guidance via the Drosophila Derailed receptor. Nature 422: 583-588.

Zallen JA, Yi BA, Bargmann CI. 1998. The conserved immunoglobulin superfamily member SAX-3/Robo directs multiple aspects of axon guidance in *C. elegans*. Cell 92: 217-227.

Zorn AM. 2001. Wnt signalling: antagonistic Dickkopfs. Curr Biol 11: R592-595.

Zou Y, Stoeckli E, Chen H, Tessier-Lavigne M. 2000. Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord. Cell 102: 363-375.

# 17 Opioid Receptor Genes and Their Regulation

*L.-N. Wei · H. H. Loh*

**Abstract:** Each opioid receptor (OR) is encoded by a single gene, namely *MOR* for the μ , *DOR* for the δ and *KOR* for the κ receptors. The three OR genes share a highly conserved genomic structure, with three major exons spanning most of the protein-coding region of each gene. Regulation of OR gene expression involves transcriptional and posttranscriptional events. Transcriptional regulation of these three OR genes employs both common and unique pathways and involves both positive and negative regulatory elements. Posttranscriptionally, multiple processes regulate the maturation of OR messenger RNAs (mRNAs), and therefore the production of their proteins. These include the generation of multiple RNA variants that produce, primarily, the same receptor protein for each gene, as well as regulation during RNA stability, splicing, translation, polyadenylation, and transport in neurons. The pharmacological implication of OR gene regulation at these transcriptional or posttranscriptional levels remains to be explored.

**List of Abbreviations:** DOR, δ opioid receptor; DP, distal promoter; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; Ik, Ikaros; KOR, κ opioid receptor; *lacZ*, β-galactosidase gene; MOR, μ opioid receptor; NO, nitric oxide; NRSE, neurorestrictive silencer element; OR, opioid receptor; PP, proximal promoter; Pr1, promoter 1; Pr2, promoter 2; RA, retinoic acid; UTR, untranslated region

## 1 Introduction

Three types of opioid receptors (ORs), i.e., μ (MOR), δ (DOR), and κ (KOR), were found on the cell membranes; each was named according to its selectivity toward specific ligands (Chang and Cuatrecasas, 1979; Chang et al., 1979) and cross-tolerance of its ligands (Schultz et al., 1980; Porreca et al., 1982). While subtypes were documented for each OR, complementary DNA (cDNA) and genomic DNA cloning revealed a single gene for each receptor. According to the DNA and protein sequences, it was concluded that all three ORs belong to the superfamily of G-protein-coupled receptors (GPCRs) (Law et al., 2000) that are characterized by the presence of seven transmembrane domains. The predicted seven transmembrane domains and intracellular loops are highly conserved (73–100%) whereas the N and C termini diverge significantly (9–20%) (Neer, 1995; Offermans and Simon, 1996).

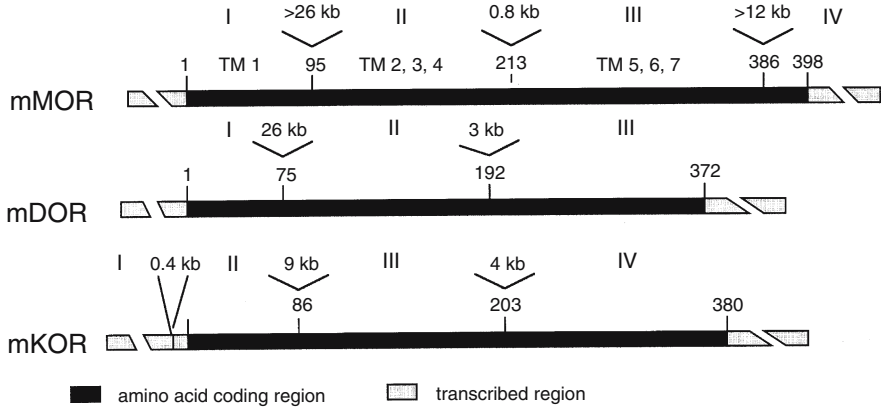
Alignment of the messenger RNA (mRNA) sequences to their genomic sequences indicated that each OR gene produced multiple mature mRNA isoforms, or variants (for review see Wei and Loh, 2002; Wei et al., 2004). While pharmacological subtypes of ORs were detected in animals, attempts to correlate these mRNA-splicing-variant protein products with receptor subtypes were largely unsuccessful. As such, the biological implication of these multiple mRNA variants required further investigation, and the molecular identity of OR subtypes remained to be determined. Interestingly, the untranslated sequences of KOR mRNA variants, in both the 5′- and the 3′-ends, were found to play important roles in regulating the production of KOR (Wei et al., 2000; Hu et al., 2002; Bi et al., 2003). Although their genomic structures and promoters share some common features, the three OR genes are subjected to distinct transcriptional controls (for review, see Wei and Loh, 2002). This chapter is devoted to the three ORs with respect to their gene structures and regulatory mechanisms underlying the production of OR proteins, including both transcriptional and posttranscriptional events.

## 2 Genomic Structures of the Three OR Genes

The three OR genes share a very similar genomic structure where three similarly positioned exons encode the major portion of the amino acid-coding region of each gene. The sequence of the seven transmembrane domains is most highly conserved, whereas both the amino- and the carboxy-terminal regions are significantly different. The common features of the mouse MOR (Min et al., 1994), DOR (Simon et al., 1994; Augustin et al., 1995), and KOR (Liu et al., 1995) genes are shown in ❯ *Figure 17-1*. Splicing junctions, i.e., the positions of introns, within the coding region of each gene are located at similar positions on each cDNA, suggesting a common ancestral origin for the three genes. Further, their promoters share several common features: TATA-less, a high G/C content, and the presence of common transcription factors like

**Figure 17-1**

**Genomic alignment of the mouse MOR (mMOR), the mouse DOR (mDOR), and the mouse KOR (mKOR) genes. The transcribed region is aligned according to the translation initiation codon (number *1* above each gene). Exons are numbered above each gene map with the approximate sizes of introns indicated at the amino acid residues where splicing events occur. In the coding region, numbers represent the amino acid positions. The *dotted broken bars* represent the 5′- and 3′-UTR of each gene. (Wei and Loh, 2002)**



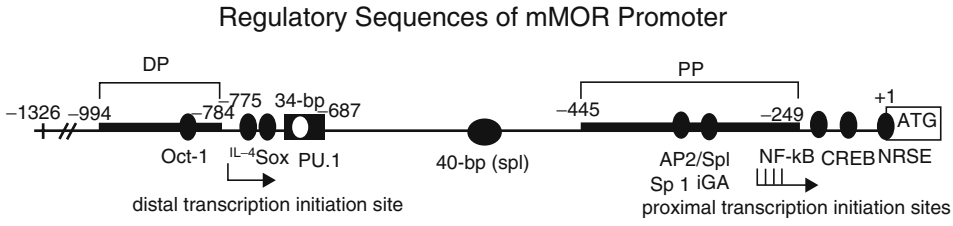

SP-1, Ikaros (Ik), E-box factors, and AP1/AP2 (Wei and Loh, 2002). However, each gene is also differentially regulated by some unique transcription factors. Apparently, the three OR genes have evolved to employ both common and unique pathways for transcriptional regulation of their expression. In agreement with the features of their promoters, the distribution of the three ORs also exhibit both common (or overlapping) and unique patterns as revealed by various detection methods, such as ligand binding (Folwer and Fraser, 1994), immunohistochemistry (Elde et al., 1995), in situ hybridization (Mansour et al., 1995), and transgenic reporters (Hu et al., 1999).

## 3 Transcriptional Regulation

### 3.1 MOR Gene

Two functional promoters have been identified for the mouse MOR (mMOR) gene (Ko et al., 1997), one initiating at −784 position (relative to translation initiation codon, denoted as DP (distal promoter) and the second initiating from a cluster of four sites between −291 and −268 positions, denoted as PP (proximal promoter). In terms of tissue specificities, PP is preferentially used in most animal tissues. Multiple regulatory elements for MOR promoters have been identified, including an inverted GA motif between −340 and −300 positions that contains several Sp1-binding sites (Ko et al., 1998), a single-stranded DNA-binding protein binding site between −340 and −400 positions (Ko et al., 2001), an AP2/Sp1 site between −400 and −450 positions (Ko et al., 2003, 2004), an Oct-1-binding site (Liang and Carr, 1996), an IL-4-binding site (Kraus et al., 2001), a binding site for Sox18 or Sox21 (Im et al., 2001; Hwang et al., 2003), and a 34-bp silencing region for Pu.1-binding (Choe et al., 1998; Hwang et al., 2004). Regulatory sequences were also found in its 5′-untranslated region (5′-UTR), such as an NF-κB site (originally identified from the human gene) (Kraus et al., 2003), a cAMP response element binding (CREB) site (Lee and Lee, 2003), and a suppressive sequence homologous to the neurorestrictive silencer element (NRSE) (Andria and Simon, 2001; Kim et al., 2004). The known regulatory DNA elements of the mMOR gene are depicted in ❯ *Figure 17-2*.

**Figure 17-2**
**Regulatory DNA elements and their corresponding transcription factors of the mouse MOR (mDOR) gene. *DP* distal promoter, *PP* proximal promoter**

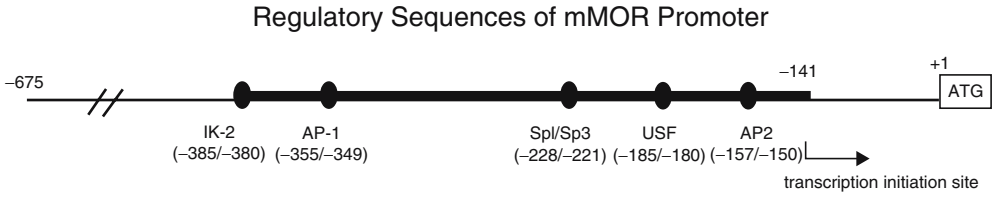



The functional activity of a 4-kb DNA fragment from the MOR promoter has been demonstrated in a transgenic mouse model in this laboratory (L. N. Wei and H. H. Loh, unpublished data) where the pattern of a MOR–*lacZ* reporter recapitulates most of the endogenous MOR expression pattern as assessed by in situ hybridization and immunohistochemistry. It remains to be confirmed if and how these transcription factors are involved in transcriptional regulation of MOR gene in a physiological context such as in different tissues and under various conditions.

## 3.2 DOR Gene

Two major transcription initiation sites have been identified for the single promoter of the mouse DOR (mDOR) gene, which is located between –324 and −142 positions with respect to the ATG initiation codon (Augustin et al., 1995). A C/G-rich region of this promoter, i.e., between −141 and −262 positions, is highly methylated and bound by transcription factors Sp3 and methylated DNA-binding protein MBD2 (Smirnov et al., 2001; Wang et al., 2003). Multiple regulatory elements for the DOR promoter include an AP-2-binding site (Woltje et al., 2000), an USF-binding E box (Liu et al., 1995), an Ets-binding site (Sun and Loh, 2001), an AP-1 site (Woltje et al., 2000), and an Ik-2-binding site (Sun and Loh, 2002, 2003). The known regulatory DNA elements of the mDOR gene are depicted in *Figure 17-3*. Likewise, their physiological relevance remains to be established.

**Figure 17-3**
**Regulatory DNA elements and their corresponding transcription factors of the mouse DOR (mDOR) gene**

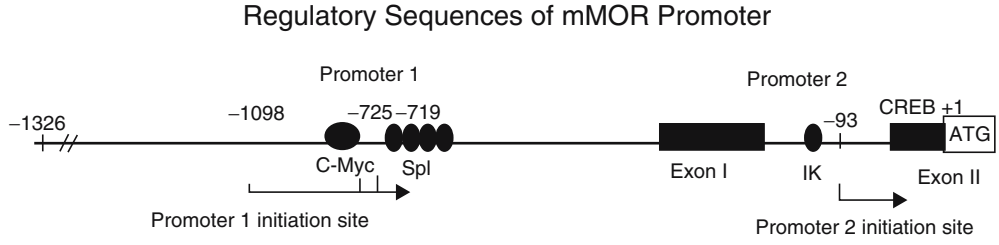



## 3.3 KOR Gene

Two functional KOR promoters have been identified, separated by a noncoding exon near the 5′-end of the cDNA, and are denoted as promoter 1 (Pr1) and promoter 2 (Pr2) (Lu et al., 1997). Pr1 initiates transcription from a cluster of residues located between −1098 and −719 positions with respect to the ATG codon. Pr2 initiates transcription from a single site at the −93 position (Lu et al., 1997) and is located within the first intron. This intron is immediately upstream from the first protein-coding exon where

alternative splicing can occur to generate at least two mRNA variants initiated from Pr1 (Wei et al., 2000). Multiple positive and negative regulatory regions are known for KOR promoters, such as binding sites for Sp1 (Li et al., 2002), c-Myc (Park and Wei, 2003), and Ik (Hu et al., 2001). The known regulatory DNA elements of the mouse KOR (mKOR) gene are depicted in *Figure 17-4*. As in the case of MOR promoters,

**Figure 17-4**
**Regulatory DNA elements and their corresponding transcription factors of the mouse KOR (mKOR) gene**




the functional significance of KOR promoters has been confirmed in a KOR–*lacZ* transgenic mouse model (Hu et al., 1999). By using this animal model, we first identified vitamin A, or its active ingredient retinoic acid (RA), in KOR transcriptional regulation (Bi et al., 2001). Regulation of KOR gene by RA was further validated in a cell culture model where the pathway mediating the action of RA can be delineated (see the following).

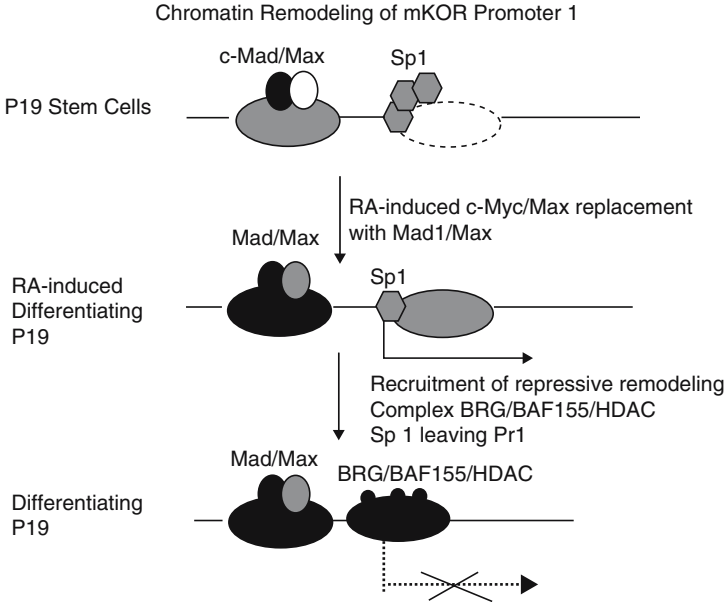
In the P19 embryonal carcinoma cell differentiation model, RA activates transcription factor Ik, whose expression in turn suppresses KOR gene transcription by recruiting histone deacetylases to condense the chromatin of KOR promoters (Bi et al., 2001; Hu et al., 2001). In the P19 embryonic stem cell line that constitutively expresses a low level of KOR, RA serves as a negative signal for KOR gene in the initial phase of cell differentiation. Recently, we have determined the chromatin structure of the mKOR gene promoter, and found multiple regularly spaced nucleosomes in both promoters of this gene in RA-induced, differentiating P19 cells (Park et al., 2005). This is in line with the suppressive effects of RA on KOR transcription in the initial phase of RA-induced P19 differentiation. Without RA, the KOR gene promoter, in particular Pr1, exists in an open configuration, consistent with the constitutive activity of this promoter in P19 stem cells. However, a high concentration of RA ($10^{-6}$ M) also transiently activates KOR gene, mediated by rapid dephosphorylation of Sp1 due to the blockage of the ERK pathway by a transient surge in RA concentration (Li et al., 2002). It is tempting to speculate that RA, at physiological concentrations, can act as a silencing factor for KOR transcription in differentiating stem cells by inducing chromatin remodeling that results in the formation of tightly organized nucleosomes in the promoter region. The transient RA surge, as caused by pharmacological manipulations, may contribute to a rapid upregulation of KOR transcription in more mature, committed cells where KOR gene promoters are in an open configuration and ready to be further induced. A model is proposed in *Figure 17-5* to describe how KOR gene transcription may be regulated by both chromatin remodeling factors and general transcription factors elicited by RA.

In addition to RA, a second signal input for KOR transcriptional control is the gas molecule, nitric oxide (NO). This gas suppresses KOR transcription by inactivating NF-κB (Park et al., 2002), which is an activator of c-Myc (Park et al., 2003). By inactivating c-Myc, an activator for KOR transcription, NO ultimately suppresses KOR gene transcription. As c-Myc has been shown to recruit histone-modifying enzymes such as histone acetyl transferases, it is highly possible that NO signal is also capable of triggering chromatin remodeling on this gene promoter. It remains to be determined in what context this gas molecule exerts its physiological function toward the control of KOR gene transcription.

The extensive documentation of some common transcription factors for all three OR genes suggests a fundamentally similar mechanism of transcriptional regulation for these genes. This is in line with the fact that these three genes share a common genomic structure and a very similar promoter feature, and appear to have evolved from the same ancestral gene. The known DNA regulatory sequences and their

**Figure 17-5**

**Retinoic acid (RA)-induced chromatin remodeling of mouse KOR (mKOR) gene promoter 1. In P19 stem cells (*top panel*), the chromatin structure of promoter 1 of the KOR gene is arranged in an open configuration, with a loose nucleosome (*gray oval shape*) covering the E box for c-Myc/Max binding and an open (no nucleosome) region spanning the GC boxes for Sp1 binding and the transcription initiation site (*dashed oval shape*). The promoter is actively transcribed in these cells (indicated by *thick arrow*). In cells that are induced by RA (*middle and bottom panels*), c-Myc/Max is replaced with Mad/Max on the E box, which recruits histone deacetylase (HDAC) and other repressive remodeling complexes such as BRG and BAF155. Chromatin is modified and a tight nucleosome is formed on the E box, with a new nucleosome beginning to form on the GC boxes where Sp1 binds (*middle panel*). Transcription of this promoter is slowed down (indicated by a *thin arrow*). Ultimately, a second tight nucleosome is formed on the GC boxes after full remodeling of this promoter in the differentiated cells (*bottom panel*). Transcription from this promoter is stopped (indicated by a *dashed arrow*)**




corresponding transcription factors mMOR, mDOR, and mKOR are summarized in *Table 17-1*. These common regulatory controls might be responsible for their somewhat overlapping expression patterns, such as neuron or immune cell specificity. However, each gene has also evolved to employ certain unique transcription factors, which might contribute to the difference in their cell type specificity in the nervous system. Despite the efforts to search for the so-called cell-specific transcription factors of these genes, it remains unknown as to whether these OR genes can be regulated by "cell-specific" transcription factors. To this end, it is tempting to speculate that a mechanism involving the combination of multiple transcription regulators and chromatin remodeling factors regulates the control of a specific OR gene transcription in particular cell types.

## 4 Posttranscriptional Regulation of OR Expression

### 4.1 mRNA Variants of OR Genes

All three OR genes exhibit extensive alternative splicing patterns, and all three genes can utilize alternative polyadenylation signals (Hu et al., 2002). A total of 14 RNA variants have been reported for the MOR gene (Pan et al., 2000, 2001; Pan, 2003). However, our recent Northern blot analyses revealed only one mature

**Table 17-1**
**Summary of transcription factors acting on opioid receptor genes**

| MOR | DOR | KOR | References |
|---|---|---|---|
| AP2 | AP-1/AP-2 | | Ko et al. (2003), Ko and Loh (2004) |
| | | c-Myc | Park et al. (2003) |
| CREB | | | Lee and Lee (2003) |
| | Ets | | Sun and Loh (2001) |
| | Ikaros | Ikaros | Hu et al. (2001), Sun and Loh (2002, 2003) |
| IL-4 | | | Kraus et al. (2001) |
| | MBD2 | | Wang et al. (2003) |
| NF-κB | | | Kraus et al. (2003) |
| NRSE | | | Andria and Simon (2001), Kim et al. (2004) |
| Oct-1 | | | Liang and Carr (1996) |
| PU.1 | | | Choe et al. (1998), Hwang et al. (2004) |
| SS DBP | | | Ko and Loh (2001) |
| Sox | | | Hwang et al. (2003, 2004); Im et al. (2001) |
| Sp1 | Sp1/Sp3 | Sp1 | Ko et al. (1998), Li et al. (2002); Smirnov et al. (2001) |
| | USF | | Liu et al. (1995) |

mouse MOR mRNA species, approximately 12 kb in size, which could be detected by both exon 1 (encoding the extracellular domain required for ligand binding) and exon 3 (encoding the transmembrane domain) probes. A functional poly(A) was also confirmed for this mature mouse MOR (L. N. Wei, unpublished data). Further, this MOR mRNA species could not be detected with probes prepared from the putative exons of reported MOR variants as predicted from polymerase chain reaction studies. This result confirmed that only one type of mature mouse mRNA that had the capacity to encode a functional mouse MOR protein was produced.

For the KOR gene, six mature mRNA variants have been identified (Wei et al., 2000; Hu et al., 2002). For the DOR gene, at least one splicing variant has been found (Gaveriauxruff et al., 1997) that generates an in-frame stop codon and, potentially, a truncated receptor. However, most of these splicing variants are predicted to generate the same receptor for the same gene. Experimental data suggest that the production of these mRNA variants probably serves to modulate the expression of these proteins after the gene is transcribed. This is best exemplified by the six KOR mRNA variants, each generating the same protein but with a distinct profile in terms of mRNA stability, translation efficiency, and transport in neurons.

The three KOR mRNA 5′-variants A, B, and C are differentially expressed in different brain areas (Bi et al., 2001), suggesting a regulatory role for the variable sequences of these KOR RNA variants. For instance, variant A is most widely expressed, whereas variant C is detected primarily in the central nervous system (CNS) and only after birth. RA regulates not only the transcription of KOR gene (see earlier), but also the splicing of this gene (Bi et al., 2001). In addition to RA treatment, using a mechanical allodynia animal pain model, we have found differential responses of these KOR mRNA variants in animals exhibiting different pain behaviors (Sung et al., 2000), i.e., variant A is better correlated with nerve-injury-induced pain. It remains to be examined whether a causal relationship exists between KOR RNA variant A and pain sensation. Recently, it was also found that immunoreactivity of DOR in rat spinal cord was influenced by peripheral nerve injury (Stone et al., 2004). It is plausible that the association of altered OR expression with specific forms of pain could be a common phenomenon.

## 4.2 RNA Stability, Translation Efficiency, and Polyadenylation

The half-life of KOR mRNA variants has been examined in the P19 culture model (Wei et al., 2000). It is estimated that variant A has a half-life of approximately 12 h and variant B's half-life is approximately 8 h. It

is amazing that the difference between variants A and B lies merely at an insertion of 30 nucleotides in the 5′-UTR for variant B. Apparently, RNA structure must be altered significantly as a result of this short insertion.

To address the translation efficiency of these KOR 5′-variants, in vitro translation and in vivo reporter systems have been used. Variants B and C, which share an extensive 5′-UTR for translation control, are comparable in terms of translation efficiency. On the other hand, variant A, which contains a 30-nucleotide insertion in the region for translational control, is least efficient in terms of translation (Wei et al., 2000).

Two functional PA signals, PA1 and PA2, have been identified for KOR, separated by approximately 2 kb (Hu et al., 2002). The two PAs are differentially regulated by RA in the P19 system, where PA2 is preferred. Extensive studies are needed to identify molecules or sequences that regulate the selection of a particular PA site in specific cell types, and how that may contribute to the property of each KOR mRNA variant.

Despite the documentation of these posttranscriptional events that contribute to differential expression of KOR RNA variants, none of these events have been directly linked to any pharmacological properties of the κ receptor. Therefore, it remains a major challenge to establish a pharmacological or physiological relevance in terms of the generation of these KOR mRNA variants in the context of whole animals.

### 4.3 RNA transport

Discrepancy between the localization of receptor proteins and their mRNA distribution has been noticed for all three ORs. While this could be attributed to technical factors, recent observation of posttranscriptional regulation of KOR suggests a potentially important biological basis for this discrepancy. The initial observation of KOR mRNA variants being detected at different levels in neuronal cell bodies and their dissected fibers suggested that KOR mRNAs might be differentially transported in neurons (Bi et al., 2003). To demonstrate and locate potential signals for transporting KOR mRNAs, a nuclear green fluorescent protein (GFP)-tagged phage RNA-binding protein motif MS2 (GFP–MS2) was used as a tracer to follow KOR mRNA that was fused to the MS2-recognizing RNA sequence (MS2–KOR). By tagging each KOR mRNA variant with the same MS2-binding sequence, different MS2–KOR variants, i.e., MS2–KORa, MS2–KORb, and MS2–KORc, were generated. Neurons, both in vitro differentiated P19 neurons and primary neurons, were cotransfected with GFP–MS2 and one of the MS2–KOR constructs, and the distribution of GFP was monitored. In this series of experiments, GFP–MS2 alone was restricted to the nuclei because of its nuclear localization signal. However, when MS2–KOR was coexpressed, GFP–MS2 would bind to MS2–KOR and exhibit a pattern reflecting the distribution of that particular MS2–KOR, i.e., the particular KOR mRNA variant used in the construct. With this reporter system, we have found that, first, the three KOR mRNA 5′-variants are differentially transported out of cell bodies into the fibers, suggesting a role of KOR mRNA sequence in the mobilization of these mRNAs. Second, the three variants are transported at different rates, suggesting differential regulation mediated by the three KOR mRNA variable regions (Bi et al., 2003). Third, KOR mRNA transport can also occur in primary neurons, such as axons of primary dorsal root ganglia, suggesting a potentially novel mRNA-based transport mechanism in the axons of certain sensory neurons (L. N. Wei and J. Bi, unpublished data). Thus, this would implicate a role of mRNA variants in the control of KOR protein production in different parts of neurons.

While it is generally thought that nonstructural proteins in the axonal compartment rely only on cargo transport of preformed cytosolic protein complexes, our results would suggest that mRNA transport in axons can occur in sensory nerves for certain nonstructural components such as KOR. Further, the fact that different KOR mRNAs are differentially transported suggests that local KOR protein expression in the remote parts of neuronal compartments may also be subjected to differential regulation by signals received at the nerve terminals. It remains an enormous challenge to address this issue in a pharmacological context.

## 5 Conclusion and Perspectives

Each OR is encoded by a different gene, but the three OR genes share a highly conserved structure, extensive homologous sequences, and some common regulatory mechanisms; however, each of them can also be

subjected to distinct transcriptional controls. For each gene, numerous mRNA variants have been found, but there were few predicted OR protein variants. Therefore, the biological significance of these OR RNA variants is probably more relevant to the regulation of these genes during RNA stability, splicing, translation, polyadenylation, and transport in neurons. Despite extensive demonstration of transcriptional regulation of these genes by specific DNA elements and transcription factors, the pharmacological implication of these transcriptional events remains to be explored. A particularly interesting issue is the demonstration of KOR RNA regulation and its axonal or dendritic transport in neurons. This could potentially determine receptor expression levels at these sites. It has been demonstrated that regulation of receptor activities varies at different axonal or dendritic spines, such as the differential regulation of MOR by morphine at different neuronal locations (Haberstock-Debic et al., 2003). This would suggest that receptor activity can be associated with its cellular localization. Differential receptor RNA transport would provide the basis for regulation of receptor production in different locations, and thus for different local activities of the same type of receptor.

Attempts to identify receptor "subtypes," as defined pharmacologically, from mRNA variants of each gene have not been rewarding. It appears to be a challenging task to identify these OR subtypes at the molecular level. Finally, it remains to be substantiated whether the demonstrated gene regulatory events, either transcriptional or posttranscriptional, using cell culture models, are physiologically significant in the context of whole animals and bear any pharmacological relevance.

## Acknowledgments

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## References

Andria ML, Simon EJ. 2001. Identification of a neurorestrictive suppressor element (NRSE) in the human μ opioid receptor gene. Mol Brain Res 91: 73-80.

Augustin LB, Felsheim RF, Min BH, Fuchs SM, Loh HH. 1995. Genomic structure of the mouse delta opioid receptor gene. Biochem Biophys Res Commun 207: 111-119.

Bi J, Hu X, Loh HH, Wei L-N. 2001. Regulation of mouse kappa opioid receptor gene expression by retinoids. J Neurosci 21: 1590-1599.

Bi J, Hu X, Loh HH, Wei L-N. 2003. Mouse κ-opioid receptor mRNA differential transport in neurons. Mol Pharmacol 64: 594-599.

Chang KJ, Cooper BR, Hazum E, Cuatrecasas P. 1979. Multiple opiate receptors: differential regional distribution in the brain and differential binding of opiates and opioid peptides. Mol Pharmacol 16: 91-104.

Chang KJ, Cuatrecasas P. 1979. Multiple opiate receptors: enkephalins and morphine bind to receptors of different specificity. J Biol Chem 254: 2610-2618.

Choe CY, Im HJ, Ko JL, Loh HH. 1998. Mouse μ OR gene expression. J Biol Chem 273: 34926-34932.

Elde R, Arvidsson U, Riedl M, Vulchanova L, Lee JH, et al. 1995. Distribution of neuropeptide receptors. New views of peptidergic neurotransmission made possible by antibodies to opioid receptors. Ann N Y Acad Sci 757: 390-404.

Fowler CJ, Fraser GL. 1994. Mu-, delta-, kappa-opioid receptors and their subtypes. A critical review with emphasis on radioligand binding experiments. Neurochem Int 24: 401-426.

Gaveriauxruff C, Peluso J, Befort K, Simonin F, Zilliox C, et al. 1997. Detection of opioid receptor mRNA by RT-PCR reveals alternative splicing for the delta- and kappa-opioid receptors. Mol Brain Res 48: 298-304.

Haberstock-Debic H, Wein M, Barrot M, Colago EE, Rahman Z, et al. 2003. Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. J Neurosci 23: 4324-4332.

Hu X, Bi J, Loh HH, Wei L-N. 2001. An intronic Ikaros binding element mediates retinoic acid suppression of kappa opioid receptor gene, accompanied by histone deacetylation on the promoters. J Biol Chem 276: 4597-4603.

Hu X, Bi J, Loh HH, Wei L-N. 2002. Regulation of mouse kappa opioid receptor gene expression by different 3′-untranslated regions and the effect of retinoic acid. Mol Pharmacol 62: 881-887.

Hu X, Cao S, Loh HH, Wei L-N. 1999. Promoter activity of mouse κ opioid receptor gene in transgenic mouse. Mol Brain Res 69: 35-43.

Hwang CK, Kim CS, Choi HS, McKercher SR, Loh HH. 2004. Transcriptional regulation of mouse μ opioid receptor gene by PU-1. J Biol Chem 279: 19764-19774.

Hwang CK, Wu X, Wang G, Kim CS, Loh HH. 2003. Mouse μ opioid receptor distal promoter transcriptional regulation by SOX proteins. J Biol Chem 278: 3742-3750.

Im HJ, Smirnov D, Yuhi T, Raghavan S, Olsson JE, et al. 2001. Transcriptional modulation of mouse μ-opioid receptor distal promoter activity by Sox 18. Mol Pharmacol 59: 1486-1496.

Kim CS, Hwang CK, Choi HS, Song KY, Law PY, et al. 2004. Neuron-restrictive silencer factor (NRSF) functions as a repressor in neuronal cells to regulate the μ opioid receptor gene. J Biol Chem 279: 46464-46473.

Ko JL, Loh HH. 2001. Single-stranded DNA binding complex involved in transcriptional regulation of mouse μ-opioid receptor gene. J Biol Chem 276: 788-795.

Ko JL, Loh HH. 2004. Identification and characterization of poly C binding protein, a single-stranded DNA binding protein, as a transcriptional regulator of mouse μ opioid receptor gene. J Neurochem In press.

Ko JL, Liu HC, Loh HH. 2003. Role of an AP-2 like element in transcriptional regulation of mouse μ opioid receptor gene. Mol Brain Res 112: 153-162.

Ko JL, Liu HC, Minnerath S, Loh HH. 1998. Transcriptional regulation of mouse μ-opioid receptor gene. J Biol Chem 273: 27678-27685.

Ko JL, Minnerath SR, Loh HH. 1997. Dual promoters of mouse mu-opioid receptor gene. Biochem Biophys Res Commun 234: 351-357.

Kraus J, Borner C, Giannini E, Hickfang K, Braun H, et al. 2001. Regulation of μ opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site. J Biol Chem 276: 43901-43908.

Kraus J, Borner C, Giannini E, Hollt V. 2003. The role of nuclear factor κB in tumor necrosis factor-regulated transcription of the human μ opioid receptor gene. Mol Pharmacol 64: 876-884.

Law PY, Wong YH, Loh HH. 2000. Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 40: 389-430.

Lee PW, Lee YM. 2003. Transcriptional regulation of μ opioid receptor gene by cAMP pathway. Mol Pharmacol 64: 1410-1418.

Li J, Park SW, Loh HH, Wei L-N. 2002. Induction of the mouse kappa opioid receptor gene by retinoic acid in P19 cells. J Biol Chem 277: 39967-39972.

Liang Y, Carr LG. 1996. Identification of an octamer-transcription factor binding site in the promoter of the mouse μ-opioid receptor gene. J Neurochem 67: 1352-1359.

Liu HC, Lu S, Augustin LB, Felsheim RF, Chen HC, et al. 1995. Cloning and promoter mapping of mouse kappa opioid receptor gene. Biochem Biophys Res Commun 209: 639-647.

Lu S, Loh HH, Wei L-N. 1997. Studies of dual promoters of mouse kappa opioid receptor gene. Mol Pharmacol 52: 415-420.

Mansour A, Cox CA, Akil H, Watson SJ. 1995. Opioid receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends Neurosci 18: 22-29.

Min BH, Augustin LB, Felsheim RF, Fuchs JA, Loh HH. 1994. Genomic structure and analysis of promoter sequence of a mouse μ opioid receptor gene. Proc Natl Acad Sci USA 91: 9081-9085.

Neer EJ. 1995. Heterotrimeric G proteins: organizers of transmembrane signals. Cell 80: 249-257.

Offermans SA, Simon MJ. 1996. Organization of transmembrane signaling by heterotrimeric G proteins. Cancer Surv 27: 177-198.

Pan YX. 2003. Identification and characterization of a novel promoter of the mouse mu opioid receptor gene (Oprm) that generates eight splice variants. Gene 295: 97-108.

Pan YX, Xu J, Bolan E, Chang A, Mahurter L, et al. 2000. Isolation and expression of a novel alternatively spliced mu opioid receptor isoform, MOR-1F. FEBS Lett 466: 337-340.

Pan YX, Xu J, Majirter L, Bolan E, Xu M, et al. 2001. Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. Proc Natl Acad Sci USA 98: 14084-14089.

Park SW, Huq MDM, Loh HH, Wei L-N. 2005. Retinoic acid-induced chromatin remodeling of mouse kappa opioid receptor gene promoter. J Neurosci 25: 3350-3357.

Park SW, Li J, Loh HH, Wei L-N. 2002. A novel signaling pathway of nitric oxide on transcriptional regulation of mouse kappa opioid receptor gene. J Neurosci 22: 7941-7947.

Park SW, Wei L-N. 2003. Regulation of c-myc gene by nitric oxide via inactivating NF-κB complex in P19 mouse embryonal carcinoma cells. J Biol Chem 278: 29776-29782.

Porreca F, Cowan A, Raffa RB, Tallarida RJ. 1982. Tolerance and cross-tolerance studies with morphine and ethylketocyclazocine. J Pharm Pharmacol 34: 666-667.

Schultz R, Wuster M, Kreness H, Herz H. 1980. Selective development of tolerance without dependence in multiple opiate receptors of mouse vas deferens. Nature 285: 242-243.

Simon F, Befort K, Gaveriaus-Ruff C, Matthes H, Nappey V, et al. 1994. The human delta-opioid receptor: genomic organization, cDNA cloning, functional expression and

distribution in human brain. Mol Pharmacol 46: 1015-1021.

Smirnov D, Im HJ, Loh HH. 2001. δ-opioid receptor gene: effect of Sp1 factor on transcriptional regulation in vivo. Mol Pharmacol 60: 331-340.

Stone LS, Vulchanova L, Riedl MS, Williams FG, Wilcox GL, et al. 2004. Effects of peripheral nerve injury on delta opioid receptor (DOR) immunoreactivity in the rat spinal cord. Neurosci Lett 361: 208-211.

Sun P, Loh HH. 2001. Transcriptional regulation of mouse δ-opioid receptor gene: role of Ets-1 in the transcription of mouse δ-opioid receptor gene. J Biol Chem 276: 45462-45469.

Sun P, Loh HH. 2002. Transcriptional regulation of mouse δ-opioid receptor gene: role of Ikaros in the stimulation of mouse δ-opioid receptor gene in activated T cells. J Biol Chem 277: 12854-12860.

Sun P, Loh HH. 2003. Transcriptional regulation of mouse δ-opioid receptor gene: Ikaros-1 and upstream stimulatory factor synergize in trans-activating mouse δ-opioid receptor gene in T cells. J Biol Chem 278: 2304-2308.

Sung B, Loh HH, Wei L-N. 2000. Association of kappa opioid receptor mRNA upregulation in dorsal root ganglia with mechanical allodynia mice following nerve injury. Neurosci Lett 291: 163-166.

Wang G, Wei L-N, Loh HH. 2003. Transcriptional regulation of mouse δ-opioid receptor gene DpG methylation. J Biol Chem 278: 40650-40656.

Wei L-N, Hu X, Bi J, Loh HH. 2000. Post-transcriptional regulation of mouse kappa opioid receptor expression. Mol Pharmacol 57: 401-408.

Wei L-N, Law PY, Loh HH. 2004. Post-transcriptional regulation of opioid receptors in the nervous system. Front Biosci 9: 1665-1679.

Wei L-N, Loh HH. 2002. Regulation of opioid receptor expression. Curr Opin Pharmacol 2: 69-75.

Woltje M, Kraus J, Hollt V. 2000. Regulation of mouse delta-opioid receptor gene transcription: involvement of the transcription factors AP-1 and AP-2. J Neurochem 74: 1355-1362.

# 18 Opioid Receptor Signaling and Regulation

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**Abstract:** Opioid receptors are transmembrane proteins that have been shown to be the targets of alkaloids isolated from the opium poppy, *Papaver somniferum*. The identification of the opioid receptor by ligand–receptor-binding assays has led to the discovery of the first class of endogenous drug molecules: enkephalin and endorphins. From the discovery of these endogenous gene products and the varied pharmacological responses, multiple opioid receptors were defined and characterized. Since the identification of the receptor by binding assay, opioid receptor has been considered to belong to the membrane receptor superfamily, the G-protein-coupled receptor (GPCR). Until the successful cloning of one of the receptor types, δ-opioid receptor (DOR), by Evans and Kieffer in 1992 (Evans et al., 1992; Kieffer et al., 1992), the eventual properties of the receptor remained elusive. Since then, much is now known about the receptor structure involved in ligand binding, signaling, and cellular control of the receptor. Several recent reviews have summarized the receptor structures/activities relationship and the ligands' selectivity studies (Law et al., 1999; Quock et al., 1999; Chaturvedi et al., 2000; Janecka et al., 2004), and others have described in detail the regulation of receptor signaling (Law et al., 2000e; Waldhoer et al., 2004). Thus, in this review, we will first briefly examine the historical perspectives in the evolution of the concepts of multiple opioid receptor types, and follow with a review on the studies of receptor signaling, with an emphasis on how chronic opioid treatment could alter the potency and efficacy of agonists.

**List of Abbreviations:** ARF, ADP-ribosylation factor; DAMGO, [D-Ala$^2$, N-Me-Phe$^4$, Gly$^5$-ol]-enkephalin; DOR, δ-opioid receptor; ERK, extracellular signal-regulated kinase; GFP, green fluorescence protein; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; Kir, G-protein-dependent inward rectifying potassium channels; KOR, κ-opioid receptor; MOR, μ-opioid receptor; PDZ, PSD-95, discs-large, ZO-1; PKC, Protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; RGS, regulator of G protein signaling

## 1 Historical Perspectives

Since Freidrich W. Serturner isolated morphine in 1805 from *Papaver somniferum* and named the alkaloid after Morpheus, the Greek god of dreams, many have hypothesized that the action of morphine is mediated by the drug binding to a receptor. Their conclusions were based on the relatively low concentration of morphine needed to relieve pain (10 mg/kg body weight) and on the stereoselective requirement for the analgesic action of the drug. Demonstration of the existence of such a receptor was not achieved until 1973. Following the guidelines established by Avram Goldstein (Goldstein et al., 1971) in determining the stereoselective high-affinity opiate-binding sites, Lars Terenius, Solomon Snyder, Eric Simon, and their coworkers simultaneously reported the identification of opiate-binding sites in synaptic membrane preparations of the rodent brain (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973). The affinities of various opiate ligands for these binding sites and the location of these binding sites by autoradiography studies correlated well with the in vivo potencies and sites of action for opiate drugs (Creese and Snyder, 1975; Pert et al., 1975; Mansour et al., 1988). Immediately, it is apparent that these mammalian binding sites could not be evolved to be the targets of plant alkaloids. This dichotomy was resolved by the isolation of endogenous opioid peptides, enkephalins, by Hughes and Kosterlitz in 1975 (Hughes et al., 1975). Enkephalins are pentapeptides with sequences of YGGFM (Met$^5$-enkephalin) or YGGFL (Leu$^5$-enkephalin) and are products of a single gene, proenkephalin A, that contains four copies of YGGFM and one copy of YGGFL. Subsequently, β-endorphin, the 32-amino- acid peptide derived from the β-lipotropin fragment of the proopiomelanocortin gene, and dynorphin derived from the prodynorphin or proenkephalin B gene, were reported by Li and Goldstein, respectively, to exhibit potent in vivo opiate properties (Loh et al., 1976; Li et al., 1977; Ghazarossian et al., 1980; Goldstein and Ghazarossian, 1980). The regional distribution of these endogenous opioid peptides in the brain parallels that of opioid receptor and neuropeptides that are known to be involved in pain transmission, such as substance P (Akil et al., 1984). Thus, it is apparent that the in vivo action of morphine and its congeners must be a reflection of these endogenous opioid peptide

actions. The presence of these multiple opioid peptide genes also implicates the probable existence of multiple opioid receptors.

## 2 The Evolution of the Multiple Opioid Receptor Concept

The concept of multiple opioid receptors was initially introduced by Bill Martin and his colleagues in 1976. Concluding from physiological responses to various opioid drugs in chronic spinal dog model, Martin and coworkers coined the multiple opioid receptor terminology based on the prototypic ligands that elicited responses, with morphine being the mu (μ) agonist, ketocyclazocine being the kappa (κ) agonist, and SKF10.047 being the sigma (σ) agonist (Martin et al., 1976). Differences in the responses to the opioid peptides in in vitro preparations of guinea pig ileum and mouse vas deferens led Kosterlitz and his coworkers to theorize the probable existence of another opioid receptor type, the delta (δ) opioid receptor (Lord et al., 1977). Initial biochemical demonstrations in the differences of peptides and alkaloid binding, and thus, the existence of multiple opioid binding sites, were originally reported by our laboratory and by K.J. Chang and his coworkers (Law and Loh, 1978; Chang et al., 1979, 1981). The biochemical characterization of the multiple opioid receptors was assisted later by the development of receptor-selective antagonists, such as naltrindole and TIPP for δ-opioid receptor, norbinaltorphimine for κ-opioid receptor, and β-funaltrexamine and CTOP for μ-opioid receptor (Takemori et al., 1981, 1988; Ward et al., 1982; Portoghese et al., 1988; Bonner et al., 1997; Schiller et al., 1999). The conclusions from binding studies in establishing the existence of multiple opioid receptors were substantiated by pharmacological cross-tolerance studies in which the loss of response after chronic treatment with a selective agonist to an opioid receptor type was not observed after treatment with a selective agonist to another receptor type (Schulz et al., 1980). The existence of multiple opioid receptors was also demonstrated by differences in their regional distribution in the brain with the original autoradiographic studies (Mansour et al., 1988). However, the unequivocal demonstration for the existence of multiple opioid receptors was not achieved until the molecular cloning of these receptor types. The cloning of the mouse δ-opioid receptor by Evans, Kieffer, and their coworkers (Evans et al., 1992; Kieffer et al., 1992), and the subsequent cloning of μ- and κ-opioid receptors based on reported sequence of δ-opioid receptor (Chen et al., 1993a, b; Meng et al., 1993; Minami and Satoh, 1995) have led to the unequivocal classification of the opioid receptors as the GPCR superfamily of receptors. The μ-, δ- and κ-opioid receptors (henceforth abbreviated as MOR, DOR, and KOR, respectively) all have the putative structure of seven transmembrane domains for GPCRs, with an extracellular N terminus containing multiple glycosylation sites, intracellular loops with multiple amphiphatic α-helixes, and the fourth intracellular loop formed by the putative palmitoylation sites at the carboxyl tails (Minami and Satoh, 1995). On the whole, these receptors are about 60% identical to each other, with the greatest identity found in the transmembrane domains (73–76%) and intracellular loops (86–100%). The greatest divergent areas were found in the N terminus (9–10%), extracellular loops (14–72%), and the C terminus (14–20%) (Minami and Satoh, 1995). The MOR, DOR, and KOR are products of distinctive genes, located in the murine chromosomes 1, 4, and 10, respectively (Kaufman et al., 1994; Kozak et al., 1994). From subsequent cloning of the σ-1 receptor (Hanner, 1996), it was apparent that the σ-opioid receptor originally defined by Martin et al. (Martin et al., 1976) does not belong to opioid receptor types. The only other GPCR with similar high sequence homology as the opioid receptors is the orphanin/FQ receptor (Bunzow et al., 1994; Chen et al., 1994; Wang et al., 1994; Wick et al., 1994). However, this orphanin receptor does not have high affinity for the opioid ligands, and only exhibits high affinity for the opiate alkaloids if a series of amino acids were mutated, especially $Ala^{213}$ to Lys in the EL2/TM5 interface, and if the $Val^{276}$-$Gln^{277}$-$Val^{278}$ residues were mutated to the IHI sequence (Meng et al., 1998). Thus, the various opioid receptor subtypes reported with pharmacological and biochemical studies do not have the equivalent species in molecular cloning experiments. Some have suggested that the putative opioid receptor subtypes are products of splice variants of these opioid receptor genes (Pan et al., 1999, 2001) or a reflection of the heterodimerization of these receptors (Jordan and Devi, 1999; Gomes et al., 2004). However, none of these spliced variants or the heterodimers reflect the reported pharmacological properties of the opioid receptor subtypes. Hence, it is likely that the opioid receptors are represented by the MOR, DOR, and KOR only.

## 3 The Signaling of the Multiple Opioid Receptors

Prior to the cloning of multiple receptors, opioid receptors were classified as prototypical "Gi/Go-coupled" receptors because their signals could be blunted by pertussis toxin (PTX)-catalyzed ADP-ribosylation and subsequent inactivation of the α-subunits of these heterotrimeric G proteins. Similar to other GPCRs that signal through Gi/Go, opioid receptors have been known to inhibit the activities of adenylyl cyclase (Sharma et al., 1975; Blume et al., 1979) and $Ca^{2+}$ channels (Hescheler et al., 1987; Jin et al., 1993b; Kim et al., 1997) and also to stimulate the $K^+$ channels (North et al., 1987) and to increase the intracellular $Ca^{2+}$ level (Jin et al., 1993a; Connor and Henderson, 1996). In addition, these receptors have been reported to regulate the mitogen-activated protein (MAP) kinase cascade (Fukuda et al., 1996; Li and Chang, 1996; Wilson et al., 1997) and the activation of phospholipase D (Mangoura and Dawson, 1993). Similar regulation of these signaling pathways and others has been reported also with the cloned opioid receptors. Details of the receptor regulation of the various signaling systems has been described in a recent review (Law et al., 2000d). Hence, we will briefly summarize the overall opioid receptor signal transduction processes.

### 3.1 The G Proteins Involved in Opioid Receptor Signal Transduction

The promiscuity of opioid receptors in coupling to various heterotrimeric G proteins is well documented. Using either $^{32}$P-azidoanilido GTP to label or cholera-toxin-mediated ADP-ribosylation to mark the agonist-induced dissociation of the G α-subunits, we have shown that MOR, DOR, and KOR could activate all the Gi/Go proteins with equal potency (Offermann et al., 1991; Roerig et al., 1992; Prather et al., 1994a, b, 1995; Chakrabarti et al., 1995b). These G proteins could be coimmunoprecipitated with MOR from brain membrane (Chalecka-Franaszek et al., 2000). However, in cells where Gz was overexpressed, opioid receptor was shown to activate PTX-insensitive heterotrimeric G proteins (Wong et al., 1992). The opioid receptors have also been shown to activate $G_{12}$- and $G_{16}$-proteins in their signal transduction processes (Lee et al., 1998; Belcheva et al., 2000; Ho et al., 2001, 2002). Even with such promiscuity, there appears to be selectivity in the G proteins involved in the activation of specific second messenger system. Studies with Gα-specific antibodies suggest that $G_i2$ mediates DOR inhibition of adenylyl cyclase in NG108-15 cells (McKenzie and Milligan, 1990), whereas Go mediates MOR inhibition of the enzyme activity in SHSY5Y cells and in brain membrane (Carter and Nedzihradsky, 1993). Similar antibody studies indicated Go-mediated MOR or DOR inhibition of the voltage-dependent $Ca^{2+}$ channels (Hescheler et al., 1987; Moises et al., 1994). The ability of opioids to inhibit the $Ca^{2+}$ channels in DRG neurons from $G_o\alpha$-subunit knockout mice was impaired (Jiang et al., 1998). Although some studies suggested opioid receptor, such as DOR, could activate one specific G protein ($G_i\alpha1$) more efficiently than the other ($G_o\alpha$) (Moon et al., 2001), these studies and others appear to support specific G protein in specific regulation of effectors. However, it appears that more than one type of G protein is mediating the same opioid receptor signal. For example, when Gz was coexpressed with either of the opioid receptors, the agonist-induced inhibition of adenylyl cyclase activity was PTX-resistant (Chan et al., 1995; Lai et al., 1995; Tsu et al., 1995b), and physical interaction between receptor and Gz was demonstrated by coimmunoprecipitation (Law and Reisine, 1997). These studies suggested that Gz, the PTX-insensitive G proteins primarily expressed in neuronal tissues, could mediate opioid receptor inhibition of the adenylyl cyclase activity in addition to the Gi/Go proteins.

Another example of more than one G protein being involved in mediating the same opioid receptor signal is the agonist-mediated activation of phospholipase C (PLCβ). Using antisense oligodeoxynucleotides, it was shown that opioid-induced intracellular $Ca^{2+}$ mobilization in ND8-47 neuroblastoma x DRG hybrid cells is mediated by $G_i2$ (Tang et al., 1995). However, coinjection of Gα and receptor RNAs into *Xenopus* oocytes suggested that $G_i\alpha1$ was required for opioid-induced $Ca^{2+}$-dependent chloride current (Ueda et al., 1995). Additionally, coexpression of MOR with Gα16 in Cos-7 cells resulted in opioid agonist-induced $Ca^{2+}$ mobilization via PLCβ activation that was PTX-insensitive (Offermann and Simon, 1995). Again, these studies and others point to the possibility that multiple G proteins are involved in transducing the same receptor signal.

One of the reasons for the involvement of multiple G proteins in transducing the same signal is in effector divergence and in the ability of both Gα and βγ subunits of the heterotrimeric G protein to regulate the effectors. DOR-mobilized intracellular $Ca^{2+}$ via the PLCβ pathway in NG108-15 cells is mediated by the βγ subunits (Yoon et al., 1999). Gβγ rather than $G_o\alpha$ is responsible for the inhibition of $Ca^{2+}$ channels (Ikeda, 1996; Herlitze et al., 1997). The stimulation of the MAPKinase cascade by opioid receptor is also mediated by the Gβγ subunits (Belcheva et al., 1998). Since different Gβ subunits determine coupling of GPCRs to the same effector (Kleuss et al., 1992), multiple G proteins could participate in the opioid receptor regulation of the same effector depending on the Gβγ composition.

Another explanation of the involvement of multiple G proteins in the same opioid receptor signal could be the compartmentalization of the receptor-signaling complexes. The partition of GPCRs into microdomains has dramatic effects on their signalings (Ostrom, 2002). In addition, cellular proteins recruited to the receptor vicinity or which function as protein scaffold could also regulate the GPCR activities (Brady and Limbird, 2002; Hall and Lefkowitz, 2002). An excellent example is the Drosophila InaD gene that codes for a protein with 5 PDZ (PSD-95, Discs-large, ZO-1) domains (Tsunoda et al., 1997). InaD associates through these PDZ domains with a light-activated $Ca^{2+}$ channel (TRP), PLCβ, and protein kinase C (PKC). The organization of these effectors by InaD allows for the efficient activation of TRP by PLCβ in response to the stimulation of rhodopsin and $G_q\alpha$, and the inactivation by the phosphorylation of TRP by PKC. The scaffoldings of signaling proteins and GPCRs via the interaction of PDZ domains have been well documented. For example, $\beta_1$-adrenergic receptor associates via its carboxyl tail with the postsynaptic density protein 95 (PSD-95) and membrane-associated guanylate kinase-like protein inverted-2 (MAGI-2) (Hu et al., 2000; Xu et al., 2001). $\beta_2$-adrenergic receptor associates with a PDZ-domain-containing protein, NHERF/EBP50 ($Na^+/H^+$ exchanger regulatory factor/ezrin binding protein 50), that could control the recycling of the receptor to the cell surface in addition to controlling agonist-induced intracellular $Na^+/H^+$ exchange (Hall et al., 1998; Cao et al., 1999). Similar interaction and function of NHERF/EBP50 have been reported with KOR (Li et al., 2002; Huang et al., 2004). In addition to static interaction with domains on scaffolding proteins to form initial signaling complexes, activation of GPCRs could recruit cellular proteins that would alter the signaling amplitude and duration. An excellent example is the recruitment of β-arrestin molecule after agonist-induced phosphorylation of GPCR. Initially, β-arrestin molecules were considered to be proteins involved in receptor desensitization (Lohse et al., 1990; Attramadal et al., 1992). Since then, β-arrestin has been demonstrated to associate with proteins involved in receptor endocytosis (Goodman et al., 1996), such as AP2 (Laporte et al., 2000, 2002), ARF6 (Claing et al., 2001), *N*-ethylmaleimide-sensitive factor (NSF) (McDonald et al. 1999), ARNO (Claing et al., 2001), Mdm2 (Shenoy et al., 2001), Src (Luttrell et al., 1999; Miller et al., 2000), c-Jun N-terminal kinase 3 (JNK3) apoptosis stimulating kinase 1 (ASK1) (McDonald et al., 2000; Miller et al., 2001), and Erk1/2 (DeFea et al., 2000; Luttrell et al., 2001). Since some of these proteins, such as Src and Erk1/2, have been reported to regulate opioid receptor phosphorylation and trafficking (Schmidt et al., 2000; Eisinger and Schultz, 2004), the organization of opioid receptor, G proteins, and other cellular proteins in microdomains could be the determinants in the G proteins used in receptor signaling (Elenko et al., 2003).

## 3.2 Opioid Receptor Regulation of Adenylyl Cyclase Activity

The ability of opioid agonist to regulate the intracellular cAMP level was initially reported by Collier and his coworkers with brain homogenates (Collier and Roy, 1974; Collier and Francis, 1975) and later substantiated by Sharma et al. with the cell line model using NG108-15 cells (Sharma et al., 1975, 1977). Acute activation of opioid receptor resulted in a PTX-sensitive decrease in the intracellular cAMP level, while chronic activation of receptor resulted in an increase of cAMP level when the agonist was removed. As discussed in Sect. 3.1, the inhibition of adenylyl cyclase activity by opioid receptor can be mediated by Gi, Go, or Gz. With at least nine isoforms of mammalian adenylyl cyclase being cloned and exhibiting diverse sensitivities to regulators like G protein α- or βγ-subunits, $Ca^{2+}$, and kinases (Tang and Hurley, 1998), it is not surprising that observations on opioid stimulation of adenylyl cyclase have been reported. Opioid agonists stimulated adenylyl cyclase in brain membranes (Puri et al., 1975), F-11 neuroblastoma–sensory

neuron hybrid cells (Cruciani et al., 1993), olfactory bulb (Olianas and Onali, 1995), and spinal cord-ganglion explants (Makman et al., 1988). These opioid stimulatory effects could be the result of the G protein βγ subunits (Gβγ) to stimulate type-2, -4, and -7 adenylyl cyclases. Many classical inhibitory receptors (e.g. $\alpha_2$-adrenergic, $D_2$ (dopamine), $A_1$ (adenosine), and chemoattractant receptors) stimulate the type-2 adenylyl cyclase via the released Gβγ (Federman et al., 1992; Tsu et al., 1995a). The stimulation of type-2 adenylyl cyclase requires the presence of GTP-bound Gαs (Federman et al., 1992; Taussig et al., 1994). Provision of activated Gαs can indeed permit all three forms of opioid receptors to stimulate cAMP accumulation in transfected cells coexpressing the type-2 adenylyl cyclase which was PTX-sensitive (Chan et al., 1995; Lai et al., 1995; Tsu et al., 1995b). This stimulatory mechanism could account for the observed increase in adenylyl cyclase activity after chronic agonist treatment.

Originally, the increase in adenylyl cyclase activity was postulated to be the biochemical correlate to morphine tolerance (Sharma et al., 1977). We have since demonstrated that the loss of response and increase in adenylyl cyclase activity are two different cellular adaptational processes (Law et al., 1982b). Crain and his colleagues suggested that such increase in adenylyl cyclase activity was from the result of a direct coupling between opioid receptor and the Gs proteins that could be regulated by GM1 gangliosides (Cruciani et al., 1993; Crain and Shen, 1996, 1998a, b). Other studies did not support such a mechanism. Using the Gβγ-stimulated type-2 adenylyl cyclase activity as an index of G protein activation, MOR could be shown to couple to six members of the Gi/Go subfamily, but not to Gs (Chan et al., 1995). However, due to the complexity in the adenylyl cyclase types, the increase in adenylyl cyclase activity or superactivation of adenylyl cyclase after chronic agonist treatment as a direct result of Gβγ effect cannot be demonstrated unequivocally. Superactivation of adenylyl cyclase has been shown to be isozyme-specific, with the ability of chronic opioid treatment to superactivate the type-1, -5, -6, and -8 enzymes but not the type-2, -3, -4, and -7 enzymes (Avidor-Reiss et al., 1996, 1997; Ammer and Christ, 2002). The complexity and versatility of the mammalian adenylyl cyclase system allows opioid agonists to modulate the enzyme activity via various routes. For example, type-1 and -8 adenylyl cyclases are activated by $Ca^{2+}$/calmodulin (Tang and Hurley, 1998). Studies indicated that the third intracellular loop of the opioid receptor contains a consensus calmodulin-binding motif and that agonist binding released the bound calmodulin (Wang et al., 1999, 2000). Thus, it is not surprising that opioid-induced elevation of intracellular cAMP level in SK-N-SH cells involves $Ca^{2+}$ entry and calmodulin activation (Sarne et al., 1998). Though such $Ca^{2+}$/calmodulin mechanism could be the basis for type-1 and -8 activation by opioids, other factors such as covalent modification of the enzyme molecules or the G protein itself during chronic agonist treatment could also account for the superactivation. Phosphorylation of the Gβγ, adenylyl cyclase, and other molecules involved in receptor desensitization, such as β-arrestin and GRK2/3, during chronic morphine treatment appeared to augment the association of these molecules and activation of the adenylyl cyclase (Chakrabarti et al., 1998b, c, 2001). Depalmitoylation of $G_s\alpha$ was observed during chronic opioid treatment resulting in the direct association of $G_s\alpha$ with adenylyl cyclase molecules preceding receptor activation (Ammer and Shultz, 1997). All of these observations and others provide alternative pathways in place of the βγ effect in the superactivation of adenylyl cyclase during chronic agonist treatment.

## 3.3 Regulation of Ion Channels

The overall action of opioid drugs is to inhibit neurotransmitter release by inhibiting the voltage-gated $Ca^{2+}$ channels and activating the G-protein-coupled inward rectifying voltage-gated potassium channels (Kir). All three opioid receptors have been shown to inhibit different types of $Ca^{2+}$ channels in various brain regions. For example, MOR and KOR inhibit N- and P/Q-type $Ca^{2+}$ channels in the nucleus tractus solitarius of the rat (Rhim and Miller, 1994; Rhim et al., 1996), while only MOR, but not DOR or KOR, is responsible for the modulation of $Ca^{2+}$ channel currents in mouse periaqueductal grey neurons (Connor et al., 1999). The cloned MOR, when expressed in NG108-15 cells, is functionally coupled to the ω-contoxin-sensitive N-type $Ca^{2+}$ channels (Morikawa et al., 1995, 1999). On the other hand, the cloned MOR and DOR inhibit voltage-activated L-type $Ca^{2+}$ channels via Gi/Go proteins in GH3 pituitary cells (Piros et al., 1995, 1996). The differences in the $Ca^{2+}$ types being regulated by the opioid receptors could

stem from the multiple $Ca^{2+}$ channel subunits that constitute the variety of voltage-gated $Ca^{2+}$ channels, i.e., L-, N-, P/Q-, R-, and T-type. Thus far, it was shown that $Ca^{2+}$ channels that consisted of $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$, or $\alpha_{1E}$ subunits were inhibited by MOR (Bourinet et al., 1996; Ottolia et al., 1998; Safa et al., 2001). Whether $Ca^{2+}$ with other α-subunits could be similarly regulated by opioid receptor or the composition of the channels' subunits predetermined the receptor coupling has not been fully addressed yet.

The involvement of $G_o$ proteins as the transducer of opioid receptor inhibition of $Ca^{2+}$ channels was demonstrated initially by Hescheler et al. (Hescheler et al., 1987) and later confirmed with $G\alpha_o$-specific antiserum (Moises et al., 1994). Now, it is accepted that the $Ca^{2+}$ channel is inhibited by the Gβγ rather than the $G\alpha_o$ subunit. Expression of Gβγ in rat sympathetic neurons mimicked GPCR-induced inhibition of $Ca^{2+}$ currents (Ikeda, 1996), and similar results were observed when Gβγ was coexpressed with $Ca^{2+}$ channel subunits in a heterologous expression system (Herlitze et al., 1996). The Gβγ-binding domain on the $Ca^{2+}$ channel has been mapped to the intracellular loop connecting domains I and II of the $\alpha_1$ subunit (Herlitze et al., 1997). This site contains the Q-X-X-E-R motif that is believed to form part of the Gβγ docking site. Intranuclear injections of DNA of different Gβ subunits into rat superior cervical ganglion neurons suggest that $G\beta_1$ and/or $G\beta_2$ subunits account for most of the voltage-dependent inhibition of N-type $Ca^{2+}$ channels, while $G\beta_5$ produces weak inhibition, and both $G\beta_3$ and $G\beta_4$ are ineffective (Garcia et al., 1998). These data and others suggest that the exact composition of G-protein-heterotrimers is important in determining the specificity of GPCR-induced inhibition of $Ca^{2+}$ channels (Kleuss et al., 1991). Although the Gβγ subunits are responsible for mediating the inhibition of $Ca^{2+}$ channels, the $G\alpha_o$ subunit is indispensable for coupling the opioid receptors to the channels. This conclusion is supported by $G\alpha_o$ knockout mice studies, in which the ability of opioid agonists to inhibit $Ca^{2+}$ channels in the DRG neurons was significantly impaired (Jiang et al., 1998).

In addition to inhibiting various voltage-dependent $Ca^{2+}$ channels, opioid agonists prevent neuronal excitation or propagation of the action potentials by hyperpolarizing the postsynaptic membrane via the activation of $K^+$ channels. Electrophysiological studies in the rat locus coeruleus have shown that both MOR and DOR can activate Kir channels via PTX-sensitive G proteins (North et al., 1987). Activation of KOR in the same preparation did not produce Kir currents. However, KOR can be shown to activate the same Kir channels as seen in the intracellular recordings of substantia gelatinosa neurons. In this preparation, all three opioid receptor types activate Kir currents (Grudt and Williams, 1993; Schneider et al., 1998). The ability of KOR to regulate the inward rectifying Kir channels was demonstrated by the coexpression of KOR and Kir3.1 in *Xenopus* oocytes. The KOR agonists activate this inward rectifying $K^+$ channel via PTX-sensitive G proteins (Henry et al., 1995; Ma et al., 1995). These channels that are located in the periaqueductal gray neurons can be activated by μ-opioid agonists and are shown to be involved in acute opioid analgesia (Han et al., 1999; Ikeda et al., 2000). The cardioprotective effects of some of the opioid agonists, such as TAN-67 for $\delta_1$-opioid receptor, were shown to be mediated by the activation of Kir channels via $G_i$ proteins (Schultz et al., 1998). The gating properties of these $K^+$ channels were altered after chronic morphine treatment (Chen et al., 2000). The identities of the Kir subunits involved in opioid functions were demonstrated clearly with Kir knockout mice. The acute inhibitory effects of opioids at locus coeruleus neurons were mediated by Kir3.2 and Kir3.3 (Torrecilla et al., 2002). The antinociceptive effects of morphine are likely to be mediated by Kir3.1 and Kir3.2 (Marker et al., 2002, 2004).

Similar to the inhibition of various $Ca^{2+}$ channels, activation of Kir3 channels appears to be mediated via the Gβγ subunits (Wickman et al., 1994). For a review on Gβγ regulation of Kir channels, please refer to Yamada et al. (Yamada et al., 1998). At least 12 distinct channel subunits are responsible for the complexity and diversity of inward rectifying $K^+$ channels, with Kir3.1 being a major subunit. Using fusion proteins containing glutathione S-transferase and different N- and C-terminal deletion mutants of Kir3.1, two Gβγ binding sites have been identified (Huang et al., 1995, 1997). At the C terminus of Kir3.1, the Gβγ binding domain is composed of two segments (Huang et al., 1995), one of which contains the N-X-X-E-R motif observed in type-2 adenylyl cyclase shown to be critical for Gβγ interaction (Chen et al., 1995). Interaction of the C-terminal domains with a small segment on the N terminus of Kir3.1 resulted in a synergistic binding of Gβγ. There is evidence to suggest that different Gβ subunits have distinct efficacy in interacting with the N-terminal domain of Kir3.1 (Yan and Gautam, 1996). Since the Kir3 subunits have similar Gβγ interaction domains, any differences in opioid receptors in activating the Kir3 channels could be the result

of different Gβ subunits associating with the G proteins activated by these receptors. However, this is unlikely since the specificity of Gβ subunit interaction with Kir disappeared as demonstrated by different combination of Gβγ to activate the Kir3.1 expressed in *Xenopus* oocytes (Lim et al., 1995). Hence, other mechanisms such as the PIP2 regulation of Gβγ-induced activation of Kir channels (Huang et al., 1998) could participate in the opioid receptor regulation of these channels.

## 3.4 Activation of MAPkinase Cascades

Similar to the large number of GPCRs that regulate cell growth, survival and death, opioid receptor activation results in the stimulation of MAPkinase cascades. The MAPkinase pathways are comprised of three protein kinase cascades, i.e., the extracellular-signal regulated kinases (ERKs), Jun N-terminal kinases (JNKs), and p38 kinases (reviewed by Garrington and Johnson (Garrington and Johnson, 1999)). As expected, activation of endogenous or heterologous expressed MOR, DOR, or KOR in various cell models has resulted in the activation of Erk1 and 2 (Burt et al., 1996; Fukuda et al., 1996; Li and Chang, 1996; Wilson et al., 1997; Hawes et al., 1998; Bohn et al., 2000a; Tso et al., 2000; Shoda et al., 2001). The opioid activation of Erk1/2 occurs through the Gβγ subunit and in a Ras-dependent manner (Belcheva et al., 1998). In jurkat cells stably expressing DOR, the opioid activation of Erk1/2 was Ras independent (Shahabi et al., 1999). The G protein most likely involved is the Go protein (Zhang et al., 2003). However, there appears to be multiple mechanisms in the opioid receptor activation of Erk1/2. Coscia and coworkers have suggested that, similar to $\beta_2$-adrenergic receptor, opioid receptor internalization is a prerequisite for Erk1/2 activation (Ignatova et al., 1999; Bohn et al., 2000b). However, several laboratories reported that opioid activation of these kinases did not require agonist-induced receptor internalization (Li et al., 1999; Whistler and von Zastrow, 1999; Kramer and Simon, 2000; Trapaidze et al., 2000b). Coscia and coworkers also suggested that opioid activation of Erk1/2 is mediated by a calmodulin-dependent transactivation of the epidermal growth factor receptor (Belcheva et al., 2001, 2002, 2003). Such transactivation as reflected in phosphorylation of the tyrosine receptor kinases was not observed by others (Kramer et al., 2002). Nevertheless, opioid receptor has been reported to regulate Erk1/2 activity in the brain as observed from the opioid activation of Erk1/2 in preparations of ventral tegmental area in vitro (Lesscher et al., 2003) or from distinct brain regions in vivo (Schultz and Hollt, 1998; Narita et al., 2002a; Eitan et al., 2003). The in vivo studies suggested that Erk1/2 activity was diminished with chronic morphine treatment, but was dramatically increased during morphine withdrawal. The Erk1/2 activation also appears to participate in morphine rewarding effect in mice (Ozaki et al., 2004). These studies implicated the role of Erk1/2 in the expression of chronic opioid effects. This was demonstrated by the ability of Erk1/2 inhibitor, PD98059, to attenuate opioid receptor desensitization, receptor phosphorylation, and internalization in cell model (Polakiewicz et al., 1998b; Schmidt et al., 2000). However, persistent activation of Erk1/2 with glutamate and paclitaxel resulted in the blockade of agonist-induced DOR internalization (Eisinger and Schultz, 2004). There does not appear to be a correlation between Erk1/2 activation and the superactivation of adenylyl cyclase activity after chronic agonist treatment (Tso and Wong, 2001). Therefore, the exact role of Erk1/2 in chronic opioid effect remains to be determined.

The activation of Erk1/2 has been linked to cell survival and proliferation. Opioid receptors have been shown to be involved in both apoptosis and cell survival (Law et al., 1997; Yin et al., 1997; Hauser and Mangoura, 1998; Chatzaki et al., 2001; Singhal et al., 2002; Zagon et al., 2002; Iglesias et al., 2003; Persson et al., 2003a, b). Thus, it should follow that opioid receptor activation should alter the activities of Erk1/2 cascades. Among the multiple kinases activated in the Erk1/2 signaling cascades, opioid agonists have been reported to activate the phosphoinositide-3-phosphate (PI3)-dependent kinase, Akt (PKB), and the p70 and p85 S6 kinases (Wilson et al., 1997; Polakiewicz et al., 1998a; Goswami et al., 2000; Narita et al., 2002b; Tan et al., 2003). In addition, the adapter protein p52 Shc was tyrosine phosphorylated upon DOR activation in Rat-1 fibroblast cells (Mullaney et al., 1997). Collectively, the activation of Erk1/2, S6 kinase, PI3kinase, and Shc proteins provides a strong mitogenic signal for opioids to regulate cell growth. Activation of Erk1/2 and subsequent kinases could also be the basis for the observed cardioprotection effect of opioid pretreatment (Fryer et al., 2001a–c).

## 3.5 Activation of Phospholipases and Intracellular $Ca^{2+}$ Homeostasis

The inability of PTX-sensitive Gα subunit to directly activate phospholipase PLCβ is well documented (Rhee, 2001). Thus, the initial observation on DOR activation in NG108-15 cells to mobilize intracellular $Ca^{2+}$ via the activation of PLCβ was totally unexpected (Jin et al., 1993a). Since then, the ability of opioid agonists to stimulate $IP_3$ production and mobilize intracellular $Ca^{2+}$ was demonstrated in human neuroblastoma SHSY5Y cells (Smart et al., 1994; Connor and Henderson, 1996; Smart and Lambert, 1996), in human epithelial tumor cells (Diao et al., 2000), smooth muscle (Murthy and Makhlouf, 1996), and spinal cord (Sanchez-Blazquez et al., 1999). Similar activation of phospholipase C, and thus an increase in intracellular $Ca^{2+}$ transient was observed with the heterologous expression of cloned opioid receptors in neuroblastoma cells (Spencer et al., 1997), in CHO cells (Smart et al., 1997), $Ltk^-$ cells (Tsu et al., 1995b), and HEK293 cells (Quillan et al., 2002). All of these opioid responses were sensitive to PTX pretreatment, suggesting the involvement of Gi/Go in the regulation of PLC activities. The G proteins involved were identified as Gi2 in the ND8-47 neuroblastoma x DRG hybrid cells with antisense studies (Tang et al., 1995), or as $G_i1$ when receptor and Gα subunits mRNAs were coinjected into *Xenopus* oocytes and chloride current was measured (Ueda et al., 1995), or by reconstitution studies with guinea pig cerebellum (Misawa et al., 1995). Since relatively high $EC_{50}$ values of opioid agonists were needed to activate PLC in these systems, and Gi/Go α-subunits have a low affinity for the PLCβ, it has been accepted that this PTX-sensitive opioid receptor response is mediated by the Gβγ subunits. The involvement of Gβγ subunits was demonstrated both in the opioid-activated PLCβ activity in intestinal smooth muscle (Murthy and Makhlouf, 1996) and by blockade of the opioid response after injection of Gβγ-binding peptide (QEHA), but not Gq-binding peptide (QLKK) into NG108-15 cells (Yoon et al., 1999).

Opioid-induced intracellular $Ca^{2+}$ increase might involve mechanisms other than Gβγ-activated PLC. In single-cell fluorescence measurements using $Ca^{2+}$ sensitive dye, a majority of the cells do not respond to the agonist addition. Only when chimeric G proteins such as Gq/Gi or promiscuous G proteins such as $G\alpha_{16}$ are used, robust responses to opioid agonists were observed (Offermann and Simon, 1995; Lee et al., 1998; Joshi et al., 1999; Ho et al., 2001). Such opioid activation of the PLC activities was PTX-insensitive. Recent reports suggested that Gi/Go-coupled receptors, such as the opioid receptors, increase intracellular $Ca^{2+}$ release only in the presence or after preactivation of Gq-coupled receptors (Chan et al., 2000; Yeo et al., 2001). Since Gβγ subunits have been implicated in opioid receptor action, the coincident signaling between Gq-coupled receptor and opioid receptor suggests that the binding of Gβγ subunits at the N-terminal PH domain affects the interaction of Gq α-subunit with the C2 domain of the PLCβ3, thus potentiating Gq α-subunit activity. However, an increase in IP3 production in SHSY5Y cells did not parallel intracellular $Ca^{2+}$ increase (Yeo et al., 2001). Hence, a mechanism other than coactivation of PLC could be involved in opioid-induced $Ca^{2+}$ increase. $Ca^{2+}$ influx via the L-type $Ca^{2+}$ channels was suggested to be the mechanism for MOR-mediated PLC activation in SHSY5Y cells (Smart et al., 1995). DOR regulation of intracellular $Ca^{2+}$ transient in human neuroblastoma SK-N-BE cells appears to be mediated by the ryanodine receptor and was PTX-insensitive (Allouche et al., 1996). These are the probable pathways involved. However, a more plausible explanation for the coincident signaling between Gi/Go-coupled receptors and Gq-coupled receptors lies in the regulation of IP3-receptor activity. Phosphorylation of IP3 receptor by PKC resulted in the ability of Gβ to activate $Ca^{2+}$ release from the IP3-sensitive $Ca^{2+}$ stores (Patterson et al., 2004). Thus, opioid agonist could regulate the intracellular $Ca^{2+}$ pools without directly activating PLC. Whether this is the mechanism remains to be demonstrated.

The physiological relevance of opioid-induced stimulation of PLC is not immediately apparent. There appears to be cross-talk between the PLC pathway and other opioid receptor regulated pathways, such as adenylyl cyclase (Fan et al., 1998; Wu et al., 1998). PLCβ has been implicated in the antinociceptive effects of opioids by antisense oligonucleotide studies (Sanchez-Blazquez and Garzon, 1998; Narita et al., 2000) or with PLCβ knockout mice studies (Xie et al., 1999a). A PTX-sensitive PLC pathway appears to mediate the arrhythmogenic effect of κ-agonists in isolated rat heart (Bian et al., 1998). In T cells, activation of DOR stimulates $Ca^{2+}$ mobilization (Sharp et al., 1998) and enhances IL-2 secretion (Hedin et al., 1997). Since an increase in activities of both PKC and $Ca^{2+}$-dependent protein kinases usually follows an increase in intracellular $Ca^{2+}$, stimulation of the activities of these kinases has been suggested to be involved in chronic

opioid drug action. The activity of $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMK II) in the rat hippocampus is stimulated by morphine (Lou et al., 1999), and CaMK II was implicated in the phosphorylation and subsequent desensitization of MOR (Koch et al., 1997, 2000) and DOR (Fan et al., 1997). PKC was shown to translocate and participate in MOR downregulation during chronic agonist treatment (Kramer and Simon, 1999a, b). Phosphorylation and endocytosis of DOR can be mediated by PKC (Xiang et al., 2001). The increase in the adenylyl cyclase activity during chronic agonist treatment is related to PKC activities (Rubovitch et al., 2003). However, whether such PKC-mediated events have functional roles in chronic agonist responses is debatable, due to a feedback mechanism regulating PLC activities. PKC-mediated phosphorylation of PLC$\beta_3$ has been demonstrated to rapidly attenuate opioid-induced IP3 turnover in NG108-15 cells (Strassheim et al., 1998). This feedback mechansim may limit the increase in PKC activities and subsequent involvement of PLC$\beta$ in the chronic actions of opioids.

## 4 Regulation of Receptor Signaling

Opioid receptors belong to the same subfamily of the GPCR superfamily of receptors as $\beta_2$-adrenergic receptors, the rhodopsin subfamily. Thus, many of the studies on the cellular control of opioid receptor signaling are modeled after the $\beta_2$-adrenergic receptor. According to the model for $\beta_2$-adrenergic receptor desensitization as proposed by Lefkowitz and his coworkers (Lefkowitz, 1998), agonist binding results in the rapid phosphorylation of the receptor by protein kinases including the G-protein-coupled receptor kinases (GRKs), thereby promoting the association of the cellular protein $\beta$-arrestin. Association of $\beta$-arrestin with the receptor uncouples the receptor from the respective G protein that transduces the signal, thus blunting the receptor signaling (receptor desensitization). $\beta$-arrestin is also involved in the agonist-induced, clathrin-coated vesicles mediated receptor internalization. Internalized receptor could be trafficked to other subcellular compartments, such as lyzosomes for degradation, or could be recycled to the cell surface where receptor signaling is continued. Eventually, with prolonged exposure to agonist, there is a decrease or downregulation of the overall cellular receptor content. $\beta$-arrestin itself also serves as an adapter molecule in $\beta_2$-adrenergic receptor signaling such that a receptor–src kinase complex is formed through which activation of the MAP kinases, Erk1/2, by the $\beta_2$-adrenergic receptor is accomplished (Luttrell et al., 1999). Thus, the cellular regulation of opioid receptor signaling could follow the model for $\beta_2$-adrenergic receptor desensitization, i.e., there is an agonist-induced receptor phosphorylation leading to recruitment of $\beta$-arrestin and subsequent receptor endocytosis and desensitization. We will examine these cellular events individually.

### 4.1 Receptor Phosphorylation

Concrete demonstration of opioid receptor phosphorylation was first reported by Pei et al. in DOR (Pei et al., 1995). Subsequently, phosphorylation of MOR and KOR was demonstrated by others (Arden et al., 1995; Appleyard et al., 1997). Studies with DOR (Pei et al., 1995) or MOR (Zhang et al., 1996; El Kouhen et al., 1999) suggested the phosphorylation of the opioid receptor is mediated via GRKs and not by PKC. Predictably, the ability of opioid ligand to induce receptor phosphorylation correlated to its efficacy (Yu et al., 1997). With the exception of morphine, agonists such as DAMGO or etorphine were reported to induce MOR phosphorylation. Yu et al. reported that morphine could induce MOR phosphorylation in CHO cells (Yu et al., 1997), while Arden et al. and Zhang et al. reported that morphine could not induce MOR phosphorylation in HEK293 cells (Arden et al., 1995; Zhang et al., 1998). The morphine–receptor complex is a poor GRK substrate as demonstrated by the ability of overexpressed GRK-2 to phosphorylate MOR in HEK293 cells during morphine treatment (Zhang et al., 1998). The differences between morphine–MOR and DAMGO–MOR complexes are best illustrated by our studies in which cAMP-dependent protein kinase (PKA) could phosphorylate MOR in the presence of morphine, but not in the presence of DAMGO (Chakrabarti et al., 1998a). Thus, whether morphine could induce MOR phosphorylation in neurons or in cell models will depend on the levels of specific protein kinases present in the system.

The amino acid residues within opioid receptors that are phosphorylated in the presence of agonist have been identified. Initial experiments with deletion analyses have suggested that the carboxyl tail of the opioid receptor is the site for agonist-induced receptor phosphorylation (Zhao et al., 1997; Murray et al., 1998). Subsequent systematic mutations of the Ser/Thr residues within the carboxyl tail sequences identified $Thr^{358}$ and $Ser^{363}$ residues in DOR that were phosphorylated in the presence of agonist (Guo et al., 2000; Maestri-El Kouhen et al., 2000). Further, the agonist-induced phosphorylation was hierarchical. Phosphorylation of $Ser^{363}$ and DOR must occur prior to the phosphorylation of $Thr^{358}$ residue (Maestri-El Kouhen et al., 2000). For KOR, $Ser^{369}$ residue within the carboxyl tail domain was the site for agonist-induced phosphorylation (McLaughlin et al., 2003).

In contrast, the identities of the amino acid residues that are phosphorylated in MOR have been controversial. There are reports suggesting phosphorylation of Tyr residues in addition to Ser/Thr residues (Pak et al., 1999; Kramer et al., 2000; McLaughlin and Chavkin, 2001). Pak et al. reported the mutation of $Thr^{394}$ to Ala in the rat MOR resulted in the blunting of the agonist-induced receptor desensitization (Pak et al., 1997). Subsequently, Deng et al. reported that $Thr^{394}$ was indeed phosphorylated in the presence of agonist by GRKs (Deng et al., 2000). However, using similar mutational analyses, we could not demonstrate the phosphorylation of $Thr^{394}$ in HEK293 cells. Instead, agonist induced the phosphorylation of two residues within the carboxyl tail motif of MOR: $Thr^{370}$ and $Ser^{375}$ (El Kouhen et al., 2001). Comparison of the amino acid residues of MOR and DOR being phosphorylated in the presence of agonist suggests the presence of a consensus motif. In both MOR and DOR, the Ser residue immediately downstream from a Pro residue is phosphorylated. The phosphorylated Thr residue is five-amino-acid residues upstream from the Ser residue, or $\sim$two $\alpha$-helical turns. Thus, the consensus agonist-induced phosphorylation motif for the opioid receptor is defined by the sequence T-X-X-X-P-S, where X is any amino acid. $Thr^{394}$ located at the carboxyl terminal of MOR lies outside of the consensus motif.

The protein kinases that most likely participate in agonist-induced receptor phosphorylation are members of GRKs. Expression of the dominant negative mutant of GRK or overexpression of GRK5 resulted in the attenuation or potentiation of agonist-dependent phosphorylation of DOR (Pei et al., 1995). Using purified GRK2, we could demonstrate phosphorylation of the $Ser^{375}$ but not the $Thr^{370}$ residue of MOR (unpublished observations). Further, purified GRK5 could not phosphorylate the carboxyl tail domain of the receptor. These studies and others support the involvement of GRK2 in opioid receptor phosphorylation. However, other protein kinases might be involved in agonist-induced receptor phosphorylation. Our data suggest that overexpression of the GRK2 has a minimal effect on DAMGO-induced MOR phosphorylation, but could potentiate both etorphine- or morphine-induced receptor phosphorylation (Zhang et al., 1998; El Kouhen et al., 1999). Mutation of $Ser^{261}$ and $Ser^{266}$, two putative CaM kinase II sites in the third intracellular loop of MOR, could block the agonist-induced receptor desensitization with a parallel decrease in receptor phosphorylation (Koch et al., 1997). However, whether these two sites are being phosphorylated by PKC is debatable. Both truncation and cyanogen bromide cleavage studies indicate that agonist-induced phosphorylation sites were located at the carboxyl tail domain of the receptor (Murray et al., 1998; El Kouhen et al., 2001). Although opioid receptor could be phosphorylated in the absence of agonist, such basal phosphorylation sites are also located at the carboxyl tail domain. Basal phosphorylation site of DOR by PKC has been demonstrated to be a $Ser^{344}$ residue (Xiang et al., 2001). Thus, the significance of PKC phosphorylation of opioid receptor is not obvious.

## 4.2 Receptor Desensitization

From the $\beta_2$-adrenergic model discussed earlier, it can be seen that receptor phosphorylation leads to receptor desensitization. A causal relationship between opioid receptor phosphorylation and desensitization appears to exist. Desensitization of DOR was reported to correlate with the phosphorylation of the receptor protein in the SK-N-BE cells (Hasbi et al., 1998). Overexpression of GRK or its dominant negative mutant, or the mutation of the putative phosphorylation sites, could modulate DOR and MOR desensitization (Pei et al., 1995; Kovoor et al., 1997). Mutation of $Ser^{369}$ to Ala in KOR also resulted in blunting of the agonist-induced desensitization process (McLaughlin et al., 2003). Zhang et al. reported a direct correlation

between MOR phosphorylation and desensitization (Zhang et al., 1996), while overexpression of β-arrestin resulted in rapid morphine-induced MOR desensitization and internalization (Whistler and von Zastrow, 1998; Zhang et al., 1998). The direct involvement of β-arrestin in chronic opioid action was demonstrated by the blunting of in vivo tolerance to morphine antinociceptive response in β-arrestin2 knockout mice (Bohn et al., 1999, 2000c, 2002). At the same time, there was a lack of in vitro receptor desensitization in fibroblasts isolated from these animals (Bohn et al., 2002). Interestingly, the opioid efficacy appears to be related to the ability of the opioid to recruit β-arrestin to the cell surface (Bohn et al., 2004). The use of GRK3 knockout mice also suggests that tolerance development to the κ-opioid agonist appeared to be related to receptor phosphorylation (McLaughlin et al., 2004). All these data and others support the observation that the opioid receptor desensitization fits the model of $\beta_2$-adrenergic receptor desensitization.

However, opioid receptor phosphorylation does not appear to be a prerequisite for receptor desensitization. For one, the time course for receptor phosphorylation is rapid, while the receptor desensitization, as measured by adenylyl cyclase inhibition, was slow (Law et al., 2000a). Overexpression of GRK increased the level of phosphorylated receptor but did not increase the rate of receptor desensitization (El Kouhen et al., 1999). Deletion of the last 31 amino acids of DOR resulted in the abolition of both GRK- or PKC-mediated agonist-dependent phosphorylation of the receptor, but did not block the agonist-induced receptor desensitization (Murray et al., 1998; Wang et al., 1998). The mutation of all Ser/Thr residues within the third intracellular loop and the C terminus of MOR did not prevent DAMGO-induced receptor desensitization (Capeyrou et al., 1997). Though prolonged morphine treatment could produce receptor desensitization (Chakrabarti et al., 1995a), morphine normally does not induce receptor phosphorylation. These data and others suggest that blunting of the opioid signals by β-arrestin does not require opioid receptor phosphorylation. This is not too surprising since the agonist-induced phosphorylation of other GPCRs results in increase in receptor affinities for β-arrestin. Without phosphorylation, β-arrestin could interact with the agonist–receptor complexes. The recruitment of β-arrestin by the nonphosphorylated receptor was illustrated by studies in which the putative agonist-induced phosphorylation sites i.e., after $Ser^{344}$ residue in DOR or after $Ser^{363}$ residue in MOR were removed. Agonist could induce endocytosis, which is a β-arrestin-dependent process (Murray et al., 1998; Qui et al., 2003). Opioid agonist could induce the translocation of the β-arrestin–GFP fusion protein from the cytosol to plasma membrane in the truncated carboxyl mutants of MOR or the Ser/Thr phosphorylation mutants of DOR (Law et al., 2000b; Qui et al., 2003). Similar arrestin–receptor interaction was reported by BIACORE studies (Cen et al., 2001b). It is important to note that the rate of receptor desensitization will depend on its ability to internalize and recycle. Mutation of $Thr^{394}$ in MOR that was suggested to affect the recycling of the receptor, altered the rate of receptor desensitization (Koch et al., 1998b, 2001b). Blockade of MOR recycling with monensin or receptor truncation also increases agonist-induced receptor desensitization (Law et al., 2000a; Qui et al., 2003). Thus, the process of β-arrestin-dependent receptor endocytosis has great impact in the rate of opioid receptor desensitization.

## 4.3 Receptor Trafficking

The trafficking of GPCR is a dynamic process. The recruitment of β-arrestin after receptor phosphorylation results in receptor endocytosis via a dynamin-dependent process in clathrin-coated pits that delivers the receptor containing vesicles to the early endosomes (Krupnick and Benovic, 1998; Roth et al., 1998). The receptors are further trafficked to the late endosomes where the decision for recycling or degradation takes place (Moore et al., 1999). Since receptor endocytosis is basically a process to remove active receptors from the cell surface, such a loss of receptor generally equates to signal termination. However, many studies have suggested that receptor endocytosis has other functions. The dephosphorylation and resensitization of the $\beta_2$-adrenergic and $A_2$-adenosine receptors require receptor internalization and trafficking to the endosomes (Zhang et al., 1997; Mundell and Kelly, 1998). As discussed earlier, activation of the MAPkinases by $\beta_2$-adrenergic receptor was dependent on receptor endocytosis (Daaka et al., 1998). $\alpha_2$-Adrenergic receptor-mediated MAPkinases activation was also dependent on receptor endocytosis, but appears to be cell line-specific (DeGraff et al., 1999; Schramm and Limbird, 1999; Pierce et al., 2000). The internalized

receptor could also determine the fate of the activated MAP kinases as demonstrated by the translocation of nonendocytosed mutant of PAR2-receptor-activated MAP kinases to the nucleus, whereas the endocytosed PAR2- receptor-activated MAP kinases remained in the cytosol (DeFea et al., 2000). Thus, the GPCR signaling and the consequence of the signals are influenced by the receptor trafficking.

It is clear from earlier studies that opioid receptor would internalize in the presence of agonist. Such a receptor endocytosis process was observed with endogenously expressed DOR and MOR in neuroblastoma cell lines (Chang et al., 1982; Law et al., 1982a; Zadina et al., 1993). Trafficking of the internalized receptor to endosomes, and eventually to lysosomes, was first demonstrated with an accumulation of radioactive agonist (Law et al., 1984), and subsequently with the epitope-tagged cloned opioid receptor (Ko et al., 1999). Interestingly, the internalization of the opioid receptor such as MOR is a ligand-dependent event, with etorphine inducing receptor endocytosis, while morphine could not (Keith et al., 1996). Although there are reports suggesting that DOR could recycle and resensitize after endocytosis (Trapaidze et al., 2000a), it is accepted that DOR does not recycle but is trafficked directly to lysosomes after initial endocytosis (Whistler et al., 1999). Similar to other GPCRs opioid receptor endocytosis involves the β-arrestin- and dynamin-dependent clathrin-coated pits pathway (Murray et al., 1998; Whistler and von Zastrow, 1998; Zhang et al., 1998, 1999; Li et al., 1999).

In addition to observation of opioid receptor endocytosis in in vitro cell models, in vivo opioid receptor endocytosis could be observed in organo cultures or primary neuronal cultures and also in neurons. DAMGO treatment of the longitudinal muscle-myenteric plexus preparation or the primary hippocampal neuron cultures resulted in MOR endocytosis (Sternini et al., 2000; Bushell et al., 2002). Within 15 min of an intraperitoneal injection of etorphine, MOR immunoreactivity was observed in the endosomal structures of the myenteric neurons of guinea pig ileum (Sternini et al., 1996). Rapid clustering of a MOR spliced variant, MOR-1C, was observed in the lateral septum of the mouse after intracerebroventricular injection of DAMGO (Abbadie and Pastnernak, 2001). Such studies and others extended the earlier studies in which either in vivo administration of receptor-selective ligands such as morphiceptin, endormorphin-1, or DADLE, resulted in the selective downregulation of MOR and DOR (Tao et al., 1990, 1998; Harrison et al., 2000). Thus, agonist treatment will affect the in vivo trafficking of opioid receptors.

Since opioid receptor endocytosis is dependent on receptor phosphorylation and arrestin binding, it is logical to suggest that intracellular domains will participate in the cellular trafficking of the receptors. Earlier experiments by truncating DOR after $Ser^{344}$ or mutation of $Thr^{353}$ to Ala blocked the agonist-induced receptor downregulation (Cvejic et al., 1996), while mutating the Ser/Thr residues between $Ser^{344}$ and $Ser^{363}$ retarded the rate of receptor internalization (Trapaidze et al., 1996; Maestri-El Kouhen et al., 2000). These studies supported the notion that receptor phosphorylation is a critical step for opioid receptor internalization. However, with the identification of DOR phosphorylation sites by mutational analyses (Guo et al., 2000; Maestri-El Kouhen et al., 2000), the amino acid residues previously reported to participate in receptor trafficking are not phosphorylated in the presence of agonist. These amino acids most likely participate in the receptor interaction with β-arrestin as suggested by the pull-down assay and BIACORE studies (Cen et al., 2001b, a). However, the exact amino acid sequence involved remains to be determined. Since the level of β-arrestin is critical in agonist-induced receptor internalization (Whistler and von Zastrow, 1998), it is not surprising to observe cell line dependency in agonist-induced receptor internalization. For example, the same $Ser^{344}$ truncated DOR that eliminated agonist-induced receptor phosphorylation could be internalized in HEK293 cells but not in CHO cells (Trapaidze et al., 1996; Murray et al.,1998). Overexpression of β-arrestin in HEK293 cells resulted in morphine-induced MOR internalization (Whistler and von Zastrow, 1998). The ability of morphine-activated MOR to recruit β-arrestin without receptor phosphorylation was clearly demonstrated with the fibroblasts isolated from the β-arrestin2 knockout mice (Bohn et al., 2004). These and other results indicate that receptor interaction with β-arrestin is the key for agonist-induced receptor internalization.

The importance of carboxyl tail in the regulation of opioid receptor trafficking has been implicated. The role of the carboxyl tail domain was clearly established by observations that the internalized DOR was trafficked to the lysosomal compartments in the absence of agonist (Tsao and von Zastrow, 2000), there were distinct differences between MOR and its carboxyl tail spliced variants to recycle and resensitize (Koch et al., 1998a, 2001a; Wolf et al., 1999), the MOR/DOR chimeras could be downregulated more rapidly than

the wild type (Afify et al., 1998) and that the chimeras could be internalized by morphine while wild type could not (Whistler et al., 1999). Comparison of the agonist-induced internalization of the wild-type receptors with that of MOR/DOR receptor chimeras in which respective carboxyl tail sequences were exchanged indicated that the DOR carboxyl tail carried the signals for lysosomal targeting, while MOR carboxyl tail sequence contained the recycling signals (Afify et al., 1998; Whistler et al., 1999; Wang et al., 2003). Unlike other GPCRs, such as $\beta_2$-adrenergic receptor that contain consensus PDZ-interacting domains that affect trafficking, the opioid receptors do not contain such motifs in their carboxyl tail sequence. With the exception of KOR, overexpression of EPB50, a PDZ-domain protein involved in the recycling of $\beta_2$-adrenergic receptor, could not affect the agonist-induced endocytosis of opioid receptor (Li et al., 2002). Additional receptor sequences, such as the di-leucine motif within the third intracellular loop of DOR, appear to be involved in receptor intracellular trafficking (Wang et al., 2003). Recent evidence has indicated that a unique recycling signal exists at the MOR carboxyl tail consisting of $Leu^{387}$ and $Leu^{390}$ (Tanowitz and von Zastrow, 2003). Our studies indicate that there are receptor sequences in addition to those involved in the recycling of MOR. Interestingly, all of these recycling signals do not participate in the binding of EPB50 or vacuolar protein-sorting proteins such as Tsg101 (Hislop et al., 2004). Whether such receptor sequences participate in the interaction with proteins, such as GASP, in controlling the receptor sorting after endocytosis remains to be demonstrated (Whistler et al., 2002).

## 4.4 Consequences for Receptor Endocytosis

The ability of an internalized receptor, such as the $\beta_2$-adrenergic receptor, to signal has been demonstrated. However, whether the opioid receptor could signal after endocytosis has not been established clearly. As discussed previously, there are conflicting data on the requirement of opioid receptor endocytosis in MAP kinase activation. Ignatova et al. (Ignatova et al., 1999) and Bohn et al. (Bohn et al., 2000a) have suggested that opioid receptor mediated modulation of MAP kinase activity requires the endocytosis of the receptor, while Whistler and von Zastrow (Whistler and von Zastrow, 1999), Li et al. (Li et al., 1999), Kramer et al. (Kramer and Simon, 2000), and Trapaidze et al. (Trapaidze et al., 2000b) presented data that did not support the requirement of receptor internalization for MAP kinase activation. Since MAP kinases have been implicated in chronic opioid responses (see ❯ Sect. 3.4), the question whether internalized receptor could activate MAPkinase might have significant implication in studying opioid tolerance and dependence development. The internalization and subsequent downregulation of the receptor probably has a minimal role in the development of in vivo tolerance. This is best exemplified by the ability of both chronic etorphine and morphine treatment to elicit tolerance development, while only etorphine could downregulate, morphine alone upregulates MOR (Tao et al., 1987). The noncorrelation between degree of receptor downregulation and tolerance was also observed with chronic fentanyl or clocinnamox treatment (Chan et al., 1997). Similarly, morphine and etorphine could desensitize DOR, while only etorphine could induce the downregulation of the receptor (Law et al., 1983).

It is clear that opioid receptor endocytosis is critical for the receptor to resensitize. Wolf et al. (Wolf et al., 1999) reported that the mutation of $Thr^{394}$ in MOR to Ala results in the rapid internalization and resensitization of receptor. Similar observations were reported with various spliced variants of MOR, in which the rate of desensitization appears to correlate inversely with the resensitization properties of the receptor (Koch et al., 1998b, 2001b). Such observations and others have led to a hypothesis proposed by Whistler and von Zastrow that the ability of various opioid agonists to produce tolerance is dependent on their "RAVE" values (Whistler et al., 1999; Finn and Whistler, 2001; He et al., 2002). In their hypothesis, agonist that induces rapid receptor internalization, e.g., etorphine, would develop less tolerance in animals than agonist such as morphine, which does not produce receptor internalization. Their hypothesis is based on the observation that in the chimeric receptor construct in which MOR carboxyl tail domain was replaced by the similar sequence from DOR, morphine could produce receptor internalization, and that the drug would now induce receptor desensitization (Finn and Whistler, 2001; He et al., 2002). It has also been proposed by this same group that the ability of agonist to internalize the receptor may be related to opioid dependence. Agonist, such as morphine, that does not induce receptor internalization has a greater degree

of "dependence" as measured by the increase in adenylyl cyclase activities. Meanwhile, agonist, such as etorphine, that induces rapid receptor internalization and subsequent resensitization, has a lower degree of "dependence" (Finn and Whistler, 2001). Thus, the action of opioid agonist that normally does not induce receptor internalization, such as morphine, could be affected by agonist that induces receptor internalization, such as DAMGO. Though this is an attractive model, recent and past data do not completely support such a hypothesis. For example, the magnitude of the adenylyl cyclase activities increases, a "hallmark" of dependence, in cells expressing DOR, and does not depend on the agonist used to treat the system chronically but rather on the initial receptor density (Law et al., 1994). Morphine and other partial agonists, e.g. levallorphan in the NG108-15 cells expressing DOR, could elicit similar level of increase in adenylyl cyclase activity as that of agonists such as DADLE (Law et al., 1983). Low doses of DAMGO did not potentiate the morphine-induced receptor desensitization in the locus coeruleus neurons as predicted by the "RAVE" theory (Bailey et al., 2003). Hence, whether agonist-induced receptor endocytosis and trafficking has any role in the opioid tolerance and dependence remains to be demonstrated.

## 4.5 Control of Receptor Activities by Other Methods

### 4.5.1 Ubiquitination

In addition to agonist-induced phosphorylation that could recruit cellular proteins, such as β-arrestin, and alter the receptor signaling process, covalent modification of the GPCR by conjugating polypeptides, such as ubiquitin, has been shown to control the receptor activities. Normally, multiubiquitin chains, i.e., the carboxy-termini glycine of ubiquitin, are linked to the $Lys^{48}$ of the preceding ubiquitin, to the ε-amino group of the lysine residue of the target protein, resulting in the trafficking of the modified proteins by proteasomes. However, there is accumulating evidence to suggest a role for monoubiquitination in the endocytosis of plasma membrane proteins and their trafficking to the lysosomes (Hicke, 2001a, b). In the case of growth hormone receptors, GHRs, polyubiquitination occurs prior to their recruitment to the clathrin-coated pit (van Kerkhof et al., 2001, 2002). Agonist-induced ubiquitination has been reported with the opioid receptor (Chaturvedi et al., 2001), CXCR4 receptor (Marchese and Benovic, 2001), and $\beta_2$-adrenergic receptor (Shenoy et al., 2001). Inclusion of proteasome inhibitors during chronic agonist treatment could prevent the downregulation of these receptors. In most of the receptors studied, the monoubiquitination process appears to participate in the endosomal sorting of the receptor, preventing the recycling of the proteins and shuttling of the molecules to the multivesicular bodies of the late endosomes and subsequent degradation in the lysosome. This is supported by the observations that the ubiquitination of GHR regulates the lysosomal degradation (van Kerkhof et al., 2001, 2002) but not its internalization (Govers et al., 1999). The mutation of the lysine residues within the degradative motif of CXCR4 (Marchese and Benovic, 2001) or the mutation of all 16 cytosolic lysine residues in the $\beta_2$-adrenergic receptor (Shenoy et al., 2001) did not affect the agonist-induced internalization of the receptor, but instead inhibited the degradation of these receptors.

A similar situation exists for the opioid receptor. Ubiquitination of the opioid receptor has been reported to be involved in the proteasome-mediated degradation of the incorrectly folded, deglycosylated receptor (Petaja-Repo et al., 2000, 2001). These receptors could be rescued with lipophilic opioid ligands that serve as chaperones for the receptor trafficking to the plasma membrane (Petaja-Repo et al., 2002). Although confocal microscopic studies suggested the colocalization of internalized receptors with the lysosomal markers (Ko et al., 1999; Gage et al., 2001), Chaturvedi et al. (Chaturvedi et al., 2001) reported that agonist-induced MOR and DOR downregulation were not affected by lysosomal inhibitors but were attenuated by the inhibitors of proteasome inhibitors. On the other hand, mutation of all the intracellular lysine residues did not prevent the lysosomal trafficking of DOR (Tanowitz and von Zastrow, 2002). Whether the ubiquitination of opioid receptor participates in the agonist-induced or newly synthesized receptor intracellular trafficking remains to be investigated.

In addition to direct ubiquitination of the receptor, ubiquitination of *trans*-acting endocytic protein (s) could also affect the agonist-induced receptor activity. In the case of $\beta_2$-adrenergic receptor, the

ubiquitination of β-arrestin, which also serves as the adapter molecule for the E3 ligase, is essential for the endocytosis and the recycling of the receptor (Shenoy and Lefkowitz, 2003). Our initial studies suggested that activation of opioid receptor also results in the ubiquitination of the β-arrestin molecules. Further, the duration of β-arrestin also depends on whether the receptor was recycled after endocytosis. Thus, the ubiquitination of *trans*-acting endocytic proteins could affect the opioid receptor activity.

### 4.5.2 Receptor–Receptor Oligomers

The ability of GPCRs to homo- or heterodimerize has implications in the functions of the receptors. Dimerization of the receptors has been reported for the class A GPCRs such as the adenosine (Ciruela et al., 1995), adrenergic (Hebert et al., 1996; Angers et al., 2000), angiotensin (Monnot et al., 1996), dopamine (Ng et al., 1996), muscarinic (Zheng and Wess, 1999), vasopressin (Hebert and Bouvier, 1998), and opioid (Cvejic and Devi, 1997; Jordan and Devi, 1999; George et al., 2000; McVey et al., 2001a; Gomes et al., 2002) receptors and the class C GPCRs such as the calcium-sensing (Bai et al., 1998), metabotropic glutamate receptors (Kunishima et al., 2000), and γ-amino-n-butyric acid type B ($GABA_B$) receptors (Jones et al., 1998; Kaupmann et al., 1998). The homo- and heterodimerization of these receptors have been demonstrated by coimmunoprecipitation experiments (Cvejic and Devi, 1997; Salim et al., 2002), and recently by fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) techniques (Angers et al., 2000; McVey et al., 2001b; Ramsay et al., 2002). The heterodimerization of the GPCRs was shown to be selective, with formation of heterodimers with some but not all subtypes of the receptors (Jordan and Devi, 1999; Rocheville et al., 2000b). Most importantly, there are functional differences between the monomers and the homo- and heterodimers of the GPCRs. The classic example is the inability of individual $GABA_{B1}$ and $GABA_{B2}$ subunit to form a functional receptor (Jones et al., 1998; Kaupmann et al., 1998). Alteration in the GPCR function or expression was also observed with the heterodimerization of 5HT1B and 5HT1D (Xie et al., 1999b), dopamine D1 and adenosine A1 (Gines et al., 2000), muscarinic M2 and M3 (Sawyer and Ehlert, 1999), dopamine, and somatostatin (Rocheville et al., 2000a) receptors. Heterooligomerization of the GPCRs with other receptor types, such as the ionotropic $GABA_A$ receptor, has been observed, resulting in the alteration in the ion-gating properties of the channels (Liu et al., 2000).

There is accumulating evidence to support the homo- and heterodimerization of the opioid receptors. Oligomerization of the opioid receptors appears to alter or control receptor function. Homodimerization of DOR was reported with immunoprecipitation, and agonist-induced receptor internalization appears to be related to the formation of dimers (Cvejic and Devi, 1997). The agonist-induced change in DOR oligomerization was not observed with BRET experiments (McVey et al., 2001a). KOR is reported to exist as homodimers and could heterodimerize with DOR but not with MOR (Jordan and Devi, 1999). The heterodimerization of DOR and KOR has resulted in a decrease in the affinities of receptor-selective ligands (Jordan and Devi, 1999). The DOR and KOR could also heterodimerize with the $\beta_2$-adrenergic receptor, resulting in an alteration of $\beta_2$-adrenergic receptor functionality (Jordan et al., 2001). The most interesting of the heterodimers is the reported heterodimerization of the MOR and DOR receptors. Using different epitope-tagged receptors, both George et al. and Gomes et al. reported the ability of MOR and DOR to heterodimerize (George et al., 2000; Gomes et al., 2000). Both groups reported a change in functionality of the receptor, with George et al. reporting the heterodimers' function appearing to be insensitive to pertussis toxin pretreatment, implying coupling to G proteins other than Gi/Go (George et al., 2000). Using the DOR knockout mice, the putative heterodimerization of MOR and DOR appears to influence in vivo morphine analgesic activity (Gomes et al., 2004). All these data and others suggest that the opioid receptor activities could be influenced by the oligomerization of the receptors.

### 4.5.3 Receptor–Cellular Protein Complexes

Target inactivation analyses of the agonist receptor size suggests an interaction between opioid receptor and cellular proteins other than G proteins or RGS (Ott et al., 1988). The apparent mol.wt. of the

receptor complex, ~200, could not be accounted for by the size of receptor monomer and the heterotrimeric G protein. There is evidence to suggest the physical association of a ~25kDa protein labeled by $^{125}$I-β-endorphin (Law et al., 2000c). These opioid receptor–cellular proteins interactions could involve consensus motifs found in the receptor sequences. Within the seventh transmembrane domain of the opioid receptor, a highly conserved $NP(X)_{2-3}Y$ motif is present that has been identified as the consensus binding sequence for ARF (Michell et al., 1998). The interaction of rhodopsin with Rho and ARF via this sequence could be the basis for phospholipase D activation (Pronin et al., 1997). Activation of phospholipase D by opioid agonists has been reported (Mangoura and Dawson, 1993) and is ARF dependent (Koch et al., 2003). The direct interaction of phospholipase PLD2 with the receptor appears to participate in this enzyme activation, since association with phospholipase is demonstrated with yeast two-hybrid assays using carboxyl tail domain as the bait (Koch et al., 2003). A consensus sequence for calmodulin binding was also reported to be located at the third intracellular loop. The agonist activation of the receptor resulted in the dissociation of calmodulin–receptor complex (Wang et al., 1999, 2000). The functional significance of the receptor–calmodulin interaction is magnified by the presence of a single nucleotide polymorphism at this interaction domain that could alter basal G protein coupling and calmodulin binding (Wang et al., 2001).

In addition to cellular proteins associating with consensus motifs found in opioid receptor, interaction with cellular proteins that could alter the function of the receptor has been reported. The probable association of the phosphatidylethanolamine binding protein (PBP) with receptor is demonstrated by the isolation of PBP from a morphine affinity column, and the ability of heterologous expression of PBP to enhance opioid receptor and G protein coupling (Kroslak et al., 2001). Other proteins associating with the receptor are those that have roles in receptor trafficking. Using the yeast two-hybrid system, Whistler et al. reported specific interaction between the protein GASP (G-protein-coupled receptor-associated sorting protein) and DOR in its lysosomal trafficking (Whistler et al., 2002). Similar yeast two-hybrid approaches using MOR carboxyl tail domain have identified filamin A (Onoprishvili et al., 2003) or PKC interacting protein (PKCI) (Guang et al., 2004) that modulates the endocytosis and desensitization of the receptor. Thus, by associating with these cellular proteins and other yet to be identified proteins, the function of opioid receptor could be modulated.

## 5 Perspective

Since the successful cloning of the opioid receptors, many of the questions on opioid receptor signaling have been resolved. It is unequivocal that activation of the receptor results in the generation of two separate messengers from the heterotrimeric G proteins, the Gα and Gβγ subunits. With these two messengers, a myriad of effectors activated by the agonists has expanded continuously. The possibilities of coincident signaling and modulation of the signals activities are limitless. In addition to the modulation of the signals by proteins such as RGS, neuron-composition-dependent signaling has also added to the complexity of opioid receptor signaling. An excellent example for this is the differential regulation of adenylyl cyclase subtypes by the opioid receptors. Furthermore, there is accumulating evidence supporting the recruitment of cellular proteins upon opioid receptor activation. The scaffolding of cellular proteins will create microdomains within the proximity of the receptors, thus modulating the signals. An excellent example for protein scaffolding is the ability of two proteins that are known to interact with the receptor, β-arrestin and Gβγ, to recruit cellular proteins. β-arrestin has been reported to serve as adapter molecule for the various kinases, such as c-Src (Luttrell et al., 1999), and the Gβγ subunits have been shown to interact with the PH domain of the PLCβ among other proteins (Rhee, 2001). The recruitment of molecules, such as PLCβ3, and protein kinases to the receptor vicinity would provide a rapid control mechanism for the opioid receptor signaling. Hence, it is critical to identify cellular proteins that participate in opioid receptor signaling, other than the heterotrimeric G proteins. The use of specific receptor domains such as the carboxyl tail domains in the yeast two-hybrid approaches has identified some candidates such as GASP, PLD2, or PKCI that modulate receptor function. However, the yeast two-hybrid approach used thus far is limited to the interaction between the bait (receptor carboxyl tail domain) and the target in the yeast nucleus. The hydrophobic nature of the opioid receptor has prevented such interaction. Thus, the

secondary and tertiary structure of the receptor involved in cellular protein interaction cannot be considered in such assays. Further, the proteins that are recruited to the receptor vicinity after agonist activation could not be addressed. Thus, a better method, such as the proteomic approach, should be used to identify the cellular proteins involved in opioid receptor signaling. With the identification of such proteins, the question of whether they are the basis for receptor oligomerization or the receptor–protein scaffold is the basis for receptor subtypes can then be addressed.

The complexity in the opioid receptor signaling also obstructs the eventual understanding of cellular control and adaptation to the receptor activation. The current model of receptor phosphorylation and β-arrestin recruitment in turning off the signals appears to be applicable to opioid receptor regulation. This model is supported by the β-arrestin-2 knockout mice studies, in which morphine tolerance was blunted. However, it is increasingly clear that factors other than β-arrestin participate in the development of morphine tolerance. An excellent example is that the coadministration of NMDA antagonists with morphine could block tolerance development to the drug (Trujillo and Akil, 1991). In addition, morphine tolerance also was blocked in mice in which either their DOR gene or proenkephalin gene had been knocked out (Zhu et al., 1999; Nitsche et al., 2002). These genetic studies support the earlier observation in which the blockade of DOR activity with receptor-selective antagonist could prevent morphine tolerance (Abdelhamid et al., 1991). If β-arrestin is the only factor involved in morphine tolerance, as suggested with the β-arrestin knockout mice studies, then chronic morphine treatment in the presence of NMDA antagonist or DOR antagonist should result in tolerance development. Thus, regulation of opioid receptor activity during chronic agonist treatment could involve other cellular components, and the regulation of receptor trafficking could have an important role in the chronic action of the drug.

Receptor endocytosis not only serves as a means to reduce the amount of active receptor from the cell surface and as a mechanism for the resensitization of the internalized receptor, but also that the internalized receptor could continue their signaling processes inside the cells. Though contrasting data have been reported on the dependency of receptor internalization and activation of the MAP kinases by opioid agonist, the possibility that the internalized receptor could continue to signal is supported by the intriguing observation on the localization of the receptor with the nuclei fraction (Belcheva et al., 1996). Thus, it is reasonable to hypothesize that the internalized receptor could contribute to the subsequent chronic responses to the drug. Regardless, the proposed RAVE theory based on the ability of agonist to induce receptor internalization could not be the basis for tolerance development (Whistler et al., 1999), because chronic morphine and etorphine treatment could produce tolerance and dependence in rodents, while chronic morphine treatment would upregulate and chronic etorphine treatment downregulate the opioid receptor content in various brain areas (Tao et al., 1987).

In conclusion, activation of opioid receptor has resulted in the generation of two immediate second messengers, Gα and Gβγ. These second messengers form the basis for the ability of opioid receptor to regulate multiple effector systems within a single cell. Further, the ability to regulate multiple effectors provides the opportunity for coincident signaling, amplification of the signals, and the basis for drug efficacy variations among individual effector systems as reported in the literature. Control of opioid receptor signaling is further complicated by the existence of protein scaffolds via adapter proteins such as β-arrestin. The protein scaffolds modulate the receptor signaling and could be the reason for the multiple opioid receptor subtypes reported with pharmacological studies, but not with receptor gene cloning studies. In addition to the possible existence of protein scaffolds, trafficking of the receptor complexes also could determine the amplitude, frequency, and content of the signals. Thus, in order to better understand the mechanism of opioid receptor signaling and its regulation, the composition of the receptor-signaling complex must be delineated. With the advance in proteomic technology, the identity of the complex can now be more easily addressed.

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## References


Abbadie C, Pasternak GW. 2001. Differential in vivo internalization of MOR-1 and MOR-1C by morphine. Neuroreport 12: 3069-3072.

Abdelhamid EE, Sultana M, Portoghese PS, Takemori AE. 1991. Selective blockage of delta opioid receptors prevents the development of morphine tolerance and dependence in mice. J Pharmacol Exp Ther 258: 299-303.

Afify EA, Law PY, Riedl M, Elde R, Loh HH. 1998. Role of carboxyl-terminus of μ- and δ-opioid receptor in agonist-induced desensitization and down-regulation. Mol Brain Res 54: 24-34.

Akil H, Watson SJ, Young E, Lewis ME, Khachaturian H, et al. 1984. Endogenous opioids: biology and function. Annu Rev Neurosci 7: 223-255.

Allouche S, Polastron J, Jauzac P. 1996. The delta-opioid receptor regulates activity of ryanodine receptors in the human neuroblastoma cell line SK-N-BE. J Neurochem 67: 2461-2470.

Ammer H, Christ TE. 2002. Identity of adenylyl cyclase isoform determines the G protein mediating chronic opioid-induced adenylyl cyclase supersensitivity. J Neurochem 83: 818-827.

Ammer H, Shultz R. 1997. Enhanced stimulatory adenylyl cyclase signaling during opioid dependence is associated with a reduction in palmitoylated Gs alpha. Mol Pharmacol 52: 993-999.

Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, et al. 2000. Detection of β2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci USA 97: 3684-3689.

Appleyard SM, Patterson TA, Jin W, Chavkin C. 1997. Agonist-induced phosphorylation of the κ-opioid receptor. J Neurochem 69: 2405-2412.

Arden JR, Segredo V, Wang A, Lameh J, Sadee W. 1995. Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged μ-opioid receptor expressed in HEK293 cells. J Neurochem 65: 1636-1645.

Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, et al. 1992. β-Arrestin2, a novel member of the arrestin/β-arrestin gene family. J Biol Chem 267: 17882-17890.

Avidor-Reiss T, Nevo I, Levy R, Pfeuffer T, Vogel Z. 1996. Chronic opioid treatment induces adenylyl cyclase V superactivation. Involvement of Gbetagamma. J Biol Chem 271: 21309-21315.

Avidor-Reiss T, Nevo I, Saya D, Bayerwitch M, Vogel Z. 1997. Opiate-induced adenylyl cyclase superactivation is isozyme-specific. J Biol Chem 272: 5040-5047.

Bai M, Trivedi S, Brown EM. 1998. Dimerization of the extracellular calcium-sensing receptor (CαR) on the cell surface of CαR-transfected HEK 293 cells. J Biol Chem 272: 23605-23610.

Bailey CP, Couch D, Johnson E, Griffiths K, Kelly E, et al. 2003. μ-Opioid receptor desensitization in mature rat neurons: lack of interaction between DAMGO and morphine. J Neurosci 23: 10515-10520.

Belcheva MM, Haas PD, Tan Y, Heaton VM, Coscia CJ. 2002. The fibroblast growth factor receptor is at the site of convergence between mu-opioid receptor and growth factor signaling pathways in rat C6 glioma cells. J Pharmacol Exp Ther 303: 909-918.

Belcheva MM, Ignatova EG, Young EC, Coscia CJ. 1996. Agonist-induced desensitization and down-regulation of delta opioid receptors alter the levels of their 125I-beta-endorphin cross-linked products in subcellular fractions from NG108-15 cells. Biochem 35: 14818-14824.

Belcheva MM, Szucs M, Wang D, Sadee W, Coscia CJ. 2001. mu-Opioid receptor-mediated ERK activation involves calmodulin-dependent epidermal growth factor receptor transactivation. J Biol Chem 276: 33847-33853.

Belcheva MM, Tan Y, Heaton VM, Clark AL, Coscia CJ. 2003. mu-Opioid transactivation and down-regulation of the epidermal growth factor receptor in astrocytes: implications for mitogen-activated protein kinase signaling. Mol Pharmacol 64: 1391-1401.

Belcheva MM, Vogel Z, Ignatova E, Avidor-Reiss T, Zippel R, et al. 1998. Opioid modulation of extracellular signal-regulated protein kinase activity is ras-dependent and involves Gbetagamma subunits. J Neurochem 70: 635-645.

Belcheva MM, Wong YH, Coscia CJ. 2000. Evidence for transduction of mu but not kappa opioid modulation of extracellular signal-regulated kinase activity by G(z) and G(12) proteins. Cell Signal 12: 481-489.

Bian JS, Zhang WM, Xia Q, Wong TM. 1998. Phospholipase C inhibitors attenuate arrhythmias induced by kappa-receptor stimulation in the isolated rat heart. J Mol Cell Cardiol 30: 2103-2110.

Blume A, Lichtshtein D, Boone G. 1979. Coupling of opiate receptors to adenylate cyclase: requirement for Na+ and GTP. Proc Natl Acad Sci USA 76: 5626-5630.

Bohn LM, Belcheva MM, Coscia CJ. 2000a. Mitogenic signaling via endogenous kappa-opioid receptors in C6 glioma cells: evidence for the involvement of protein kinase C and the mitogen-activated protein kinase signaling cascade. J Neurochem 74: 564-573.

Bohn LM, Belcheva MM, Coscia CJ. 2000b. mu-Opioid agonist inhibition of kappa-opioid receptor-stimulated extracellular signal-regulated kinase phosphorylation is

dynamin-dependent in C6 glioma cells. J Neurochem 74: 574-581.

Bohn LM, Dykstra LA, Lefkowitz RJ, Caron MG, Barak LS. 2004. Relative opioid efficacy is determined by the complements of the G protein-coupled receptor desensitization machinery. Mol Pharmacol 66: 106-112.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. 2000c. mu-Opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature 408: 720-723.

Bohn LM, Lefkowitz RJ, Caron MG. 2002. Differential mechanisms of morphine antinociceptive tolerance revealed in (beta)arrestin-2 knock-out mice. J Neurosci 22: 10494-10500.

Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, et al. 1999. Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 286: 2495-2498.

Bonner GG, Davis P, Stropova D, Ferguson R, Yamamura HI, et al. 1997. Opioid peptides: simultaneous delta agonism and mu antagonism in somatostatin analogues. Peptides 18: 93-100.

Bourinet E, Soong TW, Stea A, Snutch TP. 1996. Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. Proc Natl Acad Sci USA 93: 1486-1491.

Brady AE, Limbird LE. 2002. G protein-coupled receptor interacting proteins: emerging roles in localization and signal transduction. Cell Signal 14: 297-309.

Bunzow JR, Saez C, Mortrud M, Bouvier C, Williams JT, et al. 1994. Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. FEBS Lett 347: 284-288.

Burt AR, Carr IC, Mullaney I, Anderson NG, Milligan G. 1996. Agonist activation of p42 and p44 mitogen-activated protein kinases following expression of the mouse delta opioid receptor in Rat-1 fibroblasts: effects of receptor expression levels and comparisons with G-protein activation. Biochem J 320: 227-235.

Bushell T, Endoh T, Simen AA, Ren D, Bindokas VP, et al. 2002. Molecular components of tolerance to opiates in single hippocampal neurons. Mol Pharmacol 61: 55-64.

Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M. 1999. A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor. Nature 401: 286-290.

Capeyrou R, Riond J, Corbani M, Lepage JF, Bertin B, et al. 1997. Agonist-induced signaling and trafficking of the mu-opioid receptor: role of serine and threonine residues in the third cytoplasmic loop and C-terminal domain. FEBS Lett 415: 200-205.

Carter BD, Medzihradsky F. 1993. Go mediates the coupling of the μ opioid receptor to adenylyl cyclase in cloned neural cells and brains. Proc Natl Acad Sci USA 90: 4062-4066.

Cen B, Xiong Y, Ma L, Pei G. 2001a. Direct and differential interaction of beta-arrestins with the intracellular domains of different opioid receptors. Mol Pharmacol 59: 758-764.

Cen B, Yu Q, Guo J, Wu Y, Ling K, et al. 2001b. Direct binding of beta-arrestins to two distinct intracellular domains of the delta opioid receptor. J Neurochem 76: 1887-1894.

Chakrabarti S, Law PY, Loh HH. 1995a. Neuroblastoma Neuro2A cells stably expressing a cloned μ-opioid receptor: a specific cellular model to study acute and chronic effects of morphine. Mol Brain Res 30: 269-278.

Chakrabarti S, Law PY, Loh HH. 1998a. Distinct differences between morphine– and [δ-Ala2,N-MePhe4,Gly-ol5]enkephalin–mu-opioid receptor complexes demonstrated by cyclic AMP-dependent protein kinase phosphorylation. J Neurochem 71: 231-239.

Chakrabarti S, Oppermann M, Gintzler AR. 2001. Chronic morphine induces the concomitant phosphorylation and altered association of multiple signaling proteins: a novel mechanism for modulating cell signaling. Proc Natl Acad Sci USA 98: 4209-4214.

Chakrabarti S, Prather PL, Yu L, Law PY, Loh HH. 1995b. Expression of the μ-opioid receptor in CHO cells: ability of μ-opioid ligands to promote $^{32}$P-α-azidoanilido GTP labeling of multiple G protein α-subunits. J Neurochem 64: 2534-2543.

Chakrabarti SR, Rivera M, Yan S-Z, Tang W-J, Gintzler AR. 1998c. Chronic morphine augments G/Gs stimulation of adenylyl cyclase: relevance to opioid tolerance. Mol Pharmacol 54: 655-662.

Chakrabarti S, Wang L, Tang W-J, Gintzler AR. 1998b. Chronic morphine augments adenylyl cyclase phosphorylation: relevance to altered signaling during tolerance/dependence. Mol Pharmacol 54: 949-953.

Chalecka-Franaszek E, Weems HB, Crowder AT, Cox BM, Cote TE. 2000. Immunoprecipitation of high-affinity, guanine nucleotide-sensitive, solubilized mu-opioid receptors from rat brain: coimmunoprecipitation of the G proteins G (alpha o), G(alpha i1), and G(alpha i3). J Neurochem 74: 1068-1078.

Chan JSC, Chiu TT, Wong YH. 1995. Activation of type II adenylyl cyclase by the cloned μ-opioid receptor: coupling to multiple G proteins. J Neurochem 65: 2682-2689.

Chan KW, Duttory A, Yoburn BC. 1997. Magnitude of tolerance to fentanyl is independent of mu-opioid receptor density. Eur J Pharmacol 319: 225-228.

Chan JSC, Lee JW, Ho MK, Wong YH. 2000. Preactivation permits subsequent stimulation of phospholipase C by G (i)-coupled receptors. Mol Pharmacol 57: 700-708.

Chang KJ, Cooper BR, Hazum E, Cuatrecasas P. 1979. Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptides. Mol Pharmacol 16: 91-104.

Chang KJ, Eckel RW, Blanchard SG. 1982. Opioid peptides induce reduction of enkephalin receptors in cultured neuroblastoma cells. Nature 296: 446-448.

Chang KJ, Hazum E, Killian A, Cuatrecasas P. 1981. Interactions of ligands with morphine and enkephalin receptors are differentially affected by guanine nucleotide. Mol Pharmacol 20: 1-7.

Chaturvedi K, Bandari P, Chinen N, Howells RD. 2001. Proteasome involvement in agonist-induced down-regulation of mu and delta opioid receptors. J Biol Chem 276: 12345-12355.

Chaturvedi K, Christoffers KH, Singh K, Howells RD. 2000. Structure and regulation of opioid receptors. Biopolymers 55: 334-346.

Chatzaki E, Makrigiannakis A, Margioris AN, Kouimtzoglou E, Gravanis A. 2001. The Fas/FasL apoptotic pathway is involved in kappa-opioid-induced apoptosis of human endometrial stromal cells. Mol Hum Reprod 7: 867-874.

Chen J, Devivo M, Dingus J, Harry A, Li J, et al. 1995. A region of adenylyl cyclase 2 critical for regulation by G protein βγ subunits. Science 268: 1166-1169.

Chen Y, Fan Y, Liu J, Mestek A, Tian M, et al. 1994. Molecular cloning, tissue distribution and chromosomal localization of a novel member of the opioid receptor gene family. FEBS Lett 347: 279-283.

Chen X, Marrero HG, Murphy R, Lin YJ, Freedman JE. 2000. Altered gating of opiate receptor-modulated K+ channels on amygdala neurons of morphine-dependent rats. Proc Natl Acad Sci USA 97: 14692-14696.

Chen Y, Mestek A, Liu J, Hurley JA, Yu L. 1993a. Molecular cloning and functional expression of a μ-opioid receptor from rat brain. Mol Pharmacol 44: 8-12.

Chen Y, Mestek A, Liu J, Yu L. 1993b. Molecular cloning of a rat κ-opioid receptor reveals sequence similarities to the μ and δ opioid receptors. Biochem J 295: 625-628.

Ciruela F, Casado V, Mallo J, Canela EE, Lluis C, et al. 1995. Immunological identification of A1 adenosine receptors in brain cortex. J Neurosci Res 42: 818-828.

Claing A, Chen W, Miller WE, Vitale N, Moss J, et al. 2001. Beta-arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis. J Biol Chem 276: 42509-42513.

Collier HO, Francis DL. 1975. Morphine abstinence is associated with increased brain cyclic AMP. Nature 255: 159-162.

Collier HO, Roy AC. 1974. Morphine-like drugs inhibit the stimulation of E prostaglandins of cyclic AMP formation by rat brain homogenate. Nature 248: 24-27.

Connor M, Henderson G. 1996. Delta- and mu-opioid receptor mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells. Br J Pharmacol 117: 333-340.

Connor M, Schuller A, Pintar JE, Christie MJ. 1999. μ-Opioid receptor modulation of calcium channel current in periaqueductal grey neurons from C57B16/J mice and mutant mice lacking MOR-1. Br J Pharmacol 126: 1553-1558.

Crain SM, Shen KF. 1996. Modulatory effects of Gσ-coupled excitatory opioid receptor functions on opioid analgesia, tolerance, and dependence. Neurochem Res 21: 1347-1351.

Crain SM, Shen KF. 1998a. Modulation of opioid analgesia, tolerance and dependence by Gσ-coupled, GM1 ganglioside-regulated opioid receptor functions. Trends Pharmacol Sci 19: 358-365.

Crain SM, Shen KF. 1998b. GM1 ganglioside-induced modulation of opioid receptor-mediated functions. Ann NY Acad Sci 845: 106-125.

Creese I, Snyder SH. 1975. Receptor binding and pharmacological activity of opiates in the guinea-pig intestine. J Pharmacol Exp Ther 194: 205-219.

Cruciani RA, Dvorkin B, Morris SA, Crain SM, Makman MH. 1993. Direct coupling of opioid receptors to both stimulatory and inhibitory guanine nucleotide-binding proteins in F-11 neuroblastoma-sensory neuron hybrid cells. Proc Natl Acad Sci USA 90: 3019-3023.

Cvejic S, Devi LA. 1997. Dimerization of the delta opioid receptor: implication for a role in receptor internalization. J Biol Chem 272: 26959-26964.

Cvejic S, Trapaidze N, Cyr C, Devi LA. 1996. Thr353, located within the COOH-terminal tail of the delta opiate receptor, is involved in receptor down-regulation. J Biol Chem 271: 4073-4076.

Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, et al. 1998. Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J Biol Chem 273: 685-688.

De Fea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O, et al. 2000. The proliferative and antiapoptotic effects of substance P are facilitated by formation of a β-arrestin-dependent scaffolding complex. Proc Natl Acad Sci USA 97: 11086-11091.

De Graff J, Gagnon AW, Benovic JL, Orsini MJ. 1999. Role of arrestins in endocytosis and signaling of β2-adrenergic receptor subtypes. J Biol Chem 274: 11253-11259.

Deng HB, Yu Y, Pak Y, O'Dowd BF, George SR, et al. 2000. Role for the C-terminus in agonist-induced mu opioid receptor phosphorylation and desensitization. Biochem 39: 5492-5499.

Diao CT, Li L, Lau SY, Wong TM, Wong NS. 2000. Kappa-opioid receptor potentiates apoptosis via a phospholipase C pathway in the CNE2 human epithelial tumor cell line. Biochim Biophys Acta 1499 (1–2): 49-62.

Eisinger DA, Schultz R. 2004. Extracellular signal-regulated kinase/mitogen-activated protein kinases block internalization of delta-opioid receptors. J Pharmacol Exp Ther 309: 776-785.

Eitan S, Bryant CD, Saliminejad N, Yang YC, Vojdani E, et al. 2003. Brain region-specific mechanisms for acute morphine-induced mitogen-activated protein kinase modulation and distinct patterns of activation during analgesic tolerance and locomotor sensitization. J Neurosci 23: 8360-8369.

El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang C-Y, Law PY, et al. 2001. Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates μ-opioid receptor internalization. J Biol Chem 276: 12774-12780.

El Kouhen R, Kouhen OM, Law PY, Loh HH. 1999. The absence of a direct correlation between the loss of [D-Ala2,MePhe4,Gly5-ol]enkephalin inhibition of adenylyl cyclase activity and agonist-induced mu-opioid receptor phosphorylation. J Biol Chem 274: 9207-9215.

Elenko E, Fischer T, Niesman I, Harding T, McQuistan T, et al. 2003. Spatial regulation of Galphai protein signaling in clathrin-coated membrane microdomains containing GAIP. Mol Pharmacol 64: 11-20.

Evans CJ, Keith DE, Morrison H, Magendzo K, Edwards RH. 1992. Cloning of a delta opioid receptor by functional expression. Science 258: 1952-1955.

Fan GH, Zhang WB, Yao CP, Pei G. 1997. Modulation by calcium/calmodulin-dependent protein kinase II of functional response of delta opioid receptor in neuroblastoma × glioma hybrid (NG108-15) cells. Neuropharmacol 36: 1763-1769.

Fan GH, Zhou TH, Zhang WB, Pei G. 1998. Suppression of phospholipase C blocks Gi-mediated inhibition of adenylyl cyclase activity. Eur J Pharmacol 341: 317-322.

Federman AD, Conklin BR, Schrader KA, Reed RR, Bourne HR. 1992. Hormonal stimulation of adenylyl cyclase through Gi-protein βγ subunits. Nature 356: 159-161.

Finn AK, Whistler JL. 2001. Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. Neuron 32: 829-839 [comment].

Fryer RM, Hsu AK, Gross GJ. 2001a. ERK and p38 MAP kinase activation are components of opioid-induced delayed cardioprotection. Basic Res Cardiol 96: 136-142.

Fryer RM, Patel HH, Hsu AK, Gross GJ. 2001b. Stress-activated protein kinase phosphorylation during cardioprotection in the ischemic myocardium. Am J Physiol Heart Circ Physiol 281: H1184-H1192.

Fryer RM, Pratt PF, Hsu AK, Gross GJ. 2001c. Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. J Pharmacol Exp Ther 296: 642-649.

Fukuda K, Kato S, Morikawa H, Shoda T, Mori K. 1996. Functional coupling of the delta-, mu-, and kappa-opioid receptors to mitogen-activated protein kinase and arachidonate release in Chinese hamster ovary cells. J Neurochem 67: 1309-1316.

Gage RM, Kim KA, Cao TT, von Zastrow M. 2001. A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. J Biol Chem 276: 44712-44720.

Garcia DE, Li B, Garcia-Ferreiro RE, Hernandez-Ochoa EO, Yan K, et al. 1998. G-protein β-subunit specificity in the fast membrane-delimited inhibition of $Ca^{2+}$ channels. J Neurosci 18: 9163-9170.

Garrington TP, Johnson GL. 1999. Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol 11: 211-218.

George SR, Fan T, Xie Z, Tse R, Tam V, et al. 2000. Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. J Biol Chem 275: 26128-26135.

Ghazarossian VE, Chavkin C, Goldstein A. 1980. A specific radioimmunoassay for the novel opioid peptide dynorphin. Life Sci 27: 75-86.

Gines S, Hillion J, Torvinen M, Le Crom S, Casado V, et al. 2000. Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. Proc Natl Acad Sci USA 97: 8606-8611.

Goldstein A, Ghazarossian VE. 1980. Immunoreactive dynorphin in pituitary and brain. Proc Natl Acad Sci USA 77: 6207-6210.

Goldstein A, Lowney LI, Pal BK. 1971. Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. Proc Natl Acad Sci USA 68: 1742-1747.

Gomes I, Filipovska J, Jordan BA, Devi LA. 2002. Oligomerization of opioid receptors. Methods Enzymol 27: 358-365.

Gomes T, Gupta A, Filipovska J, Szeto HH, Pintar JE, et al. 2004. A role for heterodimerization of μ and δ opiate receptors in enhancing morphine analgesia. Proc Natl Acad Sci USA 101: 5135-5139.

Gomes I, Jordan A, Gupta A, Trapaidze N, Nagy V, et al. 2000. Heterodimerization of μ and δ opioid receptors: a role in opiate synergy. J Neurosci 20: RC110 111-115.

Goodman OB, Krupnick JK, Santini F, Gurevich VV, Penn RB, et al. 1996. β-Arrestin acts a clathrin adaptor in endocytosis of the $\beta_2$-adrenergic receptor. Nature 383: 447-450.

Goswami R, Dawson SA, Dawson G. 2000. Multiple polyphosphoinositide pathways regulate apoptotic signalling in a dorsal root ganglion derived cell line. J Neurosci Res 59: 136-144.

Govers R, ten Broeker T, van Kerkhof P, Schwartz AL, Strous GJ. 1999. Identification of a novel ubiquitin conjugation

motif, required for ligand-induced internalization of the growth hormone receptor. EMBO J 18: 28-36.

Grudt TJ, Williams JT. 1993. κ-Opioid receptors also increase potassium conductance. Neurobiol 90: 11429-11432.

Guang W, Wang H, Su T, Weinstein IB, Wang JB. 2004. Role of mPKCI, a novel μ-opioid receptor interactive protein, in receptor desensitization, phosphorylation, and morphine-induced analgesia. Mol Pharmacol 66: 1285-1292.

Guo J, Wu Y, Zhang W, Zhao J, Devi LA, et al. 2000. Identification of G protein-coupled receptor kinase 2 phosphorylation sites responsible for agonist-stimulated delta-opioid receptor phosphorylation. Mol Pharmacol 58: 1050-1056.

Hall RA, Lefkowitz RJ. 2002. Regulation of G protein-coupled receptor signaling by scaffold proteins. Circ Res 91: 672-680.

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, et al. 1998. The $\beta_2$-adrenergic receptor interacts with the $Na^+/H^+$-exchanger regulatory factor to control $Na^+/H^+$ exchange. Nature 392: 626-630.

Han SH, Cho YW, Kim CJ, Min BI, Rhee JS, et al. 1999. mu-Opioid agonist-induced activation of G-protein-coupled inwardly rectifying potassium current in rat periaqueductal gray neurons. Neurosci 90: 209-219.

Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, et al. 1996. Purification, molecular cloning, and expression of the mammalian sigma1-binding site. Proc Natl Acad Sci USA 93: 8072-8077.

Harrison C, Rowbotham DJ, Grandy DK, Lambert DG. 2000. Endomorphin-1 induced desensitization and down-regulation of the recombinant mu-opioid receptor. Br J Pharmacol 131: 1220-1226.

Hasbi A, Polastron J, Allouche S, Stanasila L, Massotte D, et al. 1998. Desensitization of the delta-opioid receptor correlates with its phosphorylation in SK-N-BE cells: involvement of a G protein-coupled receptor kinase. J Neurochem 70: 2129-2138.

Hauser KF, Mangoura D. 1998. Diversity of the endogenous opioid system in development. Novel signal transduction translates multiple extracellular signals into neural cell growth and differentiation. Perspect Dev Neurobiol 5: 437-449.

Hawes BE, Fried S, Yao X, Weig B, Graziano MP. 1998. Nociceptin (ORL-1) and mu-opioid receptors mediate mitogen-activated protein kinase activation in CHO cells through a Gi-coupled signaling pathway: evidence for distinct mechanisms of agonist-mediated desensitization. J Neurochem 71: 1024-1033.

He L, Fong J, von Zastrow M, Whistler JL. 2002. Regulation of opioid receptor trafficking and morphine tolerance by receptor oligomerization. Cell 108: 271-282.

Hebert TE, Bouvier M. 1998. Structural and functional aspects of G protein-coupled receptor oligomerization. Biochem Cell Biol 76: 1-11.

Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, et al. 1996. A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. J Biol Chem 271: 16384-16392.

Hedin KE, Bell MP, Kalli KR, Huntoon CJ, Sharp BM, et al. 1997. δ-Opioid receptors expressed by Jurkat T cells enhance 1L-2 secretion by increasing AP-1 complexes and activity of the NF-AT/AP-1-binding promoter element. J Immunol 159: 5431-5440.

Henry DJ, Grandy DK, Lester HA, Davidson N, Chavkin C. 1995. κ-Opioid receptors couple to inwardly rectifying potassium channels when coexpressed by *Xenopus oocytes*. Mol Pharmacol 47: 551-557.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, et al. 1996. Modulation of $Ca^{2+}$ channels by G-proteins βγ subunits. Nature 380: 258-262.

Herlitze S, Hockerman GH, Scheuer T, Catterall WA. 1997. Molecular determinants of inactivation and G protein modulation in the intracellular loop connecting domains I and II of the calcium channel α1A subunit. Proc Natl Acad Sci USA 94: 1512-1516.

Hescheler J, Rosenthal W, Trautwein W, Schultz G. 1987. The GTP-binding protein Go, regulates neuronal calcium channels. Nature 325: 445-446.

Hicke L. 2001a. Protein regulation by monoubiquitin. Nat Rev/Mol Cell Biol 2: 195-201.

Hicke L. 2001b. A new ticket for entry into budding vesicles – ubiquitin. Cell 106: 527-530.

Hislop JN, Marley A, von Zastrow M. 2004. Role of mammalian vacuolar protein-sorting proteins in endocytic trafficking of a non-ubiquitinated G protein-coupled receptor to lysosomes. J Biol Chem 279: 22522-22531.

Ho MK, New DC, Wong YH. 2002. Co-expressions of different opioid receptor types differentially modulate their signaling via G(16). Neurosignals 11: 115-122.

Ho MK, Yung LY, Chan JS, Chan JH, Wong CS, et al. 2001. Galpha(14) links a variety of G(i)- and G(s)-coupled receptors to the stimulation of phospholipase C. Br J Pharmacol 132: 1431-1440.

Hu LA, Tang Y, Miller WE, Cong M, Lau AG, et al. 2000. Beta 1-adrenergic receptor association with PSD-95. Inhibition of receptor internalization and facilitation of beta 1-adrenergic receptor interaction with *N*-methyl-D-aspartate receptors. J Biol Chem 275: 38659-38666.

Huang C-L, Feng S, Hilgemann DW. 1998. Direct activation of inward rectifier potassium channels by PIP2 and its stabilization by Gβγ. Nature 391: 803-806.

Huang C-L, Jan YN, Jan LY. 1997. Binding of the G protein βγ subunit to multiple regions of G protein-gated inward-rectifying K+ channels. FEBS Lett 405: 291-298.

Huang C-L, Slesinger P, Casey P, Jan Y, Jan L. 1995. Evidence that direct binding of G-beta/gamma to the GIRK1 G-protein-gated inwardly rectifying K+ channel is important for channel activation. Neuron 15: 1132-1143.

Huang P, Steplock D, Weinman EJ, Hall RA, Ding Z, et al. 2004. Kappa opioid receptor interacts with Na(+)/H(+)-exchanger regulatory factor-1/ezrin-radixin-moesin-binding phosphoprotein-50 (NHERF-1/EBP50) to stimulate Na(+)/H(+) exchange independent of G(i)/G(o) proteins. J Biol Chem 279: 25002-25009.

Hughes J, Smith TW, Kosterlitz HW, Fothergill LA, Morgan BA, et al. 1975. Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 258: 577-580.

Iglesias M, Segura MF, Comella JX, Olmos G. 2003. mu-Opioid receptor activation prevents apoptosis following serum withdrawal in differentiated SH-SY5Y cells and cortical neurons via phosphatidylinositol 3-kinase. Neuropharmacol 44: 482-492.

Ignatova EG, Belcheva MM, Bohn LM, Neuman MC, Coscia CJ. 1999. Requirement of receptor internalization for opioid stimulation of mitogen-activated protein kinase: biochemical and immunofluorescence confocal microscopic evidence. J Neurosci 19: 56-63.

Ikeda SF. 1996. Voltage-dependent modulation of N-type calcium channels by G protein βγ subunits. Nature 380: 225-258.

Ikeda K, Kobayashi T, Kumanishi T, Niki H, Yano R. 2000. Involvement of G-protein-activated inwardly rectifying K (GIRK) channels in opioid-induced analgesia. Neurosci Res 38: 113-116.

Janecka A, Fichna J, Janecki T. 2004. Opioid receptors and their ligands. Curr Top Med Chem 4: 1-17.

Jiang M, Gold MS, Boulay G, Spicher K, Peyton M, et al. 1998. Multiple neurological abnormalities in mice deficient in the G protein Go. Proc Natl Acad Sci USA 95: 3269-3274.

Jin W, Lee N, Loh H, Thayer S. 1993a. Dual excitatory and inhibitory effects of opioids on intracellular calcium in neuroblastoma × glioma hybrid NG108-15 cells. Mol Pharmacol 42: 1083-1089.

Jin W, Lee N, Loh H, Thayer S. 1993b. Opioid-induced inhibition of voltage-gated calcium channels parallels expression of omega-conotoxin-sensitive channel subtype during differentiation of NG108-15 cells. Brain Res 607: 17-22.

Jones KA, Borowsky B, Tamm JA, Craig DA, Durkin MM, et al. 1998. GABAB receptors function as a heteromeric assembly of the subunits GABABR1 and GABABR2. Nature 396: 674-679.

Jordan BA, Devi LA. 1999. G-protein-coupled receptor heterodimerization modulates receptor function. Nature 399: 697-700.

Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA. 2001. Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proc Natl Acad Sci USA 98: 343-348.

Joshi S, Lee JW, Wong YH. 1999. Stimulation of phospholipase C by the cloned mu, delta and kappa opioid receptors via chimeric G alpha(q) mutants. Eur J Neurosci 11: 383-388.

Kaufman DL, Xia YR, Kieth Jr, DE Newman D, Evans CJ, et al. 1994. Localization of the δ-opioid receptor gene to mouse chromosome 4 by linkage analysis. Genomics 19: 405-406.

Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, et al. 1998. GABA (β)-receptor subtypes assemble into functional heteromeric complexes. Nature 396: 683-687.

Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, et al. 1996. Morphine activates opioid receptors without causing their rapid internalization. J Biol Chem 271: 19021-19024.

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG. 1992. The δ-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 89: 12048-12052.

Kim CJ, Rhee JS, Akaike N. 1997. Modulation of high-voltage activated $Ca^{2+}$ channels in the rat periaqueductal gray neurons by mu-type opioid agonist. J Neurophysiol 77: 1418-1424.

Kleuss C, Hescheler J, Ewel C, Rosenthal W, Schultz G, et al. 1991. Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature 353: 43-48.

Kleuss C, Scherubl H, Hescheler J, Schultz G, Wittig B. 1992. Different β subunits determine G protein interaction with transmembrane receptors. Nature 358: 424-426.

Ko JL, Arvidsson U, Williams FG, Law PY, Elde R, et al. 1999. Visualization of time-dependent redistribution of δ-opioid receptors in neuronal cells during prolonged agonist exposure. Mol Brain Res 69: 171-185.

Koch T, Brandenburg LO, Schulz S, Liang Y, Klein J, et al. 2003. ADP-ribosylation factor-dependent phospholipase D2 activation is required for agonist-induced mu-opioid receptor endocytosis. J Biol Chem 278: 9979-9985.

Koch T, Kroslak T, Averbeck M, Mayer P, Schroder H, et al. 2000. Allelic variation S268P of the human mu-opioid receptor affects both desensitization and G protein coupling. Mol Pharmacol 58: 328-334.

Koch T, Kroslak T, Mayer P, Raulf E, Hollt V. 1997. Site mutation in the rat mu-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein

kinase II in agonist-mediated desensitization. J Neurochem 69: 1767-1770.

Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, et al. 2001a. C-terminal splice variants of the mouse mu-opioid receptor differ in morphine-induced internalization and receptor resensitization. J Biol Chem 276: 31408-31414.

Koch T, Schulz S, Pfeiffer M, Klutzny M, Schroder H, et al. 2001b. C-terminal splice variants of the mouse μ-opioid receptor differ in morphine-induced internalization and receptor resensitization. J Biol Chem 276: 31408-31414.

Koch T, Schulz S, Schroder H, Wolf R, Raulf E, et al. 1998a. Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization. J Biol Chem 273: 13652-13657.

Koch T, Schulz S, Schroder H, Wolf R, Raulf E, et al. 1998b. Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization. J Biol Chem 273: 13652-13657.

Kovoor A, Nappey V, Kieffer BL, Chavkin C. 1997. Mu and delta opioid receptors are differentially desensitized by the coexpression of beta-adrenergic receptor kinase 2 and beta-arrestin 2 in *Xenopus oocytes*. J Biol Chem 272: 27605-27611.

Kozak CA, Filie J, Adamson MC, Chen Y, Yu L. 1994. Murine chromosomal location of mu and kappa opioid receptor genes. Genomics 21: 659-661.

Kramer HK, Andria ML, Esposito DH, Simon EJ. 2000. Tyrosine phosphorylation of the delta-opioid receptor. Evidence for its role in mitogen-activated protein kinase activation and receptor internalization. Biochem Pharmacol 60: 781-792.

Kramer HK, Onoprishvili I, Andria ML, Hanna K, Sheinkman K, et al. 2002. Delta opioid activation of the mitogen-activated protein kinase cascade does not require transphosphorylation of receptor tyrosine kinases. BMC Pharmacol 2: 5-15.

Kramer HK, Simon EJ. 1999a. Role of protein kinase C (PKC) in agonist-induced mu-opioid receptor down-regulation: I. PKC translocation to the membrane of SH-SY5Y neuroblastoma cells is induced by mu-opioid agonists. J Neurochem 72: 585-593.

Kramer HK, Simon EJ. 1999b. Role of protein kinase C (PKC) in agonist-induced mu-opioid receptor down-regulation: II. Activation and involvement of the alpha, epsilon, and zeta isoforms of PKC. J Neurochem 72: 594-604.

Kramer HK, Simon EJ. 2000. mu and delta-opioid receptor agonists induce mitogen-activated protein kinase (MAPK) activation in the absence of receptor internalization. Neuropharmacol 39: 1707-1719.

Kroslak T, Koch T, Kahl E, Hollt V. 2001. Human phosphatidylethanolamine-binding protein facilitates heterotrimeric G protein-dependent signaling. J Biol Chem 276: 39772-39778.

Krupnick JG, Benovic JL. 1998. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu Rev Pharmacol Toxicol 38: 289-319.

Kunishima N, Shimada Y, Tsuhi Y, Sato T, Yamamoto M, et al. 2000. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature 407: 971-977.

Lai HWL, Minami M, Satoh M, Wong YH. 1995. Gz coupling to the rat κ-opioid receptor. FEBS Lett 360: 97-99.

Laporte SA, Oakley RH, Holt JA, Barak LS, Caron MG. 2000. The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. J Biol Chem 275: 23120-23126.

Laporte SA, Miller WE, Kim KM, Caron MG. 2002. beta-Arrestin/AP-2 interaction in G protein-coupled receptor internalization: identification of a beta-arrestin binging site in beta 2-adaptin. J Biol Chem 277: 9247-9254.

Law PY, Erickson LJ, El-Kouhen R, Dicker L, Solberg J, et al. 2000a. Receptor density and recycling affect the rate of agonist-induced desensitization of mu-opioid receptor. Mol Pharmacol 58: 388-398.

Law PY, Hom DS, Loh HH. 1982a. Loss of opiate receptor activity in neuroblastoma × glioma NG108-15 hybrid cells after chronic opiate treatment: a multiple step process. Mol Pharmacol 22: 1-4.

Law PY, Hom DS, Loh HH. 1982b. Loss of opiate receptor activity in neuroblastoma × glioma NG108-15 hybrid cells after chronic opiate treatment: a multiple step process. Mol Pharmacol 22: 1-4.

Law P-Y, Hom D, Loh H. 1983. Opioid receptor down-regulation and desensitization in neuroblastoma × glioma NG108-15 hybrid cells are two separate cellular adaption processes. Mol Pharmacol 24: 413-424.

Law PY, Hom DS, Loh HH. 1984. Down-regulation of opiate receptor in neuroblastoma × glioma NG108-15 hybrid cells: chloroquine promotes accumulation of triated enkephalin in the lysosomes. J Biol Chem 259: 4096-4104.

Law PY, Kouhen OM, Solberg J, Wang W, Erickson LJ, et al. 2000b. Deltorphin II-induced rapid desensitization of delta-opioid receptor requires both phosphorylation and internalization of the receptor. J Biol Chem 275: 32057-32065.

Law PY, Loh HH. 1978. $^{3}$H-Leu$^{5}$-enkephalin specific binding to synaptic membrane-comparison with $^{3}$H-dihydromorphine and $^{3}$H-naloxone. Res Commun Chem Pathol Pharmacol 21: 409-434.

Law PY, McGinn TM, Campbell KM, Erickson LE, Loh HH. 1997. Agonist activation of δ-opioid receptor but not μ-opioid receptor potentiates fetal calf serum or tyrosine kinase receptor-mediated cell proliferation in a cell-line specific manner. Mol Pharmacol 51: 152-160.

Law P-Y, McGinn T, Wick M, Erickson L, Evans C, et al. 1994. Analysis of delta-opioid receptor activities stably expressed in CHO cell lines: function of receptor density. J Pharmacol Exp Ther 271: 1686-1694.

Law SF, Reisine T. 1997. Changes in the association of G protein subunits with the cloned mouse δ opioid receptor on agonist stimulation. J Pharmacol Exp Ther 281: 1476-1486.

Law PY, Tine SJ, McLeod LA, Loh HH. 2000c. Association of a lower molecular weight protein to the μ-opioid receptor demonstrated by $^{125}$β-endorphin cross-linking studies. J Neurochem 75: 164-173.

Law PY, Wong YH, Loh HH. 1999. Mutational analysis of the structure and function of opioid receptors. Biopolymers 51: 440-455.

Law PY, Wong YH, Loh HH. 2000d. Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 40: 389-430.

Law PY, Wong YH, Loh HH. 2000e. Molecular mechanisms and regulation of opioid receptor signaling. Annu Rev Pharmacol Toxicol 40: 389-430.

Lee JW, Joshi S, Chan JS, Wong YH. 1998. Differential coupling of mu-, delta-, and kappa-opioid receptors to G alpha16-mediated stimulation of phospholipase C. J Neurochem 70: 2203-2211.

Lefkowitz RJ. 1998. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J Biol Chem 273: 18677-18680.

Lesscher HM, Burbach JP, van Ree JM, Gerrits MA. 2003. ERK1/2 activation in rat ventral tegmental area by the mu-opioid agonist fentanyl: an in vitro study. Neurosci 116: 139-144.

Li LY, Chang KJ. 1996. The stimulatory effect of opioids on mitogen-activated protein kinase in Chinese hamster ovary cells transfected to express μ-opioid receptors. Mol Pharmacol 50: 599-602.

Li JG, Chen C, Liu-Chen LY. 2002. Ezrin-radixin-moesin-binding phosphoprotein-50/Na+/H+ exchanger regulatory factor (EBP50/NHERF) blocks U50, 488H-induced down-regulation of the human kappa opioid receptor by enhancing its recycling rate. J Biol Chem 277: 27545-27552.

Li JG, Luo LY, Krupnick JG, Benovic JL, Liu-Chen LY. 1999. U50,488H-induced internalization of the human kappa opioid receptor involves a beta-arrestin- and dynamin-dependent mechanism. Kappa receptor internalization is not required for mitogen-activated protein kinase activation. J Biol Chem 274: 12087-12094.

Li CH, Yamashiro D, Chung D, Doneen BA. 1977. Isolation, structure, synthesis and morphine-like activity of beta-endorphin from human pituitary glands. Ann NY Acad Sci 297: 158-166.

Lim NF, Dascal N, Labarca C, Davidson N, Lester HA. 1995. A G protein-gated $K^+$ channel is activated via $b_2$-adrenergic receptors and Gβγ subunits in *Xenopus oocytes*. J Gen Physiol 105: 421-439.

Liu F, Wang Q, Pristupa ZB, Yu XM, Wang YT, et al. 2000. Direct protein–protein coupling enables cross-talk between dopamine D5 and γ-aminobutyric acid A receptors. Nature 403: 274-280.

Loh HH, Tseng LF, Wei E, Li CH. 1976. Beta-endorphin is a potent analgesic agent. Proc Natl Acad Sci USA 73: 2895-2898.

Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. 1990. β-Arrestin: a protein that regulates β-adrenergic receptor function. Science 248: 1547-1550.

Lord JAH, Waterfield AA, Hughes J, Kosterlitz HW. 1977. Endogenous opioid peptides: multiple agonists and receptors. Nature 276: 495-499.

Lou L, Zhou T, Wang P, Pei G. 1999. Modulation of Ca2+/calmodulin-dependent protein kinase II activity by acute and chronic morphine administration in rat hippocampus: differential regulation of α and β isoforms. Mol Pharmacol 55: 557-563

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, et al. 1999. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283: 655-661 [comment].

Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, et al. 2001. Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. Proc Natl Acad Sci USA 98: 2449-2454.

Ma GH, Miller RF, Kuznetsov A, Philipson LH. 1995. κ-Opioid receptor activates an inwardly rectifying K+ channel by a G protein-linked mechanism: coexpression in *Xenopus oocytes*. Mol Pharmacol 47: 1035-1040.

Maestri-El Kouhen O, Wang G, Solberg J, Erickson LJ, Law PY, et al. 2000. Hierarchical phosphorylation of δ-opioid receptor regulates agonist-induced receptor desensitization and internalization. J Biol Chem 275: 36659-36664.

Makman MH, Dvorkin B, Crain SM. 1988. Modulation of adenylate cyclase activity of mouse spinal cord-ganglion explants by opioids, serotonin and pertussis toxin. Brain Res 445: 303-313.

Mangoura D, Dawson G. 1993. Opioid peptides activate phospholipase D and protein kinase C-epsilon in chicken embryo neuron cultures. Proc Natl Acad Sci USA 90: 2915-2919.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ. 1988. Anatomy of CNS opioid receptors. Trends Neurosci 11: 308-314.

Marchese AB, Benovic JL. 2001. Agonist-promoted ubiquitination of G protein-coupled receptor CXCR4 mediates lysosomal sorting. J Biol Chem 276: 45509-45512.

Marker CL, Cintora SC, Roman MI, Stoffel M, Wickman K. 2002. Hyperalgesia and blunted morphine analgesia in G protein-gated potassium channel subunit knockout mice. Neuroreport 13: 2509-2513.

Marker CL, Stoffel M, Wickman K. 2004. Spinal G-protein-gated K+ channels formed by GIRK1 and GIRK2 subunits modulate thermal nociception and contribute to morphine analgesia. J Neurosci 24: 2806-2812.

Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE. 1976. The effects of morphine- and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. J Pharmacol Exp Ther 197: 517-532.

McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, et al. 2000. Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. Science 290: 1574-1577 [comment].

McDonald PH, Cote NL, Lin FT, Premont RT, Pitcher JA, et al. 1999. Identification of NSF as a beta-arrestin1-binding protein. Implications for beta2-adrenergic receptor regulation. J Biol Chem 274: 10677-10680.

McKenzie FR, Milligan G. 1990. δ-Opioid receptor mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein Gi2. Biochem J 267: 391-398.

McLaughlin JP, Chavkin C. 2001. Tyrosine phosphorylation of the mu-opioid receptor regulates agonist intrinsic efficacy. Mol Pharmacol 59: 1360-1368.

McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, et al. 2004. Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. J Biol Chem 279: 1810-1818.

McLaughlin JP, Xu M, Mackie K, Chavkin C. 2003. Phosphorylation of a carboxyl-terminal serine within the kappa-opioid receptor produces desensitization and internalization. J Biol Chem 278: 34631-34640.

McVey M, Ramsay D, Kellett E, Rees S, Wilson S, et al. 2001a. Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human delta-opioid receptor displays constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy. J Biol Chem 276: 14092-14099.

McVey M, Ramsay D, Kellett E, Rees S, Wilson S, et al. 2001b. Monitoring receptor oligomerization using time-resolved fluorescence resonance energy transfer and bioluminescence resonance energy transfer. The human δ-opioid receptors constitutive oligomerization at the cell surface, which is not regulated by receptor occupancy. J Biol Chem 276: 14092-14099.

Meng F, Ueda Y, Hoversten MT, Taylor LP, Reinscheid RK, et al. 1998. Creating a functional opioid alkaloid binding site in the orphanin FQ receptor through site-directed mutagenesis. Mol Pharmacol 53: 772-777.

Meng F, Xie GX, Thompson RC, Mansour A, Goldstein A, et al. 1993. Cloning and pharmacological characterization of a rat kappa-opioid receptor. Proc Natl Acad Sci USA 90: 9954-9958.

Michell R, McCullock D, Lutz E, Johnson M, Mac Kenzie C, et al. 1998. Rhodopsin-family receptors associate with small G proteins to activate phospholipase D. Nature 392: 411-414.

Miller WE, Maudsley S, Ahn S, Khan KD, Luttrell LM, et al. 2000. Beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis. J Biol Chem 275: 11312-11319.

Miller WE, McDonald PH, Cai SF, Field ME, Davis RJ, et al. 2001. Identification of a motif in the carboxyl terminus of beta-arrestin2 responsible for activation of JNK3. J Biol Chem 276: 27770-27777.

Minami M, Satoh M. 1995. Molecular biology of the opioid receptors: structure, functions and distributions. Neurosci Res 23: 121-145.

Misawa H, Udea H, Katada T, Ui M, Satoh M. 1995. A subtype of opioid κ-receptor is coupled to inhibition of Gi1-mediated phospholipase C activity in the guinea pig cerebellum. FEBS Lett 36: 106-110.

Moises HC, Rusin KI, Macdonald RL. 1994. μ-Opioid receptor-mediated reduction of neuronal calcium current occurs via a Go-type GTP-binding protein. J Neurosci 14: 3842-3851.

Monnot C, Bihoreau C, Conchon S, Curnow KM, Corvol P, et al. 1996. Polar residues in the transmembrane domains of the type 1 angiotensin II receptor are required for binding and coupling. Reconstitution of the binding site by co-expression of two deficient mutants. J Biol Chem 271: 1507-1513.

Moon HE, Cavalli A, Bahia DS, Hoffmann M, Massotte D, et al. 2001. The human delta opioid receptor activates G(i1) alpha more efficiently than G(o1)alpha. J Neurochem 76: 1805-1813.

Moore RH, Tuffaha A, Millman EE, Dai W, Hall HS, et al. 1999. Agonist-induced sorting of human β2-adrenergic receptors to lysosomes during downregulation. J Cell Sci 112: 329-338.

Morikawa H, Fukuda K, Kato S, Mori K, Higashida H. 1995. Coupling of cloned μ-opioid receptor with the ω-conotoxin-sensitive $Ca^{2+}$ current in NG108-15 cells. J Neurochem 65: 1403-1406.

Morikawa H, Mima H, Uga H, Shoda T, Fukuda K. 1999. Opioid potentiation of N-type $Ca^{2+}$ channel currents via pertussis-toxin-sensitive G proteins in NG108-15 cells. Pflugers Arch – Eur J Physiol 438: 423-426.

Mullaney I, Carr IC, Burt AR, Wilson M, Anderson NG, et al. 1997. Agonist-mediated tyrosine phosphorylation of isoforms of the shc adapter protein by the delta opioid receptor. Cell Signal 9: 423-429.

Mundell SJ, Kelly E. 1998. The effect of inhibitors of receptor internalization on the desensitization and resensitization of three Gσ-coupled receptor responses. Br J Pharmacol 125: 1594-1600.

Murray SR, Evans CJ, von Zastrow M. 1998. Phosphorylation is not required for dynamin-dependent endocytosis of a truncated mutant opioid receptor. J Biol Chem 273: 24987-24991.

Murthy KS, Makhlouf GM. 1996. Opioid mu, delta, and kappa receptor-induced activation of phospholipase C-beta 3 and inhibition of adenylyl cyclase is mediated by Gi2 and G(o) in smooth muscle. Mol Pharmacol 50: 870-877.

Narita M, Ioka M, Suzuki M, Suzuki T. 2002a. Effect of repeated administration of morphine on the activity of extracellular signal regulated kinase in the mouse brain. Neurosci Lett 324: 97-100.

Narita M, Ohnishi O, Nemoto M, Yajima Y, Suzuki T. 2002b. Implications of phosphoinositide 3-kinase in the mu- and delta-opioid receptor-mediated supraspinal antinociception in the mouse. Neurosci 113: 647-652.

Narita M, Ohsawa M, Mizoguchi H, Aoki T, Suzuki T, et al. 2000. Role of the phosphatidylinositol-specific phospholipase C pathway in delta-opioid receptor-mediated antinociception in the mouse spinal cord. Neurosci 99: 327-331.

Ng GY, O'Dowd BF, Lee SP, Chung HT, Brann MR, et al. 1996. Dopamine D2 receptor dimmers and receptor-blocking peptides. Biochem Biophys Res Commun 227: 200-204.

Nitsche JF, Schuller AGP, King MA, Zengh M, Pasternak GW, et al. 2002. Genetic dissociation of opiate tolerance and physical dependence in δ-opioid receptor-1 and preproenkephalin knock-out mice. J Neurosci 22: 10906-10913.

North R, Williams T, Surprenant A, Christie M. 1987. Mu and delta receptors belong to the family of receptors that are coupled to potassium channels. Proc Natl Acad Sci USA 84: 5487-5491.

Offermann S, Schultz G, Rosenthal W. 1991. Evidence for opioid receptor-mediated activation of G-proteins, Go and Gi2 in membranes of neuroblastoma × glioma (NG108-15) hybrid cells. J Biol Chem 266: 3365-3368.

Offermann S, Simon M. 1995. Gα15 and Gα16 couple a wide variety of receptors to phospholipase C. J Biol Chem 270: 15175-15180.

Olianas MC, Onali P. 1995. Participation of δ-opioid receptor subtypes in stimulation of adenylyl cyclase activity in rat olfactory bulb. J Pharmacol Exp Ther 275: 1560-1567.

Onoprishvili I, Andria ML, Kramer HK, Ancevska-Taneva N, Hiller JM, et al. 2003. Interaction between μ opioid receptor and filamin A is involved in receptor regulation and trafficking. Mol Pharmacol 64: 1092-1100.

Ostrom RS. 2002. New determinants of receptor–effector coupling: trafficking and compartmentalization in membrane microdomains. Mol Pharmacol 61: 473-476.

Ott S, Costa T, Herz A. 1988. Sodium modulates opioid receptors through a membrane component different from G-proteins. Demonstration by target size analysis. J Biol Chem 263: 10524-10533.

Ottolia M, Platano D, Qin N, Noceti F, Birnbaumer M, et al. 1998. Functional coupling between human E-type $Ca^{2+}$ channels and mu opioid receptors expressed in *Xenopus oocytes*. FEBS Lett 427: 96-102.

Ozaki S, Narita M, Narita M, Ozaki M, Khotib J, et al. 2004. Role of extracellular signal-regulated kinase in the ventral tegmental area in the suppression of the morphine-induced rewarding effect in mice with sciatic nerve ligation. J Neurochem 88: 1389-1397.

Pak Y, O'Dowd BF, George SR. 1997. Agonist-induced desensitization of the mu opioid receptor is determined by threonine 394 preceded by acidic amino acids in the COOH-terminal tail. J Biol Chem 272: 24961-24965.

Pak Y, O'Dowd BF, Wang JB, George SR. 1999. Agonist-induced, G protein-dependent and -independent down-regulation of the mu opioid receptor. The receptor is a direct substrate for protein-tyrosine kinase. J Biol Chem 274: 27610-27616.

Pan YX, Xu J, Bolan E, Abbadie C, Chang A, et al. 1999. Identification and characterization of three new alternatively spliced mu-opioid receptor isoforms. Mol Pharmacol 56: 396-403.

Pan YX, Xu J, Mahurter L, Bolan E, Xu M, et al. 2001. Generation of the mu opioid receptor (MOR-1) protein by three new splice variants of the Oprm gene. Proc Natl Acad Sci USA 98: 14084-14089.

Patterson RL, Boehning D, Snyder SH. 2004. Inositol 1,4,5-triphosphate receptors as signal integrators. Annu Rev Biochem 73: 437-465.

Pei G, Kieffer BL, Lefkowitz RJ, Freedman NJ. 1995. Agonist-dependent phosphorylation of the mouse δ-opioid receptor: involvement of γ protein-coupled receptor kinases but not protein kinase C. Mol Pharmacol 48: 173-177.

Persson AI, Thorlin T, Bull C, Eriksson PS. 2003a. Opioid-induced proliferation through the MAPK pathway in cultures of adult hippocampal progenitors. Mol Cell Neurosci 23: 360-372.

Persson AI, Thorlin T, Bull C, Zarnegar P, Ekman R, et al. 2003b. Mu- and delta-opioid receptor antagonists decrease proliferation and increase neurogenesis in cultures of rat adult hippocampal progenitors. Eur J Neurosci 17: 1159-1172.

Pert CB, Kuhar MJ, Snyder SH. 1975. Autoradiographic localization of the opiate receptor in rat brain. Life Sci 16: 1849-1853.

Pert CB, Snyder SH. 1973. Properties of opiate-receptor binding in rat brain. Proc Natl Acad Sci USA 70: 2243-2247.

Petaja-Repo UE, Hogue M, Bhalla S, Laperriere A, Morello JP, et al. 2002. Ligands act as pharmacological chaperones and increase the efficiency of delta opioid receptor maturation. EMBO J 21: 1628-1637.

Petaja-Repo UE, Hogue M, Laperriere A, Bhalla S, Walker P, et al. 2001. Newly synthesized human delta opioid receptors retained in the endoplasmic reticulum are retrotranslocated to the cytosol, deglycosylated, ubiquitinated, and degraded by the proteasome. J Biol Chem 276: 4416-4423.

Petaja-Repo U, Hogue M, Laperriere A, Walker P, Bouvier M. 2000. Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opioid receptor. J Biol Chem 275: 13727-13736.

Pierce KL, Maudsley S, Daaka Y, Luttrell LM, Lefkowitz RJ. 2000. Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. Proc Natl Acad Sci USA 97: 1489-1494.

Piros ET, Prather PL, Law PY, Evans CJ, Hales TG. 1996. Voltage-dependent inhibition of Ca2+ channels in GH3 cells by cloned mu- and delta-opioid receptors. Mol Pharmacol 50: 947-956.

Piros ET, Prather PL, Loh HH, Law PY, Evans CJ, et al. 1995. $Ca^{+2}$ channel and adenylyl cyclase modulation by cloned μ-opioid receptors in GH3 cells. Mol Pharmacol 47: 1041-1049.

Polakiewicz RD, Schieferl SM, Dorner LF, Kansra V, Comb MJ. 1998b. A mitogen-activated protein kinase pathway is required for μ-opioid receptor desensitization. J Biol Chem 273: 12402-12406.

Polakiewicz RD, Schieferl SM, Gingras AC, Sonenberg N, Comb MJ. 1998a. mu-Opioid receptor activates signaling pathways implicated in cell survival and translational control. J Biol Chem 273: 23534-23541.

Portoghese PS, Sultana M, Takemori AE. 1988. Naltrindole, a highly selective and potent non-peptide delta opioid receptor antagonist. Eur J Pharmacol 146: 185-186.

Prather PL, Loh HH, Law PY. 1994a. Interaction of δ-opioid receptors with multiple G-proteins: a non-relationship between agonist potency to inhibit adenylyl cyclase and activation of G-proteins. Mol Pharmacol 45: 997-1003.

Prather PL, McGinn TM, Claude PA, Liu-Chen LY, Loh HH, et al. 1995. Properties of κ-opioid receptor expressed in CHO cells: activation of multiple G-proteins similar to other opioid receptors. Mol Brain Res 29: 336-346.

Prather PL, McGinn TM, Erickson LJ, Evans CJ, Loh HH, et al. 1994b. Ability of δ-opioid receptors to interact with multiple G-proteins is independent of receptor density. J Biol Chem 269: 21293-21302.

Pronin AN, Satpaev DK, Slepak VZ, Benovic JL. 1997. Regulation of G protein-coupled receptor kinases by calmodulin and localization of the calmodulin binding domain. J Biol Chem 272: 18273-18280.

Puri SK, Cochin J, Volicer L. 1975. Effect of morphine sulfate on adenylate cyclase and phosphodiesterase activities in rat corpus striatum. Life Sci 16: 759-768.

Qui Y, Law PY, Loh HH. 2003. μ-Opioid receptor desensitization. Role of receptor phosphorylation, internalization and resensitization. J Biol Chem 278: 36733-36739.

Quillan JM, Carlson KW, Song C, Wang D, Sadee W. 2002. Differential effects of mu-opioid receptor ligands on Ca (2+) signaling. J Pharmacol Exp Ther 302: 1002-1012.

Quock RM, Burkey TH, Varga E, Hosohata Y, Hosohata K, et al. 1999. The delta-opioid receptor: molecular pharmacology, signal transduction, and the determination of drug efficacy. Pharmacol Rev 51: 503-532.

Ramsay D, Kellett E, McVey M, Rees S, Milligan G. 2002. Homo- and hetero-oligomeric interactions between G protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequence. Biochem J 365: 429-440.

Rhee SG. 2001. Regulation of phosphoinositide-specific phospholipase C. Annu Rev Biochem 70: 281-312.

Rhim H, Miller RJ. 1994. Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius. J Neurosci 14: 7608-7615.

Rhim H, Toth PT, Miller RJ. 1996. Mechanism of inhibition of calcium channels in rat nucleus tractus solitarius by neurotransmitters. Br J Pharmacol 118: 1341-1350.

Rocheville M, Lange DC, Kumar U, Patel SC, Patel RC, et al. 2000a. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. Science 288: 154-157.

Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC, et al. 2000b. Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. J Biol Chem 275: 7862-7869.

Roerig SC, Loh HH, Law PY. 1992. Identification of three separate G-proteins which interact with the delta opioid receptor in NG108-15 neuroblastoma × glioma hybrid cells. Mol Pharmacol 45: 997-1003.

Roth BL, Willins DL, Kroeze WK. 1998. G protein-coupled receptor (GPCR) trafficking in the central nervous system: relevance for drug of abuse. Drug Alcohol Depend 51: 73-85.

Rubovitch V, Gafni M, Sarne Y. 2003. The mu opioid agonist DAMGO stimulates cAMP production in SK-N-SH cells through a PLC-PKC-$Ca^{++}$ pathway. Brain Res Mol Brain Res 110: 261-266.

Safa P, Boulter J, Hales TG. 2001. Functional properties of Cav1.3 (alpha1D) L-type $Ca^{2+}$ channel splice variants expressed by rat brain and neuroendocrine GH3 cells. J Biol Chem 276: 38727-38737.

Salim K, Fenton T, Bacha J, Urien-Rodriguez H, Bonnert T, et al. 2002. Oligomerization of G protein-coupled receptors shown by selective co-immunoprecipitation. J Biol Chem 277: 15482-15485.

Sanchez-Blazquez P, Garzon J. 1998. delta-Opioid receptor subtypes activate inositol-signaling pathways in the production of antinociception. J Pharmacol Exp Ther 285: 820-827.

Sanchez-Blazquez P, Rodriguez-Diaz M, Frejo MT, Garzon J. 1999. Stimulation of mu- and delta-opioid receptors enhances phosphoinositide metabolism in mouse spinal cord: evidence for subtypes of delta-receptors. Eur J Neurosci 11: 2059-2064.

Sarne Y, Rubovitch V, Fields A, Gafni M. 1998. Dissociation between the inhibitory and stimulatory effects of opioid peptides on cAMP formation in SK-N-SH neuroblastoma cells. Biochem Biophys Res Commun 246: 128-131.

Sawyer GW, Ehlert FJ. 1999. Muscarinic M3 receptor inactivation reveals a pertussis toxin-sensitive contractile response in the guinea pig colon: evidence for M2/M3 receptor interactions. J Pharmacol Exp Ther 289: 464-476.

Schiller PW, Weltrowska G, Berezowska I, Nguyen TM, Wilkes BC, et al. 1999. The TIPP opioid peptide family: development of delta antagonists, delta agonists, and mixed mu agonist/delta antagonists. Biopolymers 51: 411-425.

Schmidt H, Schulz S, Klutzny M, Koch T, Handel M, et al. 2000. Involvement of mitogen-activated protein kinase in agonist-induced phosphorylation of the mu-opioid receptor in HEK 293 cells. J Neurochem 74: 414-422.

Schneider SP, Eckert WA, Light AR. 1998. Opioid-activated postsynaptic, inward rectifying potassium currents in whole cell recordings in substantia gelatinosa neurons. J Neurophysiol 80: 2954-2962.

Schramm NL, Limbird LE. 1999. Stimulation of mitogen-activated protein kinase by G protein-coupled alpha(2)-adrenergic receptors does not require agonist-elicited endocytosis. J Biol Chem 274: 24935-24940.

Schultz S, Hollt V. 1998. Opioid withdrawal activates MAP kinase in locus coeruleus neurons in morphine-dependent rats in vivo. Eur J Neurosci 10: 1196-1201.

Schultz JE-J, Hsu AK, Nagase H, Gross GJ. 1998. TAN-67, a delta 1-opioid receptor agonist, reduces infarct size via activation of Gi/o proteins and KATP channels. Am J Physiol 274: H909-H914.

Schulz R, Wüster M, Kreuss H, Herz A. 1980. Selective development of tolerance without dependence in multiple opiate receptors of mouse vas deferens. Nature 285: 242-243.

Shahabi NA, Daaka Y, McAllen K, Sharp BM. 1999. Delta opioid receptors expressed by stably transfected Jurkat cells signal through the MAP kinase pathway in a ras-independent manner. J Neuroimmunol 94: 48-57.

Sharma SK, Klee WA, Nirenberg M. 1977. Opiate dependent modulation of adenylate cyclase activity. Proc Natl Acad Sci USA 74: 3365-3369.

Sharma S, Nirenberg M, Klee W. 1975. Morphine receptors as regulators of adenylate cyclase activity. Proc Natl Acad Sci USA 72: 590-594.

Sharp BM, McKean DJ, McAllen K, Shahabi NA. 1998. Signaling through delta opioid receptors on murine splenic T cells and stably transfected Jurkat cells. Ann NY Acad Sci 840: 420-424.

Shenoy SK, Lefkowitz RJ. 2003. Trafficking patterns of beta-arrestin and G protein-coupled receptors determined by the kinetics of beta-arrestin deubiquitination. J Biol Chem 278: 14498-144506.

Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. 2001. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. Science 294: 1307-1313.

Shoda T, Fukuda K, Uga H, Mima H, Morikawa H. 2001. Activation of mu-opioid receptor induces expression of c-fos and junB via mitogen-activated protein kinase cascade. Anesthesiology 95: 983-989.

Simon E, Hiller JM, Edelman I. 1973. Stereospecific binding of the potent narcotic analgesic ($^3$H) etorphine to rat-brain homogenate. Proc Natl Acad Sci USA 70: 1947-1949.

Singhal PC, Bhaskaran M, Patel J, Patel K, Kasinath BS, et al. 2002. Role of p38 mitogen-activated protein kinase phosphorylation and Fas–Fas ligand interaction in morphine-induced macrophage apoptosis. J Immunol 168: 4025-4033.

Smart D, Hirst RA, Hirota K, Grandy DK, Lambert DG. 1997. The effects of recombinant rat mu-opioid receptor activation in CHO cells on phospholipase C, $[Ca^{2+}]_i$ and adenylyl cyclase. Br J Pharmacol 120: 1165-1171.

Smart D, Lambert DG. 1996. Tyr-D-Arg2-Phe-sarcosine4 activates phospholipase C-coupled mu2-opioid receptors in SH-SY5Y cells. Eur J Pharmacol 305: 235-238.

Smart D, Smith G, Lambert DG. 1994. μ-Opioid receptor stimulation of inositol (1,4,5)trisphosphate formation via a pertussis toxin-sensitive G protein. J Neurochem 62: 1009-1014.

Smart D, Smith G, Lambert DG. 1995. μ-Opioids activate phospholipase C in SH-SY5Y human neuroblastoma cells via calcium-channel opening. Biochem J 305: 577-581.

Spencer RJ, Jin W, Thayer SA, Chakrabarti S, Law PY, et al. 1997. Mobilization of Ca+2 from intracellular stores in transfected Neuro2a cells by activation of multiple opioid receptor subtypes. Biochem Pharmacol 54: 809-818.

Sternini C, Brecha NC, Minnis J, D'Agostino G, Balestra B, et al. 2000. Role of agonist-dependent receptor internalization in the regulation of mu opioid receptors. Neurosci 98: 233-241.

Sternini C, Spann M, Anton B, Keith Jr, DE Bunnett NW, et al. 1996. Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. Proc Natl Acad Sci USA 93: 9241-9246.

Strassheim D, Law PY, Loh HH. 1998. Contribution of phospholipase C-β3 phosphorylation to the rapid attenuation of opioid-activated phosphoinositide response. Mol Pharmacol 53: 1047-1053.

Takemori AE, Ho BY, Naeseth JS, Portoghese PS. 1988. Norbinaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays. J Pharmacol Exp Ther 246: 255-258.

Takemori AE, Larson DL, Portoghese PS. 1981. The irreversible narcotic antagonistic and reversible agonistic properties of the fumaramate methyl ester derivative of naltrexone. Eur J Pharmacol 70: 445-451.

Tan M, Groszer M, Tan AM, Pandya A, Liu X, et al. 2003. Phosphoinositide 3-kinase cascade facilitates mu-opioid desensitization in sensory neurons by altering G-protein–effector interactions. J Neurosci 23: 10292-10301.

Tang WJ, Hurley JH. 1998. Catalytic mechanism and regulation of mammalian adenylyl cyclases. Mol Pharmacol 54: 231-240.

Tang T, Kiang JG, Cote TE, Cox BM. 1995. Antisense oligonucleotide to the Gi2 protein alpha subunit sequence inhibits an opioid-induced increase in the intracellular free calcium concentration in ND8-47 neuroblastoma × dorsal root ganglion hybrid cells. Mol Pharmacol 48: 189-193.

Tanowitz M, von Zastrow M. 2002. Ubiquitination-independent trafficking of G protein-coupled receptors to lysosomes. J Biol Chem 277: 50219-50222.

Tanowitz M, von Zastrow M. 2003. A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. J Biol Chem 278: 45978-45986.

Tao PL, Chang LR, Law PY, Loh HH. 1998. Decrease in delta-opioid receptor density in rat brain after chronic D-Ala2, D-Leu5 enkephalin treatment. Brain Res 462: 313-320.

Tao PL, Law PY, Loh HH. 1987. Decrease in delta- and mu-opioid receptor binding capacity in rat brain after chronic etorphine treatment. J Pharmacol Exp Ther 240: 809-816.

Tao PL, Lee HY, Chang LR, Loh HH. 1990. Decrease in μ-opioid receptor binding capacity in rat brain after chronic PL017 treatment. Brain Res 526: 270-275.

Taussig R, Tang WJ, Hepler JR, Gilman AG. 1994. Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. J Biol Chem 269: 6093-6100.

Terenius L. 1973. Characteristics of the "receptor" for narcotic analgesics in synaptic plasma membrane fraction from rat brain. Acta Pharmacol Toxicol 33: 377-384.

Torrecilla M, Marker CL, Cintora SC, Stoffel M, Williams JT, et al. 2002. G-protein-gated potassium channels containing Kir3.2 and Kir3.3 subunits mediate the acute inhibitory effects of opioids on locus ceruleus neurons. J Neurosci 22: 4328-4334.

Trapaidze N, Gomes I, Bansinath M, Devi LA. 2000a. Recycling and resensitization of delta opioid receptors. DNA Cell Biol 19: 195-204.

Trapaidze N, Gomes I, Cvejic S, Bansinath M, Devi LA. 2000b. Opioid receptor endocytosis and activation of MAP kinase pathway. Brain Res Mol Brain Res 76: 220-228.

Trapaidze N, Keith DE, Cvejic S, Evans CJ, Devi LA. 1996. Sequestration of the delta opioid receptor. Role of the C terminus in agonist-mediated internalization. J Biol Chem 271: 29279-29285.

Trujillo KA, Akil HA. 1991. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. Science 251: 85-87.

Tsao PI, von Zastrow M. 2000. Type-specific sorting of G protein-coupled receptors after endocytosis. J Biol Chem 275: 11130-11140.

Tso PH, Wong YH. 2001. Role of extracellular signal-regulated kinases in opioid-induced adenylyl cyclase superactivation in human embryonic kidney 293 cells. Neurosci Lett 316: 13-16.

Tso PH, Yung LY, Wong YH. 2000. Regulation of adenylyl cyclase, ERK1/2, and CREB by Gz following acute and chronic activation of the delta-opioid receptor. J Neurochem 74: 1685-1693.

Tsu RC, Allen RA, Wong YH. 1995a. Stimulation of type II adenylyl cyclase by formyl peptide and C5a chemoattractant receptors. Mol Pharmacol 47: 835-841.

Tsu RC, Chan JSC, Wong YH. 1995b. Regulation of multiple effectors by the cloned δ-opioid receptor: stimulation of phospholipase C and type II adenylyl cyclase. J Neurochem 64: 2700-2707.

Tsunoda S, Sierralta J, Sun Y, Bodner R, Suzuki E, et al. 1997. A multivalent PDZ-domain protein assembles signaling complexes in a G protein-coupled cascade. Nature 388: 243-249.

Ueda H, Miyamae T, Fukushima N, Takeshima H, Fukuda K, et al. 1995. Opioid μ- and κ-receptor mediate phospholipase C activation through Gi1 in *Xenopus oocytes*. Brain Res Mol Brain Res 32: 166-170.

van Kerkhof P, Sachse M, Klumperman J, Strous GJ. 2001. Growth hormone receptor ubiquitination coincides with

recruitment to clathrin-coated membrane domains. J Biol Chem 276: 3778-3784.

van Kerkhof P, Smeets M, Strous GJ. 2002. The ubiquitin–proteasome pathway regulates the availability of the GH receptor. Endocrinol 143: 1243-1252.

Waldhoer M, Bartlett S, Whistler JL. 2004. Opioid receptors. Annu Rev Biochem 73: 953-990.

Wang JB, Johnson PS, Imai Y, Persico AM, Ozenberger BA, et al. 1994. cDNA cloning of an orphan opiate receptor gene family member and its splice variant. FEBS Lett 348: 75-79.

Wang W, Loh HH, Law PY. 2003. Intracellular trafficking of opioid receptors directed by carboxyl tail and a di-leucine motif in Neuro2A cells. J Biol Chem 278: 36848-36858.

Wang D, Quillan JM, Winans K, Lucas JL, Sadee W. 2001. Single nucleotide polymorphisms in the human mu opioid receptor gene alter basal G protein coupling and calmodulin binding. J Biol Chem 276: 34624-34630.

Wang D, Sadee W, Quillan JM. 1999. Calmodulin binding to G protein-coupling domain of opioid receptors. J Biol Chem 274: 22081-22088.

Wang D, Surratt CK, Sadee W. 2000. Calmodulin regulation of basal and agonist-stimulated G protein coupling by the mu-opioid receptor (OP(3)) in morphine-pretreated cell. J Neurochem 75: 763-771.

Wang C, Zhou D, Cheng Z, Wei Q, Chen J, et al. 1998. The C-truncated delta-opioid receptor underwent agonist-dependent activation and desensitization. Biochem Biophys Res Commun 249: 321-324.

Ward SJ, Portoghese PS, Takemori AE. 1982. Pharmacological characterization in vivo of the novel opiate, beta-funaltrexamine. J Pharmacol Exp Ther 220: 494-498.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M. 1999. Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23: 737-746 [comment].

Whistler JL, Enquist J, Marley A, Fong J, Gladher F, et al. 2002. Modulation of postendocytic sorting of G protein-coupled receptors. Science 297: 615-620 [comment].

Whistler JL, von Zastrow M. 1998. Morphine-activated opioid receptors elude desensitization by beta-arrestin. Proc Natl Acad Sci USA 95: 9914-9919.

Whistler JL, Zastrow. von M 1999. Dissociation of functional roles of dynamin in receptor-mediated endocytosis and mitogenic signal transduction. J Biol Chem 274: 24575-24578.

Wick MJ, Minnerath SR, Lin X, Elde R, Law PY, et al. 1994. Isolation of a novel cDNA encoding a putative membrane receptor with high homology to the cloned mu, delta, and kappa opioid receptors. Brain Res Mol Brain Res 27: 37-44.

Wickman K., Iniguez-Lluhi J, Davenport P, Taussig R, Krapivinsky G, et al. 1994. Recombinant G-protein beta/gamma-subunits activate the muscarinic-gated atrial potassium channel. Nature 368: 255-267.

Wilson MA, Burt AR, Milligan G, Anderson NG. 1997. Mitogenic signalling by delta opioid receptors expressed in rat-1 fibroblasts involves activation of the p70s6k/p85s6k S6 kinase. Biochem J 325: 217-222.

Wolf R, Koch T, Schulz S, Klutzny M, Schroder H, et al. 1999. Replacement of threonine 394 by alanine facilitates internalization and resensitization of the rat mu opioid receptor. Mol Pharmacol 55: 263-268.

Wong YH, Conklin BR, Bourne HR. 1992. Gz-mediated inhibition of cAMP accumulation. Science 255: 339-342.

Wu YL, Pei G, Fan GH. 1998. Inhibition of phospholipase C blocks opioid receptor-mediated activation of Gi proteins. Neuroreport 9: 99-103.

Xiang B, Yu GH, Guo J, Chen L, Hu W, et al. 2001. Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization. J Biol Chem 276: 4709-4716.

Xie Z, Lee SP, O'Dowd BF, George SR. 1999b. Serotonin 5-HT1B and 5-HT1D receptors form homodimers when expressed alone and heterodimers when co-expressed. FEBS Lett 456 :63-67.

Xie W, Samoriski GM, McLaughlin JP, Romoser VA, Smrcka A, et al. 1999a. Genetic alteration of phospholipase C beta3 expression modulates behavioral and cellular responses to mu opioids. Proc Natl Acad Sci USA 96: 10385-10390.

Xu J, Paquet M, Lau AG, Wood JD, Ross CA, et al. 2001. Beta 1-adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. J Biol Chem 276: 41310-41317.

Yamada M, Inanobe A, Kurachi Y. 1998. G protein regulation of potassium ion channels. Am Soc Pharmacol Exp Ther 50: 724-757.

Yan K, Gautam N. 1996. A domain on the G protein β subunit interacts with both adenylyl cyclase 2 and the muscarinic atrial potassium channel. J Biol Chem 271: 17597-17600.

Yeo A, Samways DS, Fowler CE, Gunn-Moore F, Henderson G. 2001. Coincident signalling between the Gi/Go-coupled delta-opioid receptor and the Gq-coupled m3 muscarinic receptor at the level of intracellular free calcium in SH-SY5Y cells. J Neurochem 76: 1688-1700.

Yin DL, Ren XH, Zheng ZL, Pu L, Jiang LZ, et al. 1997. Etorphine inhibits cell growth and induces apoptosis in SK-N-SH cells: involvement of pertussis toxin-sensitive G proteins. Neurosci Res 29: 121-127.

Yoon SH, Lo TM, Loh HH, Thayer SA. 1999. Delta-opioid-induced liberation of Gbetagamma mobilizes $Ca^{2+}$ stores in NG108-15 cells. Mol Pharmacol 56: 902-908.

Yu Y, Zhang L, Yin X, Sun H, Uhl GR, et al. 1997. Mu opioid receptor phosphorylation, desensitization, and ligand efficacy. J Biol Chem 272: 28869-28874.

Zadina JE, Chang SL, Ge LJ, Kastin AJ. 1993. Mu opiate receptor down-regulation by morphine and up-regulation by naloxone in SHSY5Y human neuroblastoma cells. J Pharmacol Exp Ther 265: 254-262.

Zagon IS, Verderame MF, McLaughlin PJ. 2002. The biology of the opioid growth factor receptor (OGFr). Brain Res Rev 38: 351-376.

Zhang J, Barak LS, Winkler KE, Caron MG, Ferguson SS. 1997. A central role for beta-arrestins and clathrin-coated vesicle-mediated endocytosis in beta2-adrenergic receptor resensitization. Differential regulation of receptor resensitization in two distinct cell types. J Biol Chem 272: 27005-27014.

Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, et al. 1998. Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. Proc Natl Acad Sci USA 95: 7157-7162.

Zhang J, Ferguson SS, Law PY, Barak LS, Caron MG. 1999. Agonist-specific regulation of delta-opioid receptor trafficking by G protein-coupled receptor kinase and beta-arrestin. J Recep Signal Transduct Res 19: 301-313.

Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR, et al. 1996. Differential mu opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. J Biol Chem 271: 11449-11454.

Zhang WB, Zhang Z, Ni YX, Wu YL, Pei G. 2003. A novel function of Go: mediation of extracellular signal-regulated kinase activation by opioid receptors in neural cells. J Neurochem 86: 1213-1222.

Zhao J, Pei G, Huang YL, Zhong FM, Ma L. 1997. Carboxyl terminus of delta opioid receptor is required for agonist-dependent receptor phosphorylation. Biochem Biophys Res Commun 238: 71-76.

Zheng FY, Wess J. 1999. Identification and molecular characterization of m3 muscarinic receptor dimers. J Biol Chem 274: 19487-19497.

Zhu Y, King MA, Schuller AG, Nitsche JF, Reidl M, et al. 1999. Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. Neuron 24: 243-252.

# 19 CGRP: a Multifunctional Neuropeptide

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**Abstract:** It has been just over 20 years since the neuropeptide calcitonin gene-related peptide (CGRP) was unexpectedly discovered embedded within the calcitonin gene. Since then CGRP has emerged as a key regulator of multiple physiological and pathological functions. CGRP is expressed in a subset of neurons in the central and peripheral nervous system that innervate every major organ system. Within this context, CGRP is a multifunctional peptide that is a major modulator of the cardiovascular system and a key player in neurogenic inflammatory pain. Since the discovery of CGRP, a repertoire of related peptides have been identified. The expression patterns and activities of these family members are discussed briefly. We will focus on how CGRP gene transcription is controlled by extracellular signals that target the cell-specific and cAMP-regulated enhancers. An emphasis of the chapter will be the unusual mechanism by which the CGRP receptor requires the G-protein-coupled calcitonin-like receptor (CLR), receptor activity modifying protein-1 (RAMP1), and receptor component protein (RCP). We will then address the physiological activities of CGRP in the cardiovascular and smooth muscle, skeletal muscle, and cochlear systems. Finally, we will discuss two pathologies that involve CGRP in the trigeminovascular system: migraine and subarachnoid hemorrhage (SAH). The efficacy of a CGRP receptor antagonist has now established the importance of elevated CGRP in migraine. In contrast, the lack of CGRP may contribute to fatal vasospasms in SAH. Thus, the continuing development of pharmacological and genetic approaches for modulating CGRP expression and activity holds exciting promise for future therapeutic strategies.

**List of Abbreviations:** ABR, auditory brainstem response; ACh, acetylcholine; AChE, acetylcholinesterase; AChR, acetylcholine receptor; AM, adrenomedullin; bHLH-Zip, basic HLH-leucine zipper; cAMP, cyclic adenosine monophosphate; CGRP, calcitonin gene-related peptide; CLR, calcitonin-like receptor; CRSP, calcitonin receptor-stimulating peptide; CRE, cAMP response element; CREB, cAMP response element binding protein; CT, calcitonin; CTR, calcitonin receptor; DAG, diacylglycerol; DPOAE, distortion product otoacoustic emissions; DRG, dorsal root ganglia; Fox, forkhead; HLH, helix–loop–helix; 5-$HT_1$, 5-hydroxytryptamine 1; IP3, inositol-1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; MASH-1, mammalian achaete-scute homolog-1; MKP-1, MAP kinase phosphatase-1; PDGF, platelet-derived growth factor; $PIP_2$, phosphatidylinositol-4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; RAMP, receptor activity modifying protein; RCP, receptor component protein; SAH, subarachnoid hemorrhage; USF, upstream stimulatory factor; VSMC, vascular smooth muscle cell

## 1 Introduction

The topic of this chapter is the neuropeptide calcitonin gene-related peptide (CGRP). CGRP was discovered over 20 years ago as an alternative RNA splicing product from the calcitonin (CT) gene (Amara et al., 1982; Rosenfeld et al., 1983). At the time it was one of the first examples of an alternatively spliced cellular transcript. The rapid development of a specific antiserum revealed CGRP expression in discrete regions of the central and peripheral nervous system. On the basis of its distribution, CGRP was suggested to have multifunctional activities in the cardiovascular, integrative, and gastrointestinal systems (Rosenfeld et al., 1983). In fact, there are CGRP-containing nerve fibers in every major organ system of the body and CGRP has now been shown to have a number of biological activities (Preibisz, 1993; Wimalawansa, 1996; van Rossum et al., 1997; Brain and Grant, 2004). There is a growing CGRP family of structurally and functionally related peptides. The expression patterns and functional repertoire of these peptides will be briefly compared with CGRP.

CGRP is almost exclusively expressed in neurons and is controlled solely at the transcriptional level. We will describe the mechanisms controlling CGRP gene transcription, with particular emphasis on regulation by MAP kinases. We will then discuss in depth several of the physiological functions of CGRP. In particular, we will cover the actions of CGRP in the cardiovascular, smooth muscle, skeletal muscle, and cochlear systems.

A major advance in the CGRP field has been the cloning of the proteins responsible for mediating CGRP actions. An orphan G-protein-coupled receptor (GPCR) was identified in 1993 (Chang et al., 1993), which has been renamed as the calcitonin-like receptor (CLR) (Poyner et al., 2002). CLR remained an

orphan for 5 years because it was initially nonfunctional when transfected into mammalian cells (Fluhmann et al., 1995; Han et al., 1997). This paradox was resolved by the identification of receptor activity modifying proteins (RAMPs) that confer pharmacological specificity to CLR, and receptor component protein (RCP) that enables signaling (Luebke et al., 1996; McLatchie et al., 1998; Evans et al., 2000). The CGRP receptor is a complex of CLR with these two requisite accessory proteins: RCP and RAMP1. We will discuss the CGRP receptor and how our concept of a GPCR must now include a complex of proteins that are required for correct intracellular sorting, organization in the plasma membrane, and coupling to signal transduction proteins.

Finally, we will address the role of CGRP in migraine and subarachnoid hemorrhage (SAH). As predicted by its cardiovascular and nociceptive activities, CGRP has been implicated not only in these diseases, but also in aspects of hypertension and myocardial infarction (van Rossum et al., 1997; Brain and Grant, 2004). The ability of injected CGRP to induce migraine-like headache (Lassen et al., 2002), and of a CGRP receptor antagonist to provide relief in migraine clinical trials (Olesen et al., 2004), has established the involvement of CGRP in migraine (Arulmani et al., 2004; Durham, 2004a; Edvinsson, 2004). Elevated CGRP levels can also be beneficial. In the case of SAH, the lack of CGRP may account for fatal vasospasms (Juul et al., 1994; Imaizumi et al., 1996; Inoue et al., 1996; Toyoda et al., 2000b; Arulmani et al., 2004). In addition, following myocardial infarction it is believed that elevated CGRP plays a protective role during ischemia (Mair et al., 1990; Lechleitner et al., 1992; Franco-Cereceda and Liska, 2000; Roudenok et al., 2001; Kato et al., 2003). These observations support the possibility that modulation of CGRP synthesis and action will be increasingly effective therapeutic strategies in the future.

## 2 Members of the CGRP Gene Family

The parental namesake of the CGRP gene family is the hormone calcitonin (CT). CGRP was initially identified as an alternative splice product of the CT gene (CALCA or CT/CGRP) (Amara et al., 1982). In fact, the original name of CGRP was pseudo-Cal since the alternative RNA did not encode CT. Subsequently, other peptides have been discovered that have similar amino acid sequences and overlapping yet distinct biological activities and expression patterns. All the peptides share a similar predicted structure of a disulfide bridge and an amidated C-terminus.

### 2.1 Calcitonin

CT was identified in the early 1960s by Copp as a hypocalcemic hormone (Copp et al., 1962). CT is a 32-amino-acid hormone that acts to lower serum calcium levels by inhibiting bone resorption and by increasing renal calcium excretion (McDermott and Kidd, 1987; Inzerillo et al., 2002). CT acts on the G-protein-coupled CT receptor (CTR) (Goldring et al., 1987). The importance of CT in calcium homeostasis has been questioned since an absence of CT can be compensated for by parathyroid hormone and vitamin D3 in the normal adult. However, animal studies have suggested that it may play important roles under times of calcium stress and it is an effective therapeutic for Paget's disease of the bone and certain types of osteoporosis and hypercalcemia (McDermott and Kidd, 1987; Copp, 1992; Inzerillo et al., 2002). Furthermore, the unexpected finding that CT/CGRP knockout mice have increased bone mass suggests additional roles for CT in bone formation, possibly during embryogenesis (Hoff et al., 2002).

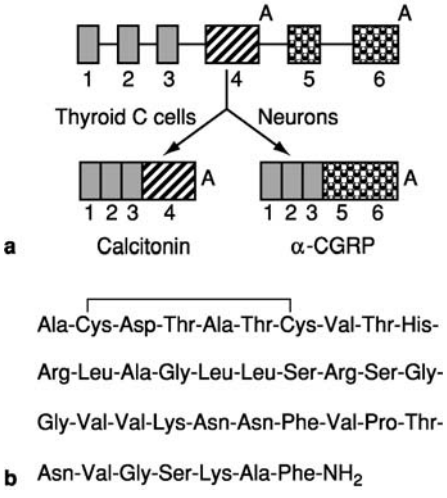
CT is produced almost exclusively by the parafollicular or C cells of the thyroid. There are reports of CT in other cell types. In particular, there are low levels of CT messenger RNA (mRNA) and peptide in the liver and adipose tissue (Bracq et al., 1997; Russwurm et al., 2001; Linscheid et al., 2003). There is also a report of relatively high levels of CT in the human prostate (Davis et al., 1989). In addition, CT-like peptides have been observed in the brain and pituitary for a number of years and this remains an enigma (Hilton et al., 1998; Pondel, 2000). Interestingly, there is massive expression of the unprocessed pro-CT in a wide range of tissues during septic shock, and pro-CT has been implicated in the pathology of sepsis (Muller et al., 2001; Becker et al., 2003; Christ-Crain et al., 2004).

## 2.2 Production of Calcitonin and α-CGRP from the Same Gene

The primary RNA transcript of the CT/CGRP gene is processed to produce CT mRNA by splicing of exons 1–4 and polyadenylation after exon 4 (*Figure 19-1*). In the thyroid C cell, ~95% of the primary transcript

**Figure 19-1**

**Schematic of the calcitonin/α-CGRP gene. (a) Alternative processing of the primary transcript in thyroid C cells yields primarily calcitonin mRNA, while CGRP mRNA is the primary product in neurons. The alternative polyadenylation sites following exons 4 and 6 are indicated. (b) Primary sequence of the human α-CGRP peptide. The disulfide bond is indicated**




is processed to CT mRNA. In contrast to the CT splicing pattern in thyroid C cells, ~99% of the CT/CGRP transcript is processed to α-CGRP in neurons (Rosenfeld et al., 1983). This processing pathway removes exon 4 and uses a downstream polyadenylation site after exon 6 to generate an mRNA containing exons 1, 2, 3, 5, and 6 (*Figure 19-1*).

While the alternative splice mechanism is not yet fully understood, a working model has emerged (Lou and Gagel, 1998). Studies in transgenic mice that ubiquitously expressed the CT/CGRP gene suggested that CT mRNA is the default choice in all tissues except for the brain and heart (Crenshaw et al., 1987). Mapping studies in cell lines subsequently identified several regulatory elements that are required for retention of exon 4 to yield CT mRNA (Adema et al., 1988; Emeson et al., 1989; Cote et al., 1992; Yeakley et al., 1993; Lou et al., 1994; van Oers et al., 1994). Among these, the best characterized is an intron enhancer located approximately 250 nucleotides downstream of CT exon 4. This novel element is similar to a 5′-splice donor site and binds a complex of splicing proteins that help direct polyadenylation of CT exon 4 (Lou et al., 1995, 1996). Thus, this element is apparently responsible for the preferential expression of CT mRNA in thyroid C cells.

## 2.3 α-CGRP

The focus of this chapter is the 37-amino-acid α-CGRP peptide. As mentioned above, it is produced by alternative processing of the CT/CGRP transcript in neurons of the peripheral nervous system and the central nervous system (CNS) (Rosenfeld et al., 1983; van Rossum et al., 1997). CGRP produced from the CT/CGRP gene is referred to as α-CGRP (or CGRP-I), although for the purposes of this review, we will refer to α-CGRP simply as CGRP unless otherwise indicated.

Within the periphery, CGRP is particularly abundant in sensory nerves of the dorsal root and trigeminal ganglia. Perhaps the most prominent pattern of CGRP expression is in perivascular nerve fibers surrounding peripheral and cerebral blood vessels (Brain et al., 1985). Physiological studies have demonstrated that CGRP is one of the most potent vasoactive neuropeptides known (Brain et al., 1985; McCulloch et al., 1986; Preibisz, 1993). It appears that a major function of CGRP released in cerebral vessels is a compensatory response to vasoconstriction (Brain et al., 1985; Goadsby and Edvinsson, 1993; Edvinsson and Goadsby, 1994). The vasodilatory activity of CGRP has been supported by findings with two of the three pedigrees of CGRP knockout mice (Lu et al., 1999; Gangula et al., 2000; Oh-hashi et al., 2001). CGRP triggers mast cell release of pro-inflammatory cytokines and compounds that contribute to neurogenic inflammation, which is coincident with vasodilation (Preibisz, 1993; Ottosson and Edvinsson, 1997). Furthermore, CGRP also modulates nociceptive input via central pathways (Cumberbatch et al., 1999). Injection of CGRP into the trigeminal nucleus of the brainstem elicits a cardiovascular response that is similar to painful stimuli (Bereiter and Benetti, 1991; Allen et al., 1996). Importantly, CGRP knockout mice have altered neurogenic inflammatory nociception in response to somatic and visceral pain (Salmon et al., 2001). Thus, CGRP acts as a potent neuromediator of vascular tone and nociception. These and other functions of α-CGRP will be discussed in greater detail in the following sections.

A focus of this review is the trigeminal ganglion, which is the major source of both CGRP and sensory nerves that connect the CNS with craniofacial structures and the cerebrovasculature (McCulloch et al., 1986; O'Connor and van der Kooy, 1986). CGRP is present primarily in unmyelinated nociceptive fibers (O'Connor and van der Kooy, 1988). The importance of trigeminovascular CGRP is highlighted by a report that human cerebral arteries are ten times more sensitive than coronary arteries to CGRP (Edvinsson et al., 2002). Notably, there are several CGRP pathologies that involve the trigeminal ganglion, which will be discussed later.

## 2.4 β-CGRP

A second gene encodes the highly homologous β-CGRP neuropeptide (CALCB, also called CGRP II) that differs by only 1–3 residues in different species (Amara et al., 1985; Steenbergh et al., 1985). As noted above, the CGRP produced from the CT/CGRP gene is α-CGRP. In contrast to the CT/CGRP gene, the β-CGRP gene does not encode a CT peptide. As expected by their similar sequences, the α and β isoforms have almost identical activities (Holman et al., 1986; Jansen-Olesen et al., 1996). The α and β isoforms of CGRP are expressed in distinct but overlapping regions of the nervous system and appear to be differentially regulated (Amara et al., 1985; Russo et al., 1988). In general β-CGRP is more predominantly expressed in motor neurons, while α-CGRP is expressed in sensory neurons (Amara et al., 1985; Mulderry et al., 1988). For example, there is ten times more α-CGRP than β-CGRP mRNA in the trigeminal ganglia, and relaxation of human cerebral arteries following trigeminal activation is apparently mediated by α-CGRP (Amara et al., 1985; Jansen-Olesen et al., 1996).

## 2.5 Adrenomedullin

About 10 years ago, the multifunctional peptide adrenomedullin (AM, also called ADM) was identified from pheochromocytomas of the adrenal medulla (Kitamura, 1993). Most of the biological activity of AM resides in the terminal 40 residues (AM13–52), which has 25% sequence similarity with human CGRP. There are several excellent reviews on AM (Hinson et al., 2000; Eto et al., 2003; Brain and Grant, 2004). AM and CGRP have overlapping activities in the vasculature and kidney (Eto and Kitamura, 2001; Nishikimi et al., 2002; Eto et al., 2003). Like CGRP, AM causes vasodilation of blood vessels. In contrast to the neuronal expression of CGRP, AM is produced by a wide variety of cell types, including vascular endothelial and smooth muscle cells, neurons, cardiomyocytes, fibroblasts, and macrophages. In the kidney, AM is a natriuretic peptide and it is believed to play a generally protective autocrine and paracrine function (Eto and Kitamura, 2001; Mukoyama et al., 2001; Nishikimi et al., 2002). AM levels are elevated

in several pathologies, including hypertension, renal failure, heart failure, and sepsis (Eto and Kitamura, 2001).

### 2.6 Amylin

Amylin is a 37-amino-acid pancreatic peptide that has 43% identity with human CGRP. Amylin was initially found when analyzing pancreatic amyloid deposits in diabetic patients (Cooper et al., 1987; Westermark et al., 1987). It is cosecreted with insulin from islet beta cells. The expression and function of amylin has been recently reviewed (Hoppener et al., 2000). While amylin has some osteoclastic and vasodilatory activity, its major function appears to be the regulation of glucose metabolism. Evidence that amylin can also decrease food intake in animals has raised interest in its potential therapeutic applications, especially for controlling obesity.

### 2.7 Other Family Members

A more distant member of the CGRP gene family is the intermedin peptide, which has been recently reviewed (Chang et al., 2004; Roh et al., 2004). There is only 15% identity between intermedin and CGRP, but up to 30% identity with AM. Intermedin acts via CLR and RAMPs 1 and 3, and shares biological activities with CGRP.

Most recently, a calcitonin receptor-stimulating peptide (termed CRSP) gene family has been identified (Katafuchi et al., 2003). Several CRSP peptides have been found in the pig, cow, dog, and horse, but the equivalent genes have not been found in humans and rodents (reviewed in Katafuchi and Minamino, 2004). The CRSP members appear to act via the CT receptor and help control calcium levels. The significance of the CRSP peptides remains to be established. Likewise, the molecular identity and biological roles of novel CT-like peptides found in the brain remains to be determined (Hilton et al., 1998; Pondel, 2000).

## 3 Control of CGRP Gene Expression

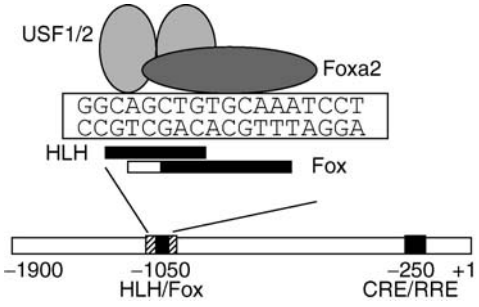
Regulation of CGRP expression in response to extracellular stimuli is controlled exclusively at the transcriptional level. While CGRP earned its early notoriety as an alternative RNA splicing product of the CT gene, splicing does not change once the cellular phenotype is established.

### 3.1 Regulated Neuronal Expression

The CT/CGRP gene is predominantly expressed in neurons and neuroendocrine cells. Transcription of the gene is enhanced by protein kinases A and C (de Bustros et al., 1986, 1990; Monla et al., 1995; Supowit et al., 1995a), and agents that act via MAP kinases (Nakagawa et al., 1987; Watson and Latchman, 1995; Durham and Russo, 1998). Transcription is inhibited by vitamin D (Peleg et al., 1993), retinoic acid (Lanigan et al., 1993), and 5-hydroxytryptamine 1 ($5\text{-HT}_1$) agonists that are commonly used as antimigraine drugs (Durham et al., 1997, 2004; Durham and Russo, 2003). Glucocorticoids can either activate or repress CT/CGRP transcription in a cell-specific manner (Russo et al., 1988; Collignon et al., 1992; Tverberg and Russo, 1992).

All agents that increase or decrease CT/CGRP transcription act through two elements (❯ *Figure 19-2*). The elements are almost identical in the rat and human CT/CGRP promoters. A distal element is the neuroendocrine cell-specific enhancer located approximately 1,000 nucleotides upstream of the transcription start site. A proximal element is responsible for cAMP- and Ras-mediated enhancement and is located approximately 250 nucleotides upstream of the start site.

**Figure 19-2**
**CGRP gene regulatory regions. The HLH/Fox cell-specific enhancer and cAMP and Ras responsive elements (CRE/RRE) and non-cell-specific enhancer sites (*hatched boxes*) that flank the HLH/Fox element are also shown. The coordinates are for the rat CGRP gene. The USF and Foxa2 proteins bind independently to overlapping motifs indicated by the *solid bars* (*open bar* indicates a partial role). An identical motif is in the human gene**




## 3.2 Proximal cAMP-Responsive Element

The proximal element of the CT/CGRP gene contains an overlapping set of motifs that are responsive to signal transduction pathways induced by cAMP (de Bustros et al., 1986; Monia et al., 1995), nerve growth factor (NGF) (Watson and Latchman, 1995), and the activated Ras protein (Nakagawa et al., 1987; Thiagalingam et al., 1996). It contains a canonical cAMP response element (CRE) that functions as a binding site for cAMP response element binding protein (CREB) and related family members. Fine mapping revealed an additional nearby cAMP-responsive element that contains overlapping motifs: a CRE-like motif flanked by a downstream octamer homeodomain-like binding site.

It appears that the CRE-like/octamer site can function without the CREB element since mutation of the CRE reduced cAMP-induced transcription in thyroid C cell lines, but did not abolish it. Conversely, mutation of the CRE-like/octamer motif diminished the activity of the CRE site (Monia et al., 1995). The function of these cAMP-responsive elements is cell specific to some extent. Most notably, the CRE-like/octamer motif is functional only in CT/CGRP-positive thyroid C cell lines.

## 3.3 Distal Cell-Specific HLH Enhancer

CT/CGRP gene expression is restricted almost exclusively to thyroid C cells and a subset of peripheral and central neurons, along with a scattered population of neuroendocrine cells in the lung, prostate, and pituitary. A distal enhancer in the rat and human genes has been identified that is active in thyroid C cell lines (Tverberg and Russo, 1993; Lanigan and Russo, 1997), trigeminal and dorsal root ganglia (DRG) in transgenic mice (Stolarsky-Fredman et al., 1990; Baetscher et al., 1991), and neurons of trigeminal ganglia primary cultures (Durham and Russo, 2003; Durham et al., 2004). It is a complex enhancer. There are three functional CANNTG motifs that bind helix–loop–helix (HLH) proteins (Peleg et al., 1990; Stolarsky-Fredman et al., 1990; Ball et al., 1992; Tverberg and Russo, 1992, 1993). The HLH motifs are flanked by elements that bind cell-specific and non-cell-specific transcription factors. A 1.25-kb promoter fragment is able to direct reporter gene expression predominantly to neurons. Approximately 90% of the cells that express the reporter were neurons (Durham et al., 2004). These data document that the CGRP promoter is preferentially active in neurons and neuroendocrine cells.

Mapping studies have identified a key 18-bp element within the distal cell-specific enhancer. The 18-bp enhancer retains cell specificity in thyroid C cells and sensory neurons (Tverberg and Russo, 1993; Lanigan and Russo, 1997; Durham and Russo, 2003; Durham et al., 2004). This enhancer has overlapping HLH and forkhead (Fox) motifs that are required for synergistic activation of transcription (*Figure 19-2*) (Tverberg

and Russo, 1993; Lanigan and Russo, 1997; Viney et al., 2004). Mutation of a single HLH site within the enhancer greatly reduces promoter activity even in the context of the remaining HLH sites (Tverberg and Russo, 1993; Lanigan and Russo, 1997). In the neuronal-like thyroid C cell lines, a heterodimer of the basic HLH-leucine zipper (bHLH-Zip) proteins upstream stimulatory factor (USF)-1 and -2 and the cell-specific protein Foxa2 bind the HLH site in the 18-bp enhancer. USFs are ubiquitous proteins, yet paradoxically they can contribute to cell-specific gene expression (Massari and Murre, 2000). The cell-specific HLH transcription factor mammalian achaete-scute homolog (MASH-1) was initially considered as a candidate cell-specific transcription factor since it is expressed in normal C cells and C cell lines (Ball, 1992; Lanigan et al., 1998). However, it is now clear that MASH-1 is not required for expression of the CT/CGRP gene (Guillemot, 1995; Lanigan et al., 1998). We have proposed that the suboptimal USF binding site in the 18-bp enhancer is needed to allow optimal binding of Foxa2 to the overlapping site (Lanigan and Russo, 1997; Viney et al., 2004). A similar paradigm has been reported for USF control of an islet-specific gene (Martin et al., 2003). Whether USF synergizes with a Fox protein in neurons remains an open question. While we can rule out a role for Foxa2 since it is not expressed in trigeminal ganglia, there are other neuronal Fox proteins, which raises the possible involvement of another Fox family member (Viney et al., 2004) in CT/CGRP transcription.

## 3.4 MAP Kinase Control of CGRP Transcription

CGRP gene expression is activated by MAP kinases. The major MAP kinase families, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, are at the convergence of signaling cascades that transduce extracellular signals to the nucleus (Seger and Krebs, 1995). The first evidence that MAP kinases control CGRP gene expression was from studies by Nelkin and coworkers using Ras, an upstream activator of MAP kinases (Nakagawa et al., 1987). Ras was shown to act through an element near the CRE that binds a novel zinc-fingered protein (Thiagalingam et al., 1996). Later studies found that the cell-specific 18-bp enhancer is also activated by MAP kinases and chemical depolarization (Durham and Russo, 1998, 2000, 2003). Physiological stimuli that activate MAP kinases, such as NGF and depolarization, have been shown to stimulate CGRP enhancer activity. NGF treatment also increases CGRP gene expression in postnatal trigeminal and DRG (Lindsay and Harmar, 1989; Durham and Russo, 2003). In cell lines, NGF acts in a cell-specific manner through the CRE and flanking elements and upstream sequences that include the distal enhancer (Lindsay and Harmar, 1989; Watson and Latchman, 1995; Durham and Russo, 1998, 2003). The possibility that signals from these factors may target USF at the enhancer is supported by reports that USF is involved in $Ca^{2+}$ activation of other promoters (Tabuchi et al., 2002; Chen et al., 2003) and that USF can be phosphorylated by p38 MAP kinase (Galibert et al., 2001).

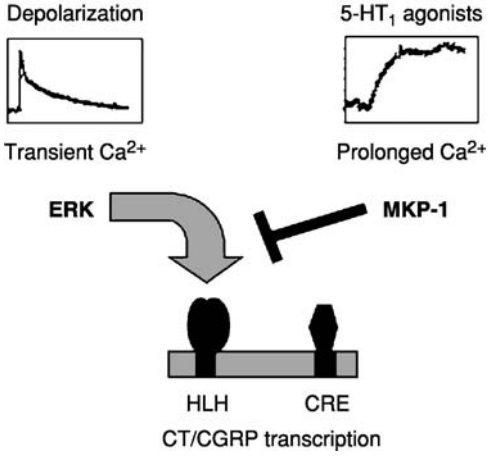
5-$HT_1$ receptor agonists that are currently used as antimigraine drugs can inhibit CGRP promoter activity (Durham and Russo, 2003; Durham et al., 2004). In patients, these drugs lower CGRP levels and relieve migraine pain (Ferrari, 1998). Both ERK MAP kinase stimulation and repression by 5-$HT_1$ agonists were mapped to the 18-bp cell-specific enhancer (Durham and Russo, 1998, 2003). We therefore reasoned that 5-$HT_1$ agonists might act by inhibiting MAP kinases. Indeed, a 5-$HT_1$ agonist can induce MAP kinase phosphatase-1 (MKP-1) expression in a neuronal-like thyroid C cell line (Durham and Russo, 2000), and MKP-1 is sufficient to repress enhancer activity in neurons (Russo, unpublished data). MKP-1 is a dual-specific phosphatase that can inactivate multiple MAP kinases (Keyse, 1995). The angiotensin type 2 and possibly insulin receptors have also been shown to repress MAP kinase activity by elevating MKPs (Horiuchi et al., 1997; Kusari et al., 1997).

An unexpected finding was that activation of 5-$HT_1$ receptors caused a sustained increase in intracellular $Ca^{2+}$ in neurons that was sufficient to repress the CGRP promoter (Durham and Russo, 2000, 2003). The effect of intracellular $Ca^{2+}$ on gene expression is determined by many parameters, including signal amplitude and duration (Berridge et al., 2003). A transient increase in intracellular $Ca^{2+}$, such as following depolarization, stimulates MAP kinase activation of the CGRP enhancer. Such activation has been widely seen with other MAP kinase-responsive genes (Rosen et al., 1994; Ghosh and Greenberg, 1995; MacArthur and Eiden, 1996). What is less appreciated is that a prolonged elevation of $Ca^{2+}$ can have the opposite effect

and decrease MAP kinase activity. Meloche and coworkers demonstrated that increased intracellular $Ca^{2+}$ was necessary and sufficient for induction of MKP-1 expression in a fibroblast cell line (Scimeca et al., 1997). In trigeminal neurons and thyroid C cell lines, we propose that this prolonged elevation of $Ca^{2+}$ induces a negative feedback loop due to MKP-1 induction, which leads to repression of the CT/CGRP gene. Importantly, we were able to use ionomycin treatment to demonstrate that the prolonged $Ca^{2+}$ elevation is sufficient to induce MKP-1 expression and repress CGRP gene expression (Durham and Russo, 2000, 2003). Thus, there appears to be a dynamic balance between MAP kinases and phosphatases that control the CGRP gene (*Figure 19-3*).

**Figure 19-3**

**Model of CGRP gene regulation by differential calcium signals. Transient calcium signals, such as following depolarization, can activate ERK MAP kinase to stimulate CGRP transcription. Conversely, a prolonged calcium signal, such as following 5-$HT_1$ agonist treatment, can elevate MKP-1, which inhibits MAP kinases. This leads to decreased CGRP transcription**

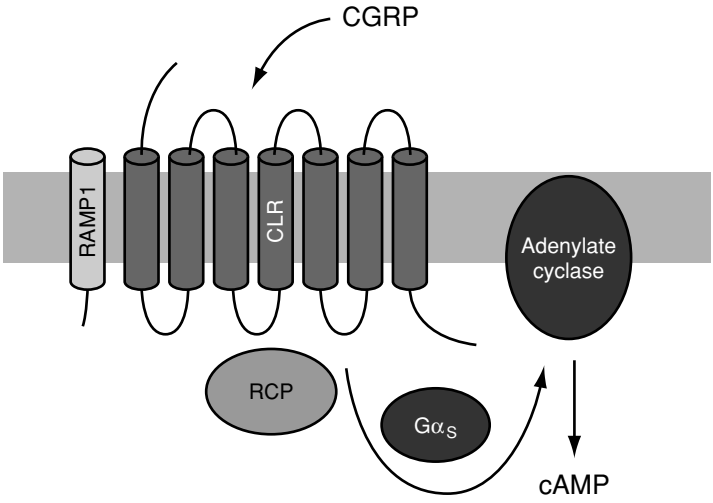



## 4 CGRP Receptor

The receptor for CGRP has proven an elusive quarry. This has been in part due to its unusual subunit requirements, which is discussed below (*Figure 19-4*). CGRP receptors have been reported in many tissues, including brain, heart, lung, kidney, spleen, and skeletal muscle (Poyner, 1992; van Rossum et al., 1997). Two original ligands were used to distinguish subtypes of CGRP receptors. An agonist, [acetoamidomethylcysteine2,7]CGRP ([Cys(ACM)2,7]CGRP), is a linear analog of CGRP in which the two cysteine residues at positions 2 and 7 have been covalently reduced. An antagonist, CGRP (8–37), represents the carboxyl 30 amino acids of mature CGRP, including the carboxylamide group. Two types of CGRP receptors were initially described using these CGRP analogs. Type I receptors were relatively insensitive to [Cys(ACM)2,7]CGRP until micromolar concentrations were used, but are sensitive to the antagonist CGRP (8–37) at nanomolar concentrations (Dennis et al., 1989, 1990; Longmore et al., 1994). Type I CGRP receptors were reported in atria, spleen, and in the SK-N-MC neuroblastoma cell line (Van Valen et al., 1990; van Rossum et al., 1997). In contrast, [Cys(ACM)2,7]CGRP was reported to be a potent agonist at type II CGRP receptors, with an $EC_{50}$ of approximately 70 nM (Dennis et al., 1989). CGRP (8–37) was not reported to be as effective at type II receptors, requiring micromolar concentrations to be effective (Chiba et al., 1989; Dennis et al., 1990). Type II CGRP receptors were described in vas deferens, liver, and kidney (Chiba et al., 1989; Dennis et al., 1990). A distinction between CGRP receptors was also reported in the

**Figure 19-4**

**CGRP receptor. Three proteins are required for CGRP receptor function: Calcitonin-like receptor (CLR) contains seven hydrophobic domains predicted to be transmembrane domains associated with the topology of a stereotypic G-protein-coupled receptor (GPCR). Receptor activity modifying protein (RAMP1) contains a single transmembrane domain and determines pharmacologic specificity of CLR for CGRP, acting as a chaperone for routing CLR to the cell surface. RAMP1 is also dependent on CLR for trafficking to the cell surface. The CGRP receptor component protein (RCP) is an intracellular peripheral membrane protein that is required for CLR/RAMP1 signal transduction**




vasculature, where CGRP was found to be a more potent agonist on small-caliber vessels than on large-caliber vessels (Foulkes et al., 1991). Furthermore, the antagonist CGRP (8–37) was less effective in the large-caliber vessels. Similarly, the cerebral arteries have been reported to be ten times more sensitive to CGRP than coronary arteries (Edvinsson et al., 2002), suggesting distinct CGRP receptor populations. However, despite data suggesting two classes of receptor for CGRP, as described below, the molecular identity has only been defined for the type I CGRP receptor pharmacology. Thus, there are either additional undiscovered CGRP receptors or the pharmacology of the known receptor can be modified by its expression itself or by the expression of accessory proteins.

## 4.1 Calcitonin-like Receptor

Application of CGRP to target tissues and cells often results in elevated levels of intracellular cAMP (Edvinsson et al., 1985; Hirata et al., 1988; Chiba et al., 1989; Van Valen et al., 1990; Crook and Yabu, 1992), suggesting the presence of a GPCR. Consistent with this GPCR hypothesis it was discovered that CGRP binding to receptors in cerebellum and cardiac myocytes was inhibited by GTPγS (Chatterjee et al., 1991, 1993; Chatterjee and Fisher, 1995). However, while straightforward tissue-culture-based expression cloning strategies identified a complementary DNA (cDNA) for the calcitonin receptor (CTR) (Lin et al., 1991), similar efforts targeting the CGRP receptor were unsuccessful. A polymerase chain reaction (PCR) cloning strategy did identify a candidate CGRP receptor cDNA from rat cerebellum using PCR primers based on conserved regions in the CTR (Chang et al., 1993). However, this receptor, called the calcitonin receptor-like receptor (CRLR), did not recognize CGRP; it and a subsequently identified human homolog (Fluhmann et al., 1995) were therefore described initially as orphan receptors without known ligands. An additional GPCR named RDC1 was described at one time as a CGRP receptor (Kapas and Clark, 1995), but

subsequent studies have been unable to confirm the initial finding, and RDC1 is currently classified as an orphan GPCR.

In 1996 Aiyar et al. used expressed sequence tag analysis to identify a cDNA named CGRP1 from a human synovial cDNA library (Aiyar et al., 1996). CGRP1 had a sequence identical to the previously identified CRLR, and when transfected into human embryonic kidney (HEK293) cells, CGRP1 resulted in expression of high-affinity type I CGRP receptors, which responded with an increase in intracellular cAMP to incubation with CGRP, consistent with the earlier pharmacologic studies. In contrast, CGRP1 was not effective when transfected into COS cells, a finding confirmed by Han et al. (1997). These transfection studies suggested that CGRP1/CRLR required additional protein factors for function, and that these proteins were not expressed in COS cells. The CGRP receptor nomenclature has subsequently been condensed, and CRLR (CGRP1) is now referred to as the calcitonin-like receptor (CLR) (*Figure 19-4*) (Poyner et al., 2002). CLR is a member of the family B (class II) GPCRs, having most homology to the receptors for vasoactive intestinal peptide, growth hormone releasing hormone, pituitary adenylate cyclase activating peptide, glucagon, glucagon-like peptide, secretin, and calcitonin (Harmar, 2001).

## 4.2 Receptor Activity Modifying Protein

One protein required for CLR function is RAMP1. RAMP1 was discovered in a *Xenopus laevis* oocyte-based expression cloning strategy (McLatchie et al., 1998), and the RAMP1 cDNA encodes a small (148-amino-acid) single transmembrane protein with a large extracellular amino terminus and a short intracellular carboxyl tail. RAMP1 cDNA confers CGRP responsiveness when expressed alone in oocytes, but not when transfected alone into tissue culture cells. However, when the RAMP1 cDNA was cotransfected with the CLR cDNA in cell culture, a high-affinity CGRP receptor was observed. Interestingly, fluorescence activated cell sorter (FACS) analysis in these studies indicated that CLR was not efficiently transported to the cell surface when expressed alone; nor was RAMP1. These data suggest that RAMP1 and CLR require each other for correct folding and export to the cell surface. Two additional RAMPs were identified (RAMP2 and RAMP3), which, while sharing only 30% identity with RAMP1, were found to also interact with CLR and resulted in expression of AM receptors when cotransfected with CLR in cell culture (McLatchie et al., 1998; Muff et al., 1998; Buhlmann et al., 1999; Kamitani et al., 1999). RAMPs can also work in conjunction with CTR, where they result in an amylin receptor (Christopoulos et al., 1999; Muff et al., 1999; Tilakaratne et al., 2000; Zumpe et al., 2000). Interestingly, the combination of CTR/RAMP1 can also bind CGRP, with an $IC_{50}$ approximately tenfold less sensitive than for amylin (Christopoulos et al., 1999). This cross-reactivity between CGRP and CTR/RAMP1 may explain earlier findings that CGRP could compete with labeled amylin for binding in the nucleus accumbens (Beaumont et al., 1993; van Rossum et al., 1994).

How the RAMPs dictate pharmacologic specificity of CLR is still unclear. Initial studies indicated that the glycosylation state of CLR could affect ligand specificity (McLatchie et al., 1998). Expression of CLR and RAMP1 resulted in detection of CLR with an apparent molecular weight of 66 kDa with CGRP binding, while expression of CLR and RAMP2 resulted in expression of a 58-kDa form of CLR with AM binding. Both these forms of CLR could be reduced to 48 kDa by treatment with endoglycosidase F, although only the 58-kDa form of CLR was sensitive to treatment with endoglycosidase H. These results suggested differential glycosylation that was RAMP-dependent. This led to early thoughts that the glycosylation state of CLR could regulate ligand specificity. However, cotransfection of CLR and either RAMP1 or RAMP2 into *Drosophila* Schneider 2 cells resulted in expression of a 58-kDa endoglycosidase-sensitive form of CLR with both RAMP1 and RAMP2, while maintaining the CGRP and AM specificity of the CLR/RAMP1 and CLR/RAMP2 dimers (Aldecoa et al., 2000). These data suggested that it was expression of the RAMP that guided CLR specificity, and not the glycosylation state of the receptor. Interestingly, a subsequent study (Hilairet et al., 2001b) found that while the major CLR species identified upon cotransfection with RAMP2 was the 58-kDa form observed previously by McLatchie et al. (McLatchie et al., 1998), the 66-kDa form was faintly observed in total extract, and, importantly, was the predominant species at the cell surface. This suggested that the glycosylation state of the cell surface form of CLR in a CLR/RAMP2 dimer was fully glycosylated, as observed for the CLR/RAMP1 dimer. In the later studies, coexpression of

CLR and RAMP3 resulted in approximately equal expression of the 66- and 58-kDa form of CLR in both total extract and surface membrane populations, while coexpression of CLR/RAMP1 resulted in the 66-kDa form of CLR in both total and membrane fractions. In these studies, cells were transfected with CLR and either RAMP1 or RAMP2, incubated with either $^{125}$I-CGRP or $^{125}$I-AM, cross-linked, and CLR was immunoprecipitated. The immunoprecipitate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, and labeled CGRP coimmunoprecipitated primarily with cells transfected with CLR/RAMP1, and AM coimmunoprecipitated from cells transfected with CLR/RAMP2. Importantly, only the 66-kDa form of CLR was identified by autoradiography in these studies, and therefore postulated to interact with the labeled ligand.

RAMP1 can be divided into the extracellular amino-terminal domain, the transmembrane domain, and the cytoplasmic carboxyl domain. The requirement for each of these domains has been tested by several investigators. The extracellular domain of RAMP1 contains six cysteine residues, and RAMP1 dimers are observed that are susceptible to reducing agents such as dithiothreitol (Hilairet et al., 2001a). Interestingly, when transfected into cell culture RAMP1 is observed primarily as intracellular dimers, but coexpression with CLR results in a shift from primarily RAMP1 dimers to monomers, and a concomitant translocation to the cell surface (Hilairet et al., 2001a). There are six conserved cysteine residues in the 90-residue extracellular domain of RAMP1 and, of these, four are required for trafficking to the cell surface and formation of a signaling- and binding-competent CGRP receptor (Steiner et al., 2003). These data suggest that RAMP1 requires disulfide bond formation for creation of a functional extracellular domain.

The extracellular amino terminus of the RAMP molecule governs the pharmacologic specificity of the RAMP/CLR dimers. Chimeric RAMP molecules were made that contained the extracellular amino terminus of RAMP2 fused with the transmembrane and intracellular domain of RAMP1 (RAMP2/RAMP1) or the extracellular amino terminus of RAMP1 fused with the transmembrane and cytoplasmic domain of RAMP2 (RAMP1/RAMP2). Upon cotransfection with CLR the chimeric RAMP2/RAMP1 molecules conferred AM receptor in cell culture, and RAMP1/RAMP2 conferred CGRP receptors (Fraser et al., 1999; Zumpe et al., 2000; Hilairet et al., 2001b).

Expression of the extracellular domain of RAMP1 as a soluble protein or as a fusion protein with the membrane-spanning and cytoplasmic domains of the PDGF receptor resulted in interaction with CLR, and facilitated CLR trafficking to the cell surface, CGRP-mediated signaling, and CGRP binding (Fitzsimmons et al., 2003). In these experiments the soluble form of the RAMP1 extracellular domain was significantly less effective than the membrane-delimited forms, possibly due to the fact that the soluble form was secreted, thereby decreasing the probability of the two molecules (RAMP1 extracellular domain and CLR) interacting at the cell membrane. These results agree with earlier experiments that identified residues 91–103 of the RAMP1 extracellular domain as important for generating the CGRP receptor phenotype when coexpressed with CLR (Kuwasako et al., 2003a). However, these results disagree with a previous study that found the transmembrane domain to be required for CLR signaling and trafficking (Steiner et al., 2002). In the previous study, progressive deletions were made from the carboxyl end of the human RAMP1 cDNA extending into the transmembrane domain, and the carboxyl-shortened mutants were cotransfected with CLR into cell culture for analysis. The carboxyl tail of RAMP1 could be deleted without affecting the $EC_{50}$ for CGRP-stimulated cAMP production, but once deletions extended into the transmembrane domain, signaling was significantly inhibited. Interestingly, addition of a consensus endoplasmic reticulum (ER)-retention signal to a transmembrane deletion could restore signaling to control levels, suggesting that increased time in the ER may enhance association of RAMP1 with CLR. When RAMP1 trafficking was analyzed by microscopy, it was noted that most of the carboxyl-deletion mutants had lost the dependence on CLR for cell surface expression. In particular, deleting the first four residues from the carboxyl end of RAMP1 maintained CLR dependence, but deletions that included an additional four residues from the carboxyl end of RAMP1 were expressed on the cell surface, as were further deletions into the transmembrane domain. This suggests the presence of an ER-retention signal in the four residues immediately adjacent to the cell membrane, which when deleted allow rapid export of RAMP1 from the ER to the cell surface. The basis for the difference in the role of the RAMP1 transmembrane domain in these two studies is not clear, although expression of additional proteins required for receptor function (such as RCP, see below) may vary between the cell lines used by the two research groups.

Recently, a nonpeptide antagonist named BIBN4096BS has been developed to the CGRP receptor (Doods et al., 2000). Interestingly, BIBN4096BS exhibits species selectivity, binding to the human CGRP receptor with 100-fold higher affinity than the CGRP receptor in other species (Doods et al., 2000; Edvinsson et al., 2001). The species selectivity appears to be due to BIBN4096BS interactions with the RAMP1 molecule. When combinations of rat or human CLR were cotransfected into cell culture with rat or human RAMP1, high-affinity binding was associated with the human RAMP1, whether cotransfected with rat or human CLR (Mallee et al., 2002; Hershey et al., 2005). Furthermore, in an elegant series of experiments, two chimeric RAMP1 cDNAs were constructed, which replaced either the first 66 amino acids of the extracellular domain of rat RAMP1 with the corresponding human sequence, or the first 112 amino acids of human sequence with rat. When cotransfected with CLR into cell culture, only the chimeric RAMP1 containing the 112-amino-acid human substitution had a high affinity for BIBN4096BS similar to human RAMP1, suggesting that the interacting domain resided between residues 66 and 112. An alignment of RAMP1 from multiple species identified a variable amino acid at position 74, which was a tryptophan in humans and marmosets (high-affinity BIBN4096BS binding) and a basic residue in rats, mice, or pigs (low-affinity BIBN4096BS binding). When the rat RAMP1 cDNA was mutated to change lysine$^{74}$ to tryptophan$^{74}$, high-affinity BIBN4096BS binding was revealed upon cotransfection with CLR. Thus, the species specificity of BIBN4096BS was largely dependent on a single amino acid residue in the extracellular domain of RAMP1, irregardless of which species of receptor was cotransfected with the chimeric RAMP.

The CLR/RAMP1 protein complex is internalized following exposure to ligand. A chimeric CLR was constructed with a carboxyl fusion to the green fluorescent protein (GFP). When transfected alone into cell culture the CLR–GFP was found primarily in a cytoplasmic pool presumed to be ER (Kuwasako et al., 2000). However, when cotransfected with RAMP1, the fraction of CLR–GFP found on the cell surface increased, suggesting that RAMP1 facilitated the trafficking of CLR–GFP to the cell surface. CLR–GFP was removed from the cell surface following exposure to CGRP, and this loss was inhibited by pretreatment with hypertonic medium, suggesting that CLR was internalized by clathrin-coated pits (Daukas and Zigmond, 1985; Zigmond et al., 1985). A second study found that addition of CGRP to cells transfected with RAMP1 and CLR resulted in a time-dependent loss of surface CLR and RAMP1, as expected for receptors undergoing internalization (Hilairet et al., 2001a). Interestingly, in these studies the kinetics of loss of cell surface CLR and RAMP1 were quite similar, yet the level of CLR and RAMP1 coimmunoprecipitation from cell lysate did not change after exposure to CGRP, suggesting that the internalized CLR was still in association with RAMP1. Internalization of the CLR/RAMP1 complex was inhibited by coexpression of dominant-negative mutants of β-arrestin, suggesting that internalization occurred via clathrin-coated pits.

## 4.3 Regulation of CLR Function by RAMP1

Increased RAMP1 and RAMP3 expression has been detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting in rat atria and ventricle in failing hearts following aortic banding (Cueille et al., 2002). In these studies no change in CLR expression was detected, suggesting the possibility of increased CGRP receptor function if the upregulated RAMP was working with existing CLR.

RAMP1 mRNA may also be upregulated in the rat uterus during pregnancy. Messenger RNA was isolated from the uterus of pregnant and control rats, and CLR and RAMP1 expression was monitored by RT-PCR (Thota et al., 2003). In these studies expression of both RAMP1 and CLR increased in the pregnant state.

## 4.4 Receptor Component Protein

A second protein required for CLR function is the CGRP-RCP. RCP is a small (148-amino-acid) hydrophilic intracellular peripheral membrane protein that was cloned using a *Xenopus* oocyte-based expression cloning assay (Luebke et al., 1996; Evans et al., 2000; Prado et al., 2001). RCP is highly conserved between humans, mice, rabbits, and guinea pigs (Prado et al., 2002), yet contains no obvious protein motifs that

suggest function. RCP is expressed in most immortalized cell lines, although it is not yet known if this is a cause or a result of the immortalization process. RCP expression was inhibited in cell culture using an antisense strategy, and in the RCP-depleted cells CGRP and AM maximal signal transduction was significantly diminished with minimal effect on $EC_{50}$, while signalings at other GPCRs endogenous to the host cell line were unchanged (Evans et al., 2000; Prado et al., 2001). No change in either affinity for CGRP or receptor density was observed in these studies using $^{125}$I-CGRP binding, suggesting that RCP was coupling the CLR/RAMP1 complex to the cellular signaling pathway. RCP coimmunoprecipitated with CLR and RAMP1 from cell culture and from cerebellar extracts in these studies, suggesting that the CGRP receptor was a complex of the three proteins. RCP can be removed from cell membranes by incubation with salt, suggesting an ionic interaction between RCP and the CLR/RAMP1 complex (Evans et al., 2000; Prado et al., 2001).

In contrast to cell culture, RCP is expressed in defined cell types in vivo. Using in situ hybridization, RCP was found in discrete cell types in the guinea pig brain, with highest expression in the anterior olfactory nucleus, the granular layer of the cerebellum, and the gyrus, CA1, CA2, and CA3 regions of hippocampus (Oliver et al., 1999). Similar in situ expression patterns have been reported for rat brain (Oliver et al., 2001). Using immunohistochemistry, expression of RCP was investigated in rat brain, where it was found juxtaposed with CGRP immunoreactivity (Ma et al., 2003). The protein expression of RCP in these immunohistochemistry studies agreed with the previous in situ data on RCP mRNA expression (Oliver et al., 1999, 2001). Interestingly, approximately 40% of the CGRP-immunoreactive neurons in the DRG were also RCP-immunoreactive, as were the majority of motoneurons of the lumbar spinal cord and the hypoglossal nucleus, suggesting a presynaptic CGRP receptor (Ma et al., 2000). A correlation between CGRP-immunoreactive cells and RCP-immunoreactive cells has also been observed in the dorsal horn of the rat spinal cord (Pokabla et al., 2002). CGRP-immunoreactive cells were detected primarily in laminae I and II of the dorsal horn, while RCP immunoreactivity was detected primarily in laminae II but also in laminae III, with nerve terminals juxtaposed primarily in laminae II. Double immunostaining of DRG neurons in these studies also detected cells that exhibited cytoplasmic CGRP immunoreactivity with a membrane-associated RCP immunoreactivty, suggesting a population of DRG neurons that expressed presynaptic CGRP receptors.

CGRP is expressed in trigeminal nerve fibers that innervate the anterior eye, and CGRP-binding sites have been reported in the ciliary process and iris of the eye (Malminiemi and Malminiemi, 1992; Heino et al., 1995). CGRP can mediate the increased intraocular pressure, breakdown of the blood–aqueous barrier, and increased levels of cAMP in the aqueous humor associated with the ocular neurogenic inflammatory response (Unger et al., 1985; Unger, 1989, 1990; Krootila et al., 1992). These data suggest that CGRP and its receptors can mediate the inflammatory response in the eye. RCP expression was detected in rabbit eye by immunohistochemistry in the epithelial cells and the blood vessels of the ciliary processes and the iris (Rosenblatt et al., 2000). The vascular localization is consistent with the increased blood flow attributed to CGRP in the neurogenic inflammatory response (Krootila et al., 1988; Oksala, 1988; Almegard and Andersson, 1993). Nerve fibers that release CGRP are in close proximity to the ciliary epithelial cells (Terenghi et al., 1985, 1986; Uusitalo et al., 1989), and RCP immunoreactivity suggests the presence of CGRP receptors in the ciliary epithelial cells. This anatomic colocalization of CGRP-containing neurons and RCP-immunoreactive ciliary epithelial cells is consistent with actions of intraocular CGRP, which causes increased secretion of aqueous humor from the ciliary epithelium and a concomitant rise in intraocular pressure (Unger, 1989, 1992). As the tight junctions of the ciliary epithelial cells are the anatomic site of the blood–aqueous barrier, CGRP may be acting directly on these cells to disrupt the blood–aqueous barrier. RCP was also observed in the lens in these studies, although a role for CGRP in the lens is not currently understood.

## 4.5 Regulation of CGRP Receptor Function by RCP

Expression of CGRP-RCP is regulated in vivo, and as such has the potential to regulate CLR function. During pregnancy, the myometrial smooth muscle of the uterus undergoes a dramatic change, from weak

contractions during gestation to strong synchronized contractions at parturition. CGRP has been characterized as an inhibitor of smooth muscle contraction (Samuelson et al., 1985; Shew et al., 1990; Tritthart et al., 1992), and CGRP neurons innervate the uterus, where CGRP inhibits evoked and spontaneous contractions (Samuelson et al., 1985; Haase et al., 1997). The ability of CGRP to inhibit myometrial contractions was shown to vary during gestation in mice in direct proportion to RCP expression. By using dissected myometrial strips that were assayed for contractile ability and then analyzed for RCP mRNA and protein expression, it was shown that the ability of CGRP to inhibit acetylcholine (ACh)-induced contractions correlated with expression of RCP protein, suggesting that RCP could regulate myometrial CGRP receptor function (Naghashpour et al., 1997).

A similar correlation between RCP expression and myometrial function was observed during the estrus cycle. In these experiments, myometrial strips were isolated from mice during the estrus cycle, and analyzed for ability of CGRP to inhibit KCl-induced contraction, and the strips were then assayed for RCP mRNA and protein expression (Naghashpour and Dahl, 2000). The inhibitory ability of CGRP was found to again correlate with RCP protein expression, with a maximal inhibition at metestrus, followed by diestrus, proestrus, and estrus. Dose–response curves comparing CGRP inhibition of contraction at estrus and metestrus showed an inhibition of maximal effect in metestrus, with minimal effect on $EC_{50}$, similar to the inhibition of maximal signal transduction observed in the RCP antisense cell lines described earlier (Evans et al., 2000; Prado et al., 2001). No correlation was observed between RAMP1 and CGRP efficacy in these experiments. To determine the role of estrogen and progesterone on CGRP responsiveness, myometrial strips were analyzed from either ovarectomized mice or ovarectomized mice supplemented with estrogen or progesterone. Ovarectomized mice had a diminished myometrial response to CGRP (less inhibition of KCl-induced contraction), and the myometrial response was regained after treatment with progesterone (Naghashpour and Dahl, 2000). RCP expression was diminished in the ovarectomized mice, and was enhanced in the progesterone-treated animals in proportion to CGRP responsiveness. Interestingly, RCP protein expression can be uncoupled from mRNA expression, as the observed changes in RCP protein were not reflected in RCP mRNA during either pregnancy or estrus (Naghashpour et al., 1997; Naghashpour and Dahl, 2000). Thus, RCP may be regulated at a posttranscriptional level, and should be analyzed at the protein level to quantify bioactivity.

RCP also affects CGRP systems in the nervous system. Following intrathecal infusion of the CGRP receptor antagonist CGRP (8–37), RCP immunoreactivity was increased in rat DRG and dorsal horn neurons (Ma et al., 2003). It had been previously shown that administration of CGRP (8–37) did not increase CGRP immunoreactivity or CGRP-binding sites in the dorsal horn (Menard et al., 1996). Combined with the enabling role for RCP in CGRP-mediated signal transduction (Evans et al., 2000; Prado et al., 2001) these data suggest that the function of a relatively constant number of CGRP receptors in the dorsal horn were upregulated by increased levels of RCP expression following treatment with antagonist. RCP expression was also increased in rat dorsal horn following treatment with the inflammatory agent carrageenan (Ma et al., 2003). Peripheral inflammation increases CGRP content in DRG and dorsal horn (Kar et al., 1994; Seybold et al., 1995), and upregulation of the CGRP receptor system by RCP may augment the neurogenic inflammatory effect of CGRP. Similarly, partial sciatic nerve ligation resulted in a significant decrease in RCP-immunoreactive neurons in ipsilateral dorsal horn compared with contralateral control.

Regulation of vascular CGRP receptors has recently been reported. In the rat subtotal nephrectomy (SN) model for hypertension, the vasculature was hyperresponsive to CGRP while CGRP synthesis was unchanged from normotensive control (Supowit et al., 1998, 2001). This suggested that CGRP was acting as a compensatory depressor to the hypertensive effects, via an enhanced receptor system. When the CGRP receptor proteins were examined by Western blotting in SN-salt rats, RCP expression was twofold higher than in normotensive control animals, while CLR and RAMP1 expression was unchanged (Katki et al., 2003). Thus, the mechanism of the increased vascular sensitivity to CGRP is likely due to upregulation of RCP, and hence more efficient coupling of the CLR/RAMP1 complex to the cellular signaling pathway. This is in contrast to the deoxycorticosterone-salt model for hypertension, where CGRP mRNA and CGRP immunoreactivity increased in DRG neurons, suggesting that increased expression of CGRP was a compensatory mechanism for hypertension (Supowit et al., 1995b, 1997).

A similar hyperresponsiveness to CGRP has been reported in human hypertension (Lind and Edvinsson, 2002). In these studies, small vascular segments from control and hypertensive patients were mounted on a force transducer, and contractile responses to noraderenaline and $K^+$ were recorded. No difference between hypertensive and control patients was detected with regard to the ability of noraderenaline or $K^+$ to contract either arteries or veins. However, when subcutaneous arteries were tested for their ability to dilate in response to CGRP, the maximal effect of CGRP was almost twofold higher in the hypertensive samples than in the control, while $EC_{50}$ remained unchanged. In these experiments, no change was observed in the vasodilatory response to substance P, and no difference was detected between hypertensive and control patients with regard to CGRP-induced dilation in the veins, or with regard to the vasodilatory effect of substance P. These data suggest that while the affinity of the CGRP receptors was unchanged in the arteries of the hypertensive patients, the signaling capacity of CGRP was significantly increased. This could have been due to an increase in membrane CGRP receptors, which could reflect increased expression of CLR or RAMP1, or to an increased coupling of the existing receptors due to increased expression of RCP, as described in the rat model above.

## 4.6 Additional Receptors

It remains an open question whether all of the receptors for CGRP can be accounted for by the combination of CLR/RAMP1/RCP. In the brain, autoradiography studies with $^{125}$I-CGRP have identified high levels of CGRP-binding sites in nucleus accumbens, ventral striatum, amygdala, superior and inferior colliculi, and cerebellum (Henke et al., 1985; Tschopp et al., 1985; Inagaki et al., 1986; Kruger et al., 1988, reviewed in van Rossum et al., 1997). However, by in situ hybridization, expression of CLR has been detected primarily in the caudate putamen and amygdala (Oliver et al., 1998; Stachniak and Krukoff, 2003). In situ analysis revealed that RAMP1 expression is highest in the caudate putamen and amygdala (Oliver et al., 2001). RCP expression in the brain has been detected using in situ hybridization and immunohistochemistry, and RCP expression is highest in caudate putamen, amygdala, and cerebellum, with a broad distribution of moderate or low expression throughout the brain (Oliver et al., 1999, 2001; Ma et al., 2003). Expression of the receptor proteins does not account for all the regions of CGRP binding, suggesting that there are additional receptors for CGRP. However, as CLR and RAMP1 analysis has been carried out at the level of mRNA, there remains the possibility that their protein expression may not match mRNA expression, as has been observed for RCP (Naghashpour et al., 1997). This debate will require immunohistochemical analysis of CLR and RAMP1.

Recently, functional CGRP receptors have recently been identified when the CTR2 isotype of the calcitonin receptor (CTR2) was coexpressed with the RAMPs (Christopoulos et al., 1999; Kuwasako et al., 2003b, 2004). These data suggest that an additional combination of known proteins (CTR2, RAMP) can contribute to CGRP receptor pharmacology. Additionally, it has recently been demonstrated that RAMPs can interact with additional GPCRs other than CLR and CTR (Christopoulos et al., 2003). In these studies, the authors took advantage of the observation that RAMPs require expression of CLR to get to the cell surface. They tagged the extracellular amino terminus of the three RAMP cDNAs, and cotransfected them with selected class II GPCRs into cell culture. The vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide VPAC1 receptor interacted with all three RAMPs, the parathyroid hormone PTH1 receptor and the glucagon receptor interacted with RAMP2, and the parathyroid hormone PTH2 receptor interacted with RAMP3. No differences in ligand binding were detected, but phosphoinositol signaling at the VPAC1 receptor was enhanced upon coexpression with RAMP2, while no change was observed for cAMP signal transduction. Thus, additional CGRP receptors may be elucidated from combinations of RAMPs with known and novel receptors. One candidate, novel CGRP receptor has been reported in cerebellum (Chauhan et al., 2003), but no sequence data have yet been published so this report is difficult to evaluate.

Recently, evidence for a peptide hormone system analogous to CGRP/CLR has been reported in the fruit fly *Drosophila melanogaster*. A diuretic neuropeptide hormone named $DH_{31}$ has been identified in *Drosophila* that has homology to CGRP (Furuya et al., 2000). $DH_{31}$ increases fluid secretion in the

drosophila malpighian tubule, and $DH_{31}$ binding results in elevated levels of intracellular cAMP, suggesting a *Drosophila* GPCR. A candidate GPCR named CG17415 has been identified that has homology to CLR (Johnson et al., 2005), and when transfected into NIH3T3 cells results in cAMP production upon addition of $DH_{31}$. Interestingly, no response was observed when it was transfected into COS-7 or HEK293 cells, suggesting the requirement for additional proteins, such as RCP or RAMP1, that are expressed in NIH3T3 cells. A *Drosophila* homolog of RCP (dRCP) named CG4875 was identified, and coexpression of *Drosophila* CLR homolog CG17415 in HEK293 cells with either human or *Drosophila* RCP resulted in high-affinity $DH_{31}$ receptors. While $EC_{50}$ was similar when CG17415 was coexpressed with either drosophila or human RCP, the maximal stimulation was significantly higher when CG17415 was coexpressed with the human RCP, possibly because the human RCP could interact more efficiently with the downstream signaling proteins in the HEK293 cells. No homolog of RAMP was discovered, although human RAMP1 or RAMP2 could facilitate receptor signaling in mammalian cotransfection experiments.

## 4.7 CGRP Receptor Signaling

The amino terminus of family B GPCRs can interact with peptide ligands, and an aspartate residue present in the sequence CNRTWDGWLCW (residues 69–74 of the extracellular amino terminus) of CLR is thought to be important for peptide binding (Dautzenberg et al., 1999; Harmar, 2001). When this residue was mutated to alanine, glutamate, or asparagine in CLR, the mutant receptor was correctly routed to the cell surface, but interaction with RAMP1 and CGRP-induced signal transduction were greatly impaired (Ittner et al., 2004). Interestingly, when residues 14–20 of CLR (TRNKIMT) were deleted, the mutant receptor could still signal in response to CGRP but not AM, suggesting a pharmacologic discriminatory site in CLR (Koller et al., 2004).

Proline residues in transmembrane domains (TM) are often conserved structural features of integral membrane proteins that can affect transmembrane helix interactions (Hulme et al., 1999; Gether, 2000; Cordes et al., 2002). Family B GPCRs contain conserved proline residues corresponding to $pro^{241}$ (TM4), $pro^{275}$ (TM5), $pro^{321}$ (TM6) in CLR, and additionally CLR and the CTR share conserved $pro^{244}$ (TM4) and $pro^{331}$ (TM6) residues. When alanine mutants were constructed for these conserved proline residues in CLR, only mutation of $pro^{321}$ or $pro^{331}$ in TM6 inhibited CGRP-mediated signaling (Conner et al., 2005). Of the two residues, $pro^{321}$ had the most severe phenotype, yet did not appear to alter cell surface expression of the mutant CLR. These data suggest that TM6 of CLR is important for signal transduction, and the ability of proline residues to act as molecular hinges suggests the possibility that $pro^{321}$ and $pro^{331}$ could be involved in ligand-induced conformational changes that facilitate coupling CLR to the cellular signaling pathway.

The majority of reports support the notion that the CGRP receptor couples to $G\alpha_s$, activating adenylate cyclase to increase intracellular cAMP and subsequently activating protein kinase A (PKA) (Laufer and Changeux, 1987; Van Valen et al., 1990; Fiscus et al., 1991; Zhang et al., 1994; Asahina et al., 1995). However, there are sufficient examples of $G\alpha_q$ coupling that prove CGRP signaling is probably more a function of the type of G protein expressed in the target cell rather than an inherent preference of the CGRP receptor. Activation of $G\alpha_q$ results in activation of phospholipase C, and subsequent hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PIP_2$) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3). IP3 can further bind to receptors on the ER to release $Ca^{2+}$ stores, resulting in elevated intracellular $Ca^{2+}$ levels, and DAG can activate protein kinase C (PKC). CGRP has been observed to cause a dose-dependent increase in IP3 in cultured chick myocytes (Laufer and Changeux, 1989), although increased cAMP levels have also been reported in cultured myocytes in response to CGRP (Fontaine et al., 1987; Laufer and Changeux, 1987, 1989; Rossi et al., 2003), suggesting that both signaling pathways can operate in these cells. Activation of multiple signaling pathways has been observed in HEK293 cells transfected with CLR, where both cAMP and intracellular $Ca^{2+}$ were stimulated in a dose-dependent manner by CGRP (Aiyar et al., 1999). In contrast, in the human osteosarcoma cell line, OHS-4 CGRP caused elevated levels of intracellular calcium while having no effect on cAMP production, and in these cells it was directly shown that the CGRP receptor

was coupling through $G\alpha_q$, which subsequently activated PLC-β1 (Drissi et al., 1998, 1999). CGRP receptor desensitization was also shown to be inhibited by PKC inhibitors in HEK293 cells transfected with CLR (Aiyar et al., 2000).

CGRP can also activate the mitogen-activated protein kinase (MAPK) signaling pathway. MAPKs are serine/threonine kinases, represented by ERK, p38 mitogen-activated protein kinase (p38 MAPK), JNK, that are activated by a kinase cascade, often initiated by activation of a small GTP-binding protein such as Ras or Rho in response to activation of receptor kinases (Denhardt, 1996). Ras or Rho can activate a MAPK kinase kinase (MAPKKK), which in turn phosphorylates and activates a MAPK kinase (MAPKK), which phosphorylates MAPK. MAPK signaling can also be activated by Gα and Gβγ subunits, leading to crosstalk between GPCR and MAPK signaling pathways. Several examples have been reported of CGRP affecting MAPK signaling pathways. In HEK293 cells transfected with CLR, CGRP caused a concentration-dependent increase in ERK and p38 MAPK activity, with no change in JNK activity (Parameswaran et al., 2000). Activation of ERK and p38 was inhibited by the antagonist CGRP (8–37), suggesting the involvement of CLR. Activation of adenylate cyclase with forskolin resulted in a similar ERK and p38 activation, suggesting that the effect of CGRP was mediated by cAMP, and activation of ERK and p38 was inhibited by treatment with the PKA inhibitor H89. Furthermore, incubation with the PI3 kinase inhibitor wortmannin decreased ERK but not p38 activity, suggesting two distinct CGRP-induced pathways for MAPK activation. In cultured rat DRG neurons, CGRP activated MAPK/extracellular receptor kinase kinase (MEK) in a PKA-mediated pathway (Anderson and Seybold, 2004), indicating that MAPKs can be activated by GPCR effector molecules.

Vascular smooth muscle cell (VSMC) proliferation is promoted by multiple pathways, including a MAPK named ERK1/2 (Touyz and Schiffrin, 2000). CGRP can inhibit smooth muscle proliferation and the resulting excessive thickening of the vasculature associated with hypertension and arteriosclerosis (Ross, 1993). One proliferative agent for VSMC is angiotensin-II, which activates ERK1/2. In cultured VSMC, angiotensin-II increased the viability of the smooth muscle cells and increased ERK1/2 activation (Qin et al., 2004). CGRP inhibited the angiotensin-II-induced ERK activity, suggesting a model for CGRP control of smooth muscle proliferation. CGRP can also spare VSMC from damage from reactive oxygen species (ROS). VSMCs were isolated from rat thoracic aorta and cultured in the presence of ROS, and cell viability was decreased by an apoptotic mechanism (Schaeffer et al., 2003). In ROS-treated VSMC, phosphorylation (activation) of ERK1/2 was increased, and pretreatment of VSMC with CGRP before ROS exposure resulted in a significant sparing of VSMC, and a concomitant increase in phosphorylated ERK1/2. The sparing effect of CGRP was inhibited by incubation of VSMC with kinase inhibitors of ERK1/2, indicating that the effect of CGRP was mediated through the MAPK pathway in VSMC.

## 5 Physiological Functions of CGRP

One of the hallmarks of CGRP physiology is vasodilation, where CGRP-immunoreactive neurons innervate the smooth muscle of the vasculature (for review see Brain and Grant, 2004). Other features of CGRP-immunoreactive neurons include their innervation of skeletal muscle at the neuromuscular junction and hair cells in the cochlea.

### 5.1 CGRP Innervation of Smooth Muscle

CGRP-immunoreactive neurons with their cell bodies in the DRG project centrally to the dorsal horn of the spinal cord and peripherally to the vasculature, where CGRP is a potent vasodilator (Gibson et al., 1984; Gulbenkian et al., 1986; Uddman et al., 1986; Bell and McDermott, 1996; Lundberg, 1996). Two primary mechanisms have been proposed for CGRP-induced vasodilation of the vasculature, either directly by acting on the smooth muscle or indirectly by acting on the endothelial cells juxtaposed to the smooth muscle layer. CGRP binding elicits a rise in intracellular cAMP, suggesting it is working through a GPCR such as CLR in both smooth muscle and endothelial cells (Hirata et al., 1988; Crossman et al., 1990).

In vascular smooth muscle, the increase in cAMP can result in activation of PKA and subsequent activation of $K^+$ channels, leading to hyperpolarization and subsequent relaxation of muscle tone. The effect of CGRP on arterial dilation was investigated in pig coronary arterial smooth muscle, where application of CGRP resulted in activation of inward $K^+$ currents (Wellman et al., 1998). These currents were blocked by incubation with glibenclamide, an inhibitor of ATP-sensitive potassium channels ($K_{ATP}$) channels. Furthermore, application of forskolin, a nonspecific activator of adenylate cyclase, also resulted in a glibenclamide-sensitive $K^+$ current, suggesting the involvement of the cAMP/PKA signaling pathway. CGRP-induced currents were reduced upon coapplication of PKA inhibitors, suggesting that the mechanism of CGRP action in these coronary arteriole smooth muscles is by a PKA-mediated activation of $K_{ATP}$ channels. A similar glibenclamide-sensitive relaxation was first observed in rabbit mesenteric arteries (Nelson et al., 1990). It is interesting to note that one study found a tenfold difference in the ability of CGRP to relax small- versus large-diameter rings from porcine coronary artery in an endothelium-independent manner (Foulkes et al., 1991). This may be due to a novel subset of CGRP receptors present in the small-caliber vessels, or to increased expression of an accessory protein for CLR such as RCP, which can enhance CLR signaling, and therefore a vasodilatory effect.

CGRP can also induce smooth muscle relaxation by an endothelial cell-mediated mechanism (Brain et al., 1985; Gray and Marshall, 1992a, b). In this paradigm, binding of CGRP to the endothelial CGRP receptor will activate $G\alpha_s$, resulting in a rise in intracellular cAMP and activation of PKA in the endothelial cell. PKA activation can enhance endothelial nitric oxide synthetase (eNOS) (Butt et al., 2000; Liu et al., 2004). An alternative model could involve coupling of the endothelial CGRP receptor to $G\alpha_q$, as has been described for the CGRP receptor in osteoblastic OHS-4 cells (Drissi et al., 1998). $G\alpha_q$ activation results in $IP_3$ production and subsequent elevated $Ca^{2+}$ levels, which could activate eNOS in a calmodulin-dependent manner (Nathan and Xie, 1994; Feron et al., 1998). Nitric oxide could then diffuse from the endothelial cell into the smooth muscle cell and cause cGMP-dependent relaxation (Moncada et al., 1991).

CLR and RAMP expression is detected in the endothelial cells and smooth muscle that are a part of the vasculature (Nikitenko et al., 2001, 2003; Hagner et al., 2002; Oliver et al., 2002; Cueille et al., 2005). In accordance with this vascular localization, CLR expression has been observed to be upregulated by hypoxic conditions. The promoter for the CLR gene was cloned, and it contains regulatory elements for the transcription factor hypoxia-inducible factor-1 (HIF-1) (Nikitenko et al., 2003). Luciferase reporter constructs were made with the CLR promoter and transfected into primary human dermal microvascular endothelial cells, and a twofold induction in luciferase activity was observed following a 16-h hypoxic exposure. Microvascular endothelial cells were also subjected to a time course of hypoxia for 16 h, and RT-PCR analysis determined that CLR mRNA expression was maximally induced after 4 h of hypoxia and then declined. Interestingly, RAMP2 expression was unchanged, and RAMP1 expression was undetected in these endothelial cells. The effects of hypoxia on CLR have also been studied in human coronary artery smooth muscle cells (CASMC) (Cueille et al., 2005). In these studies CASMC were exposed to hypoxic conditions for 1, 3, or 4 h, and CLR mRNA was increased maximally twofold at 4 h when analyzed by RT-PCR. RAMP1 mRNA did not increase, and RAMP2 mRNA increased slightly. Interestingly, when analyzed by Western blotting, CLR expression increased maximally after 1 h of hypoxia, increasing 3.5-fold over control, suggesting that CLR expression can be uncoupled from mRNA expression, as has been observed for RCP expression (Naghashpour et al., 1997). The effects of chronic hypoxia were also tested by keeping rats in a hypobaric chamber, and isolating the left and right ventricles of the heart for RT-PCR analysis. CLR mRNA peaked at day 14 in the right ventricle and day 18 in the left ventricle, while RAMP1 mRNA peaked at day 4 in the right ventricle and day 15 in the left ventricle (Cueille et al., 2005).

The lack of RAMP1 expression in hypoxic endothelial cells is in agreement with immunohistochemical data that found little RAMP1 in vascular endothelial cells but did find RAMP1-immunoreactive material expressed in the underlying smooth muscle (Oliver et al., 2002). These studies found RAMP2 expressed in endothelial cells and RAMP1 expressed in smooth muscle, suggesting the presence of an AM receptor in endothelial cells, with CGRP receptors in smooth muscle. Given the ability of CGRP to cross-react with AM receptors, this lack of RAMP1 in endothelial cells suggests the possibility that some or all of the

endothelial-dependent effects described for CGRP on the vasculature may be due to cross-reactivity between CGRP and endothelial AM receptors.

## 5.2 CGRP Innervation of Skeletal Muscle

CGRP is present in motoneurons innervating skeletal muscles, where it is present in dense-core vesicles at the neuromuscular junction (Matteoli et al., 1988; Peng et al., 1989; Uchida et al., 1990; Sakaguchi et al., 1991). These vesicles are distinct from the clear-core vesicles containing ACh, and their triggering mechanism for release is not well understood. CGRP affects ACh by modulating expression of the postsynaptic acetylcholine receptor (AChR) and acetylcholinesterase (AChE). Release of ACh results in activation of postsynaptic nicotinic AChRs, which are ligand-gated cation channels that cause postsynaptic depolarization (Changeux and Edelstein, 1998; Itier and Bertrand, 2001). Prolonged exposure to ACh or the agonist nicotine results in cAMP-mediated phosphorylation and internalization of the AChR and concomitant loss of postsynaptic ACh-binding sites, followed by a subsequent induction of receptor synthesis (Huganir and Greengard, 1983, 1990; Boyd, 1987; Hoffman et al., 1994; Fenster et al., 1999; Harkness and Millar, 2002). CGRP released at the neuromuscular junction can likewise cause phosphorylation and desensitization of the postsynaptic AChR (Mulle et al., 1988; Miles et al., 1989; Huganir and Greengard, 1990). CGRP can also increase AChR synthesis and cell surface expression in a cAMP-dependent mechanism (Fontaine et al., 1986, 1987; New and Mudge, 1986; Laufer and Changeux, 1987; Jennings and Mudge, 1989). CGRP can also potentiate the effect of ACh by causing an increase in the channel open time of the nicotinic AChR (Lu et al., 1993). This potentiation can be mimicked by application of dibutyryl-cAMP and inhibited by inhibitors of PKA.

AChE is the enzyme that hydrolyzes ACh and is the primary mechanism for terminating transmission at the neuromuscular synapse. CGRP can profoundly affect the expression of AChE. Incubation of rat myotubes with CGRP for 2 h resulted in a 42% increase in catalytically active AChE expression, an effect that was mimicked by incubation with forskolin (da Costa et al., 2001), suggesting the effect of CGRP was mediated through a GPCR. In contrast, after 20 h of incubation with CGRP, AChE expression was decreased by 37%. A similar loss of AChE activity (60% after a 48-h treatment with CGRP) was observed in cultured mouse myotubes (Boudreau-Lariviere and Jasmin, 1999). These data are in contrast to experiments with chick myotubes, where 48-h exposure to CGRP resulted in an increase in AChE mRNA (Choi et al., 2001), although in these experiments AChE activity did not increase. Incubation of quail myotubes with CGRP resulted in a dose-dependent increase in intracellular cAMP, and overnight incubation with CGRP or factors that increased intracellular cAMP levels resulted in decreased AChE activity, especially of the collagen-tailed form of AChE (Rossi et al., 2003), which is the catalytic form of AChE that is targeted to the neuromuscular synapse (Hall, 1973).

CGRP receptors have been identified on tissue-cultured myotubes by binding with $^{125}$I-CGRP (Jennings and Mudge, 1989; Popper and Micevych, 1989; Roa and Changeux, 1991). Biochemical evidence (increased cAMP, PKA dependence) suggests that CGRP works through a GPCR at the neuromuscular junction, and evidence for expression of CLR, RAMP1, and RCP at the neuromuscular junction has recently been obtained. ACh receptors in neuromuscular junctions were identified in quail muscle sections with fluorescent α-bungarotoxin, and AChE, RCP, CLR, and RAMP1 were shown to colocalize with the ACh receptors at the neuromuscular junction (Rossi et al., 2003). Similar results were obtained from rat gracilis muscle, where RCP and RAMP1 colocalized with α-bungarotoxin at motor endplates (Fernandez et al., 2003). In these studies $^{125}$I-CGRP binding was shown to be enriched in dissected motor endplates, as was protein expression of RCP.

A broad effect of CGRP at the neuromuscular junction appears to be strengthening of the cholinergic synapse, based on the upregulation of AChR, potentiation of AChR channel conductance, and inhibition of AChE. However, CGRP can have diametric effects depending on experimental conditions, perhaps reflecting the modulatory role of CGRP. It has been reported that neuromuscular junction development and morphology are not altered in α-CGRP knockout mice (Lu et al., 1999; Salmon et al., 1999), as might be predicted for loss of a modulatory rather than causative factor. However, compensation in these knockout mice, perhaps by β-CGRP, cannot yet be ruled out.

### 5.3 CGRP in Cochlea

All hair cell systems, including lateral-line organs, vestibular organs, and the cochlea, contain an efferent innervation originating in the brainstem and projecting to the hair cells and/or neural elements in the sensory epithelium. CGRP is almost universally found in the efferent innervation of hair cell systems, including the lateral-line organs in fish and amphibians, as well as cochlea and vestibular organs in mammals, where it is typically colocalized with cholinergic markers (Fex and Altschuler, 1986; Eybalin, 1993; Maison et al., 2003a). In the lateral line of the frog, cholinergic-mediated efferent stimulation suppresses spontaneous discharge (Russell, 1968; Sewell and Starr, 1991) whereas application of either α-CGRP or β-CGRP increases spontaneous discharge in primary afferent neurons (Bailey and Sewell, 2000a, b). CGRP application increases spontaneous discharge by acting through a type I CGRP receptor (Bailey and Sewell, 2000a, JARO), and a *Xenopus laevis* CLR has been identified (Prado et al., 2001; Klein et al., 2002).

In the mammalian cochlea, CGRP immunoreactivity is predominantly present in the unmyelinated component of the cochlear efferent innervation, the lateral olivocochlear system (Vetter et al., 1991; Eybalin, 1993; Cabanillas and Luebke, 2002; Maison et al., 2003a). The peripheral targets of these lateral olivocochlear fibers, which colocalize with cholinergic markers, are the dendrites of cochlear afferent neurons, in the region near the afferent synapses with inner hair cells (Liberman, 1980).

Until recently, the function of CGRP function in the mammalian ear was unknown. However, when the auditory function of α-CGRP knockout mice was compared with age-matched, strain-matched wild-type controls, α-CGRP depletion reduced suprathreshold sound-evoked activity of the cochlear nerve by 20% at all sound pressures and frequencies, as assessed by the amplitude of the auditory brainstem response (ABR) wave I (Maison et al., 2003b). There was no difference detected in the distortion product otoacoustic emissions (DPOAE), cochlear blood flow (Nuttall, unpublished observation), or auditory thresholds between the wild type and α-CGRP knockout mice. Thus, loss of CGRP reduced the detection threshold for hearing in the α-CGRP knockout mice. Conversely, endogenous release of CGRP would cause an increase in sound-evoked activity of the cochlear nerve. Such an increase in the cochlear nerve activity due to CGRP signaling would cause an increase in the dynamic range of sound perception and allow for an increased signal-to-noise ratio. This increase in the dynamic range is needed, since in normal cochleae, the two efferent pathways, medial (predominately cholinergic) and lateral (CGRP-containing efferents), are working in concert. Activation of the medial cholinergic efferents is inhibitory to outer hair cells, and reduces the amplification on the inner hair response. However, activation of the CGRP-containing lateral efferents acting later in the feedback pathway (the primary afferents receiving their signals from inner hair cells) could serve to boost the cochlear nerve signal after the medial efferent system has effectively filtered the noise from the signal. Approximately 30 million Americans suffer from some form of hearing loss that causes them to have difficulty understanding speech in the presence of background noises. The role of CGRP in the efferent neural feedback system hypothesized to influence a human's ability to amplify sound in the presence of background noises attests to the significance of understanding the complete CGRP receptor signaling cascade in the mammalian cochlea. However, little is known about the cochlear CGRP receptor signaling complex except that all components of the CGRP receptor (RCP, RAMP1, and CLR) are present and can be immunoprecipitated as a complex from the mammalian cochlea (Dickerson and Luebke, 2002).

## 6 Therapeutic Potential of CGRP for Trigeminovascular Pathologies

There is a growing body of evidence that abnormal regulation of CGRP levels or activity contributes to vascular disorders. In this section we focus on the role of CGRP in two such disorders involving the trigeminovascular system: migraine and subarachnoid hemorrhage (SAH).

### 6.1 Increased CGRP Levels in Migraine

Migraine is a chronic disease that affects about 12% of the population (Lipton et al., 2001). It is generally characterized as a severe pulsating headache affecting only one side of the head, although the symptoms can

be bilateral and vary considerably (Ferrari et al., 1991; Goadsby et al., 2002). The attacks often occur several times a week and generally last from 4 to 72 h. Migraine is usually associated with nausea, photophobia, phonophobia, and vomiting. In about 20% of the cases, the headache phase of migraine is preceded by an aura that is marked by acute visual or other sensory disturbances. During the headache, CGRP levels are elevated in the jugular venous outflow (Goadsby et al., 1990; Goadsby and Edvinsson, 1993). The CGRP levels return to normal upon treatment with triptan antimigraine drugs, coincident with pain relief (Goadsby et al., 1991; Goadsby and Edvinsson, 1993). The release of CGRP from afferent terminals is believed to cause vasodilation and neurogenic inflammation and efferent release is believed to be part of the nociceptive relay to the CNS (Arulmani et al., 2004; Edvinsson, 2004).

Migraine has traditionally been viewed as a vascular headache that involves blood flow changes. However, there is clearly a neurogenic involvement and it seems most likely that migraine involves both neuronal and vascular components (Goadsby et al., 2002; Parsons and Strijbos, 2003; Pietrobon and Striessnig, 2003). Current migraine models involve an initial CNS dysfunction that causes meningeal blood vessel dilation and activation of perivascular trigeminal nerves. Hence, migraine can be considered to be a neurovascular headache of the trigeminovascular system (Arulmani et al., 2004; Edvinsson, 2004).

While the initial triggering steps that lead to activation of the trigeminal ganglia and release of CGRP have remained elusive, recent studies have provided some insight. Mutations in the P/Q calcium channel and a $Na^+$, $K^+$-ATPase have been identified in patients with a rare form of inherited migraine, familial hemiplegic migraine (Miller, 1997; Goadsby et al., 2002; Parsons and Strijbos, 2003; Estevez et al., 2004). Interestingly, the P/Q channel mutant knock-in mouse is more susceptible to cortical spreading depression, which is associated with the migraine aura (van den Maagdenberg et al., 2004). Cortical spreading depression has been reported to release CGRP from trigeminal sensory afferents in an animal model (Bolay et al., 2002). Hence, this connection may tie the initial phase of migraine with trigeminal CGRP release.

## 6.2 Migraine Treatments that Lower or Prevent CGRP Activity

The role of CGRP in migraine is driven home by the effectiveness of at least two antimigraine drugs, the triptans and olcegepant (BIBN4096). The triptans lower CGRP levels. It has been shown in cell culture systems that the triptans can act directly on 5-$HT_1$ receptors on trigeminal ganglion nerves to repress CGRP secretion (Durham and Russo, 1999) and repress MAP kinase activation of the 18-bp distal CGRP enhancer (Durham and Russo, 2000, 2003). The inhibition of secretion is consistent with the time course of drug action in patients and agrees with a number of animal and in vitro studies (Durham and Russo, 2002). The promising international clinical trial with the CGRP receptor antagonist olecegepant is proof of principal evidence that CGRP plays a causal role in migraine pain (Olesen et al., 2004). The pharmacological properties of olecegepant suggest that this drug may sidestep some of the side effects of the triptans involving coronary vasoconstriction (Moreno et al., 2002; Durham, 2004b; Olesen et al., 2004).

How might regulation of CGRP gene transcription fit into migraine therapy? First, it seems likely that there is increased CGRP synthesis since migraine episodes can last for up to 72 h. While speculative, there might also be higher basal levels in individuals who are susceptible to migraine. Stimulation of CGRP gene expression by MAP kinases may underlie the elevation of CGRP levels. This could occur in response to neuronal activity alone, which can activate MAP kinases (Rosen et al., 1994; MacArthur and Eiden, 1996), and many of the inflammatory compounds that are released during migraines are known activators of MAP kinases (Buzzi et al., 1995). Indeed, it has been reported that inflammation of peripheral joints leads to increased CGRP peptide and mRNA levels in the DRG (Donaldson et al., 1992). The role of CGRP in arthritis is supported by decreased nociception in a CT/CGRP knockout mouse (Zhang et al., 2001). A decrease in CGRP production would thus potentially limit neurogenic pain.

In this regard, the time course of triptan action on CGRP synthesis is relevant. Transcriptional repression was seen after 2–4 h (Durham and Russo, 2003), which is close to the estimated half-life of sumatriptan and within the 12 h required for clearance from the body, especially with the newer triptans (Fowler et al., 1991). However, there is headache relief within 30 min, which indicates that transcriptional

repression of the CGRP gene is not involved in the initial drug action. While it is risky to extrapolate from cells to people, we speculate that transcriptional repression might lower CGRP levels over long-term drug use. This may be relevant in triptan overuse syndromes, possibly during the withdrawal headache (Limmroth et al., 1999, 2002).

### 6.3 Decreased CGRP Levels and Risk of Vasospasm in Subarachnoid Hemorrhage

SAH can be defined as bleeding into the subarachnoid space between the brain and the skull. This is the space between the spider web-like arachnoid membrane and the surface of the brain. It is normally filled with cerebrospinal fluid that acts as a cushion to protect the brain from trauma. SAH is most commonly caused by trauma or rupture of a vessel by an aneurysm.

Immediately following SAH, CGRP is released from sensory afferent nerve endings of the trigeminal ganglion in a reflex-like response similar to that believed to occur during migraine (Tran Dinh et al., 1994). Indeed, the initial presentation of SAH involves an excruciating headache. Over the next 1–2 days there is a depletion of CGRP from perivascular nerve endings in animal models and postmortem human tissue (Nozaki et al., 1989; Edvinsson et al., 1991). For example, in the middle cerebral arteries, there is a 90% reduction of CGRP following SAH in postmortem patients. This decrease correlates with the risk period of vasospasm. Cerebral vasospasm is a major complication of SAH. Delayed and prolonged arterial constriction can lead to brain ischemia (stroke) and death. Vasospasm with ischemia occurs in 30–40% of patients, with peak incidence 4–12 days after SAH (Juul et al., 1994; Macdonald and Weir, 1994). Thus, vasospasm following SAH is a serious risk factor.

Could the depletion of CGRP contribute to the spasms? It seems likely given the importance of trigeminal nerves and CGRP in controlling cerebral blood flow (McCulloch et al., 1986). The importance of trigeminovascular CGRP is further highlighted by reports from Edvinsson et al. (2002) that human cerebral arteries are ten times more sensitive than coronary arteries to CGRP. Pharmacologically, the cerebral and peripheral CGRP receptors are indistinguishable (Juaneda et al., 2000). The basis of this increased sensitivity of the cerebrovasculature to CGRP is not known, but may be due to increased receptor number, more efficient coupling to downstream signaling pathways, or unidentified compensatory mechanisms. The sensitivity of the cerebrovasculature to CGRP, and its absence following SAH, suggests that the absence of CGRP could be a contributing factor to SAH vasospasms. This has spurred studies on treatments to restore CGRP levels.

### 6.4 CGRP Treatment and Gene Transfer for SAH

The activity of CGRP as a compensatory vasodilator has led to efforts to use CGRP as a post-SAH treatment. Importantly, the vasodilator responses to CGRP are preserved or enhanced after SAH (Edvinsson et al., 1991; Ahmad et al., 1996; Imaizumi et al., 1996; Inoue et al., 1996; Sobey et al., 1996). As predicted, restoration of CGRP by intrathecal administration (Imaizumi et al., 1996; Inoue et al., 1996) can prevent vasospasm in animal models following SAH. A new step in CGRP therapy will be to alter vasomotor function by gene transfer in patients. Toward this goal, an adenoviral vector has been used to direct overexpression of CGRP in the perivascular adventia (Toyoda et al., 2000a). Injection of this vector into the cerebrospinal fluid was able to inhibit vasospasm after SAH in a rabbit model (Toyoda et al., 2000b). Whether therapeutic CGRP gene transfer for SAH will be applicable to human patients remains to be determined. There are clear limitations, but the possibility of CGRP gene transfer remains an exciting prospect (Toyoda et al., 2003).

## 7 Summary

The major goal of this chapter has been to provide an appreciation of the physiological and pathological significance of the neuropeptide CGRP. An understanding of the mechanisms by which CGRP levels are

regulated and how CGRP acts on target cells will allow the development of new therapeutic strategies. For example, the elevation of CGRP in painful disorders, such as migraine, has already led to drugs that lower CGRP levels and block its action at the receptor. The role of CGRP is of particular significance due to the prevalence of migraine alone. It is estimated that 18 million Americans, including almost one in five women, suffer an estimated 4 million attacks every week (Goadsby et al., 2002). The chronic pain often precipitates other serious conditions such as depression (Merikangas et al., 1990; Juang et al., 2000) and there is an enormous financial burden in the billions per year (Hu et al., 1999; Goadsby et al., 2002; Pietrobon and Striessnig, 2003; Elston Lafata et al., 2004). A large number of people also suffer from other craniofacial pains that may involve CGRP, with estimates ranging from 5% to 12% of the population (Lipton et al., 1993). Likewise, the serious risk of debilitating vasospasm following SAH supports the need for understanding how to maintain CGRP expression. Because of the high incidence and generally poor efficacy of current treatments for these disorders, there is a need for improved therapeutic and preventative measures. Future studies on the CGRP gene and its receptor should provide important insights into potential therapeutic strategies.

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## References

Adema GJ, Bovenberg RA, Jansz HS, Baas PD. 1988. Unusual branch point selection involved in splicing of the alternatively processed calcitonin/CGRP-I pre-mRNA. Nucleic Acids Res 16: 9513-9526.

Ahmad I, Imaizumi S, Shimizu H, Kaminuma T, Ochiai N, et al. 1996. Development of calcitonin gene-related peptide slow-release tablet implanted in CSF space for prevention of cerebral vasospasm after experimental subarachnoid haemorrhage. Acta Neurochir (Wien)138: 1230-1240.

Aiyar N, Disa J, Dang K, Pronin AN, Benovic JL, et al. 2000. Involvement of G protein-coupled receptor kinase-6 in desensitization of CGRP receptors. Eur J Pharmacol 403: 1-7.

Aiyar N, Disa J, Stadel JM, Lysko PG. 1999. Calcitonin gene-related peptide receptor independently stimulates 3′,5′-cyclic adenosine monophosphate and $Ca^{2+}$ signaling pathways. Mol Cell Biochem 197: 179-185.

Aiyar N, Rand K, Elshourbagy NA, Zeng Z, Adamou JE, et al. 1996. A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J Biol Chem 271: 11325-11329.

Aldecoa A, Gujer R, Fischer JA, Born W. 2000. Mammalian calcitonin receptor-like receptor/receptor activity modifying protein complexes define calcitonin gene-related peptide and adrenomedullin receptors in Drosophila Schneider 2 cells. FEBS Lett 471: 156-160.

Allen GV, Barbrick B, Esser MJ. 1996. Trigeminal-parabrachial connections: possible pathway for nociception-induced cardiovascular reflex responses. Brain Res 715: 125-135.

Almegard B, Andersson SE. 1993. Vascular effects of calcitonin gene-related peptide (CGRP) and cholecystokinin (CCK) in the monkey eye. J Ocul Pharmacol 9: 77-84.

Amara SG, Arriza JL, Leff SE, Swanson LW, Evans RM, et al. 1985. Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 229: 1094-1097.

Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM. 1982. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298: 240-244.

Anderson LE, Seybold VS. 2004. Calcitonin gene-related peptide regulates gene transcription in primary afferent neurons. J Neurochem 91: 1417-1429.

Arulmani U, Maassenvandenbrink A, Villalon CM, Saxena PR. 2004. Calcitonin gene-related peptide and its role in migraine pathophysiology. Eur J Pharmacol 500: 315-330.

Asahina A, Moro O, Hosoi J, Lerner EA, Xu S, et al. 1995. Specific induction of cAMP in Langerhans cells by calcitonin gene-related peptide: relevance to functional effects. Proc Natl Acad Sci USA 92: 8323-8327.

Baetscher M, Schmidt E, Shimizu A, Leder P, Fishman MC. 1991. SV40 T antigen transforms calcitonin cells of the thyroid but not CGRP-containing neurons in transgenic mice. Oncogene 6: 1133-1138.

Bailey GP, Sewell WF. 2000a. Pharmacological characterization of the CGRP receptor in the lateral line organ of *Xenopus laevis*. J Assoc Res Otolaryngol 1: 82-88.

Bailey GP, Sewell WF. 2000b. Calcitonin gene-related peptide suppresses hair cell responses to mechanical stimulation in the Xenopus lateral line organ. J Neurosci 20: 5163-5169.

Ball DW, Compton D, Nelkin BD, Baylin SB, de Bustros A. 1992. Human calcitonin gene regulation by helix–loop–helix recognition sequences. Nucleic Acids Res 20: 117-123.

Beaumont K, Kenney MA, Young AA, Rink TJ. 1993. High affinity amylin binding sites in rat brain. Mol Pharmacol 44: 493-497.

Becker KL, Nylen ES, Snider RH, Muller B, White JC. 2003. Immunoneutralization of procalcitonin as therapy of sepsis. J Endotoxin Res 9: 367-374.

Bell D, McDermott BJ. 1996. Calcitonin gene-related peptide in the cardiovascular system: characterization of receptor populations and their (patho)physiological significance. Pharmacol Rev 48: 253-288.

Bereiter DA, Benetti AP. 1991. Microinjections of calcitonin gene-related peptide within the trigeminal subnucleus caudalis of the cat affects adrenal and autonomic function. Brain Res 558: 53-62.

Berridge MJ, Bootman MD, Roderick HL. 2003. Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4: 517-529.

Bolay H, Reuter U, Dunn AK, Huang Z, Boas DA, et al. 2002. Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. Nat Med 8: 136-142.

Boudreau-Lariviere C, Jasmin BJ. 1999. Calcitonin gene-related peptide decreases expression of acetylcholinesterase in mammalian myotubes. FEBS Lett 444: 22-26.

Boyd ND. 1987. Two distinct kinetic phases of desensitization of acetylcholine receptors of clonal rat PC12 cells. J Physiol 389: 45-67.

Bracq S, Taboulet J, Machairas M, Lasmoles F, Houssin D, et al. 1997. Calcitonin mRNA is produced in liver by two different splicing pathways. Mol Cell Endocrinol 128: 111-115.

Brain SD, Grant AD. 2004. Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol Rev 84: 903-934.

Brain SD, Williams TJ, Tippins JR, Morris HR, Mac Intyre I. 1985. Calcitonin gene-related peptide is a potent vasodilator. Nature 313: 54-56.

Buhlmann N, Leuthauser K, Muff R, Fischer JA, Born W. 1999. A receptor activity modifying protein (RAMP)2-dependent adrenomedullin receptor is a calcitonin gene-related peptide receptor when coexpressed with human RAMP1. Endocrinology 140: 2883-2890.

Butt E, Bernhardt M, Smolenski A, Kotsonis P, Frohlich LG, et al. 2000. Endothelial nitric oxide synthase (type III) is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. J Biol Chem 275: 5179-5187.

Buzzi MG, Bonamini M, Moskowitz MA. 1995. Neurogenic model of migraine. Cephalalgia 15: 277-280.

Cabanillas LA, Luebke AE. 2002. CGRP- and cholinergic-containing fibers project to guinea pig outer hair cells. Hear Res 172: 14-17.

Chang CP, Pearse RVd, O'Connell S, Rosenfeld MG. 1993. Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. Neuron 11: 1187-1195.

Chang CL, Roh J, Hsu SY. 2004. Intermedin, a novel calcitonin family peptide that exists in teleosts as well as in mammals: a comparison with other calcitonin/intermedin family peptides in vertebrates. Peptides 25: 1633-1642.

Changeux JP, Edelstein SJ. 1998. Allosteric receptors after 30 years. Neuron 21: 959-980.

Chatterjee TK, Fisher RA. 1995. Multiple affinity and guanine nucleotide sensitive forms of the calcitonin gene related peptide (CGRP) receptor. Can J Physiol Pharmacol 73: 968-973.

Chatterjee TK, Moy JA, Cai JJ, Lee HC, Fisher RA. 1993. Solubilization and characterization of a guanine nucleotide-sensitive form of the calcitonin gene-related peptide receptor. Mol Pharmacol 43: 167-175.

Chatterjee TK, Moy JA, Fisher RA. 1991. Characterization and regulation of high affinity calcitonin gene-related peptide receptors in cultured neonatal rat cardiac myocytes. Endocrinology 128: 2731-2738.

Chauhan M, Thota CS, Kondapaka S, Wimalawansa S, Yallampalli C. 2003. Evidence for the existence of a new receptor for CGRP, which is not CRLR. Peptides 24: 65-71.

Chen WG, West AE, Tao X, Corfas G, Szentirmay MN, et al. 2003. Upstream stimulatory factors are mediators of $Ca^{2+}$-responsive transcription in neurons. J Neurosci 23: 2572-2581.

Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Morishita T, et al. 1989. Calcitonin gene-related peptide receptor antagonist human CGRP-(8–37). Am J Physiol 256: E331-E335.

Choi RC, Siow NL, Zhu SQ, Wan DC, Wong YH, et al. 2001. The cyclic AMP-mediated expression of acetylcholinesterase in myotubes shows contrasting activation and repression between avian and mammalian enzymes. Mol Cell Neurosci 17: 732-745.

Christ-Crain M, Jaccard-Stolz D, Bingisser R, Gencay MM, Huber PR, et al. 2004. Effect of procalcitonin-guided treatment on antibiotic use and outcome in lower respiratory tract infections: cluster-randomised, single-blinded intervention trial. Lancet 363: 600-607.

Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, et al. 2003. Novel receptor partners and function of receptor activity-modifying proteins. J Biol Chem 278: 3293-3297.

Christopoulos G, Perry KJ, Morfis M, Tilakaratne N, Gao Y, et al. 1999. Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin receptor gene product. Mol Pharmacol 56: 235-242.

Collignon H, Laborie C, Tahri EH, el M'Selmi A, Garel JM. 1992. Effects of dexamethasone, calcium and 1,25-dihydroxycholecalciferol on calcitonin and calcitonin gene-related peptide mRNA levels from the CA-77 C cell line. Thyroid 2: 361-365.

Conner AC, Hay DL, Simms J, Howitt SG, Schindler M, et al. 2005. A key role for transmembrane prolines in calcitonin receptor-like receptor agonist binding and signalling: implications for family B G-protein-coupled receptors. Mol Pharmacol 67: 20-31.

Cooper GJ, Willis AC, Clark A, Turner RC, Sim RB, et al. 1987. Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. Proc Natl Acad Sci USA 84: 8628-8632.

Copp DH. 1992. Remembrance: calcitonin: discovery and early development. Endocrinology 131: 1007-1008.

Copp DH, Cameron EC, Cheney BA, Davidson AG, Henze KG. 1962. Evidence for calcitonin—a new hormone from the parathyroid that lowers blood calcium. Endocrinology 70: 638-649.

Cordes FS, Bright JN, Sansom MS. 2002. Proline-induced distortions of transmembrane helices. J Mol Biol 323: 951-960.

Cote GJ, Stolow DT, Peleg S, Berget SM, Gagel RF. 1992. Identification of exon sequences and an exon binding protein involved in alternative RNA splicing of calcitonin/CGRP. Nucleic Acids Res 20: 2361-2366.

Crenshaw EB III, Russo AF, Swanson LW, Rosenfeld MG. 1987. Neuron-specific alternative RNA processing in transgenic mice expressing a metallothionein–calcitonin fusion gene. Cell 49: 389-398.

Crook RB, Yabu JM. 1992. Calcitonin gene-related peptide stimulates intracellular cAMP via a protein kinase C-controlled mechanism in human ocular ciliary epithelial cells. Biochem Biophys Res Commun 188: 662-670.

Crossman DC, Dashwood MR, Brain SD, McEwan J, Pearson JD. 1990. Action of calcitonin gene-related peptide upon bovine vascular endothelial and smooth muscle cells grown in isolation and co-culture. Br J Pharmacol 99: 71-76.

Cueille C, Birot O, Bigard X, Hagner S, Garel JM. 2005. Post-transcriptional regulation of CRLR expression during hypoxia. Biochem Biophys Res Commun 326: 23-29.

Cueille C, Pidoux E, de Vernejoul MC, Ventura-Clapier R, Garel JM. 2002. Increased myocardial expression of RAMP1 and RAMP3 in rats with chronic heart failure. Biochem Biophys Res Commun 294: 340-346.

Cumberbatch MJ, Williamson DJ, Mason GS, Hill RG, Hargreaves RJ. 1999. Dural vasodilation causes a sensitization of rat caudal trigeminal neurones in vivo that is blocked by a 5-HT1B/1D agonist. Br J Pharmacol 126: 1478-1486.

da Costa VL Jr, Lapa AJ, Godinho RO. 2001. Short- and long-term influences of calcitonin gene-related peptide on the synthesis of acetylcholinesterase in mammalian myotubes. Br J Pharmacol 133: 229-236.

Daukas G, Zigmond SH. 1985. Inhibition of receptor-mediated but not fluid-phase endocytosis in polymorphonuclear leukocytes. J Cell Biol 101: 1673-1679.

Dautzenberg FM, Mevenkamp G, Wille S, Hauger RL. 1999. N-terminal splice variants of the type I PACAP receptor: isolation, characterization and ligand binding/selectivity determinants. J Neuroendocrinol 11: 941-949.

Davis NS, DiSant'Agnese PA, Ewing JF, Mooney RA. 1989. The neuroendocrine prostate: characterization and quantitation of calcitonin in the human gland. J Urol 142: 884-888.

de Bustros A, Baylin SB, Levine MA, Nelkin BD. 1986. Cyclic AMP and phorbol esters separately induce growth inhibition, calcitonin secretion, and calcitonin gene transcription in cultured human medullary thyroid carcinoma. J Biol Chem 261: 8036-8041.

de Bustros A, Lee RY, Compton D, Tsong TY, Baylin SB, et al. 1990. Differential utilization of calcitonin gene regulatory DNA sequences in cultured lines of medullary thyroid carcinoma and small-cell lung carcinoma. Mol Cell Biol 10: 1773-1778.

Denhardt DT. 1996. Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. Biochem J 318 (Pt 3): 729-747.

Dennis T, Fournier A, Cadieux A, Pomerleau F, Jolicoeur FB, et al. 1990. hCGRP8-37, a calcitonin gene-related peptide antagonist revealing calcitonin gene-related peptide receptor heterogeneity in brain and periphery. J Pharmacol Exp Ther 254: 123-128.

Dennis T, Fournier A, St Pierre S, Quirion R. 1989. Structure–activity profile of calcitonin gene-related peptide in peripheral and brain tissues. Evidence for receptor multiplicity. J Pharmacol Exp Ther 251: 718-725.

Donaldson LF, Harmar AJ, McQueen DS, Seckl JR. 1992. Increased expression of preprotachykinin, calcitonin gene-related peptide, but not vasoactive intestinal peptide messenger RNA in dorsal root ganglia during the development of adjuvant monoarthritis in the rat. Brain Res Mol Brain Res 16: 143-149.

Doods H, Hallermayer G, Wu D, Entzeroth M, Rudolf K, et al. 2000. Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. Br J Pharmacol 129: 420-423.

Drissi H, Lasmoles F, Le Mellay V, Marie PJ, Lieberherr M. 1998. Activation of phospholipase C-beta1 via Galphaq/11

during calcium mobilization by calcitonin gene-related peptide. J Biol Chem 273: 20168-20174.

Drissi H, Lieberherr M, Hott M, Marie PJ, Lasmoles F. 1999. Calcitonin gene-related peptide (CGRP) increases intracellular free $Ca^{2+}$ concentrations but not cyclic AMP formation in CGRP receptor-positive osteosarcoma cells (OHS-4). Cytokine 11: 200-207.

Durham PL. 2004a. CGRP-receptor antagonists—a fresh approach to migraine therapy? N Engl J Med 350: 1073-1075.

Durham PL. 2004b. CGRP receptor antagonists: a new choice for acute treatment of migraine? Curr Opin Investig Drugs 5: 731-735.

Durham PL, Dong PX, Belasco KT, Kasperski J, Gierasch WW, et al. 2004. Neuronal expression and regulation of CGRP promoter activity following viral gene transfer into cultured trigeminal ganglia neurons. Brain Res 997: 103-110.

Durham PL, Russo AF. 1998. Serotonergic repression of mitogen-activated protein kinase control of the calcitonin gene-related peptide enhancer. Mol Endocrinol 12: 1002-1009.

Durham PL, Russo AF. 1999. Regulation of calcitonin gene-related peptide secretion by a serotonergic antimigraine drug. J Neurosci 19: 3423-3429.

Durham PL, Russo AF. 2000. Differential regulation of mitogen-activated protein kinase-responsive genes by the duration of a calcium signal. Mol Endocrinol 14: 1570-1582.

Durham P, Russo A. 2002. New insights into the molecular actions of serotonergic antimigraine drugs. Pharmacol Ther 94: 77-92.

Durham PL, Russo AF. 2003. Stimulation of the calcitonin gene-related peptide enhancer by mitogen-activated protein kinases and repression by an antimigraine drug in trigeminal ganglia neurons. J Neurosci 23: 807-815.

Durham PL, Sharma RV, Russo AF. 1997. Repression of the calcitonin gene-related peptide promoter by 5-HT1 receptor activation. J Neurosci 17: 9545-9553.

Edvinsson L. 2004. Blockade of CGRP receptors in the intracranial vasculature: a new target in the treatment of headache. Cephalalgia 24: 611-622.

Edvinsson L, Alm R, Shaw D, Rutledge RZ, Koblan KS, et al. 2002. Effect of the CGRP receptor antagonist BIBN4096BS in human cerebral, coronary and omental arteries and in SK–N–MC cells. Eur J Pharmacol 434: 49-53.

Edvinsson L, Ekman R, Jansen I, McCulloch J, Mortensen A, et al. 1991. Reduced levels of calcitonin gene-related peptide-like immunoreactivity in human brain vessels after subarachnoid haemorrhage. Neurosci Lett 121: 151-154.

Edvinsson L, Fredholm BB, Hamel E, Jansen I, Verrecchia C. 1985. Perivascular peptides relax cerebral arteries concomitant with stimulation of cyclic adenosine monophosphate accumulation or release of an endothelium-derived relaxing factor in the cat. Neurosci Lett 58: 213-217.

Edvinsson L, Goadsby PJ. 1994. Neuropeptides in migraine and cluster headache. Cephalalgia 14: 320-327.

Edvinsson L, Sams A, Jansen-Olesen I, Tajti J, Kane SA, et al. 2001. Characterisation of the effects of a non-peptide CGRP receptor antagonist in SK–N–MC cells and isolated human cerebral arteries. Eur J Pharmacol 415: 39-44.

Elston Lafata J, Moon C, Leotta C, Kolodner K, Poisson L, et al. 2004. The medical care utilization and costs associated with migraine headache. J Gen Intern Med 19: 1005-1012.

Emeson RB, Hedjran F, Yeakley JM, Guise JW, Rosenfeld MG. 1989. Alternative production of calcitonin and CGRP mRNA is regulated at the calcitonin-specific splice acceptor. Nature 341: 76-80.

Estevez M, Estevez AO, Cowie RH, Gardner KL. 2004. The voltage-gated calcium channel UNC-2 is involved in stress-mediated regulation of tryptophan hydroxylase. J Neurochem 88: 102-113.

Eto T, Kato J, Kitamura K. 2003. Regulation of production and secretion of adrenomedullin in the cardiovascular system. Regul Pept 112: 61-69.

Eto T, Kitamura K. 2001. Adrenomedullin and its role in renal diseases. Nephron 89: 121-134.

Evans BN, Rosenblatt MI, Mnayer LO, Oliver KR, Dickerson IM. 2000. CGRP-RCP, a novel protein required for signal transduction at calcitonin gene-related peptide and adrenomedullin receptors. J Biol Chem 275: 31438-31443.

Eybalin M. 1993. Neurotransmitters and neuromodulators of the mammalian cochlea. Physiol Rev 73: 309-373.

Fenster CP, Whitworth TL, Sheffield EB, Quick MW, Lester RA. 1999. Upregulation of surface alpha4beta2 nicotinic receptors is initiated by receptor desensitization after chronic exposure to nicotine. J Neurosci 19: 4804-4814.

Fernandez HL, Chen M, Nadelhaft I, Durr JA. 2003. Calcitonin gene-related peptides: their binding sites and receptor accessory proteins in adult mammalian skeletal muscles. Neuroscience 119: 335-345.

Feron O, Saldana F, Michel JB, Michel T. 1998. The endothelial nitric-oxide synthase-caveolin regulatory cycle. J Biol Chem 273: 3125-3128.

Ferrari MD. 1998. Migraine. Lancet 351: 1043-1051.

Ferrari R, Panzali AF, Poole-Wilson PA, Anand IS. 1991. Plasma CGRP-like immunoreactivity in treated and untreated congestive heart failure [letter; comment]. Lancet 338: 1084.

Fex J, Altschuler RA. 1986. Neurotransmitter-related immunocytochemistry of the organ of Corti. Hear Res 22: 249-263.

Fiscus RR, Zhou HL, Wang X, Han C, Ali S, et al. 1991. Calcitonin gene-related peptide (CGRP)-induced cyclic AMP, cyclic GMP and vasorelaxant responses in rat thoracic aorta are antagonized by blockers of endothelium-derived relaxant factor (EDRF). Neuropeptides 20: 133-143.

Fitzsimmons TJ, Zhao X, Wank SA. 2003. The extracellular domain of receptor activity-modifying protein 1 is sufficient for calcitonin receptor-like receptor function. J Biol Chem 278: 14313-14320.

Fluhmann B, Muff R, Hunziker W, Fischer JA, Born W. 1995. A human orphan calcitonin receptor-like structure. Biochem Biophys Res Commun 206: 341-347.

Fontaine B, Klarsfeld A, Changeux JP. 1987. Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptor alpha-subunit mRNA levels by distinct intracellular pathways. J Cell Biol 105: 1337-1342.

Fontaine B, Klarsfeld A, Hokfelt T, Changeux JP. 1986. Calcitonin gene-related peptide, a peptide present in spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes. Neurosci Lett 71: 59-65.

Foulkes R, Shaw N, Bose C, Hughes B. 1991. Differential vasodilator profile of calcitonin gene-related peptide in porcine large and small diameter coronary artery rings. Eur J Pharmacol 201: 143-149.

Fowler PA, Lacey LF, Thomas M, Keene ON, Tanner RJ, et al. 1991. The clinical pharmacology, pharmacokinetics and metabolism of sumatriptan. Eur Neurol 31: 291-294.

Franco-Cereceda A, Liska J. 2000. Potential of calcitonin gene-related peptide in coronary heart disease. Pharmacology 60: 1-8.

Fraser NJ, Wise A, Brown J, McLatchie LM, Main MJ, et al. 1999. The amino terminus of receptor activity modifying proteins is a critical determinant of glycosylation state and ligand binding of calcitonin receptor-like receptor. Mol Pharmacol 55: 1054-1059.

Furuya K, Milchak RJ, Schegg KM, Zhang J, Tobe SS, et al. 2000. Cockroach diuretic hormones: characterization of a calcitonin-like peptide in insects. Proc Natl Acad Sci USA 97: 6469-6474.

Galibert MD, Carreira S, Goding CR. 2001. The Usf-1 transcription factor is a novel target for the stress-responsive p38 kinase and mediates UV-induced tyrosinase expression. EMBO J 20: 5022-5031.

Gangula PR, Zhao H, Supowit SC, Wimalawansa SJ, Dipette DJ, et al. 2000. Increased blood pressure in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. Hypertension 35: 470-475.

Gether U. 2000. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 21: 90-113.

Ghosh A, Greenberg ME. 1995. Calcium signaling in neurons: molecular mechanisms and cellular consequences. Science 268: 239-247.

Gibson SJ, Polak JM, Bloom SR, Sabate IM, Mulderry PM, et al. 1984. Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. J Neurosci 4: 3101-3111.

Goadsby PJ, Edvinsson L. 1993. The trigeminovascular system and migraine: studies characterizing cerebrovascular and neuropeptide changes seen in humans and cats. Ann Neurol 33: 48-56.

Goadsby PJ, Edvinsson L, Ekman R. 1990. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. Ann Neurol 28: 183-187.

Goadsby PJ, Lipton RB, Ferrari MD. 2002. Migraine—current understanding and treatment. N Engl J Med 346: 257-270.

Goadsby PJ, Zagami AS, Donnan GA, Symington G, Anthony M, et al. 1991. Oral sumatriptan in acute migraine. Lancet 338: 782-783.

Goldring SR, Roelke MS, Petrison KK, Bhan AK. 1987. Human giant cell tumors of bone identification and characterization of cell types. J Clin Invest 79: 483-491.

Gray DW, Marshall I. 1992a. Nitric oxide synthesis inhibitors attenuate calcitonin gene-related peptide endothelium-dependent vasorelaxation in rat aorta. Eur J Pharmacol 212: 37-42.

Gray DW, Marshall I. 1992b. Human alpha-calcitonin gene-related peptide stimulates adenylate cyclase and guanylate cyclase and relaxes rat thoracic aorta by releasing nitric oxide. Br J Pharmacol 107: 691-696.

Guillemot F. 1995. Analysis of the role of basic-helix–loop–helix transcription factors in the development of neural lineages in the mouse. Biol Cell 84: 3-6.

Gulbenkian S, Merighi A, Wharton J, Varndell IM, Polak JM. 1986. Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. J Neurocytol 15: 535-542.

Haase EB, Buchman J, Tietz AE, Schramm LP. 1997. Pregnancy-induced uterine neuronal degeneration in the rat. Cell Tissue Res 288: 293-306.

Hagner S, Stahl U, Knoblauch B, McGregor GP, Lang RE. 2002. Calcitonin receptor-like receptor: identification and distribution in human peripheral tissues. Cell Tissue Res 310: 41-50.

Hall ZW. 1973. Multiple forms of acetylcholinesterase and their distribution in endplate and non-endplate regions of rat diaphragm muscle. J Neurobiol 4: 343-361.

Han ZQ, Coppock HA, Smith DM, Van Noorden S, Makgoba MW, et al. 1997. The interaction of CGRP and adrenomedullin with a receptor expressed in the rat pulmonary vascular endothelium. J Mol Endocrinol 18: 267-272.

Harkness PC, Millar NS. 2002. Changes in conformation and subcellular distribution of alpha4beta2 nicotinic acetylcholine receptors revealed by chronic nicotine treatment and expression of subunit chimeras. J Neurosci 22: 10172-10181.

Harmar AJ. 2001. Family-B G-protein-coupled receptors. Genome Biol 2: reviews 3013.1-3013.10.

Heino P, Oksala O, Luhtala J, Uusitalo H. 1995. Localization of calcitonin gene-related peptide binding sites in the eye of different species. Curr Eye Res 14: 783-790.

Henke H, Tschopp FA, Fischer JA. 1985. Distinct binding sites for calcitonin gene-related peptide and salmon calcitonin in rat central nervous system. Brain Res 360: 165-171.

Hershey JC, Corcoran HA, Baskin EP, Salvatore CA, Mosser S, et al. 2005. Investigation of the species selectivity of a nonpeptide CGRP receptor antagonist using a novel pharmacodynamic assay. Regul Pept 127: 71-77.

Hilairet S, Belanger C, Bertrand J, Laperriere A, Foord SM, et al. 2001a. Agonist-promoted internalization of a ternary complex between calcitonin receptor-like receptor, receptor activity-modifying protein 1 (RAMP1), and beta-arrestin. J Biol Chem 276: 42182-42190.

Hilairet S, Foord SM, Marshall FH, Bouvier M. 2001b. Protein–protein interaction and not glycosylation determines the binding selectivity of heterodimers between the calcitonin receptor-like receptor and the receptor activity-modifying proteins. J Biol Chem 276: 29575-29581.

Hilton JM, Mitchelhill KI, Pozvek G, Dowton M, Quiza M, et al. 1998. Purification of calcitonin-like peptides from rat brain and pituitary. Endocrinology 139: 982-992.

Hinson JP, Kapas S, Smith DM. 2000. Adrenomedullin, a multifunctional regulatory peptide. Endocr Rev 21: 138-167.

Hirata Y, Takagi Y, Takata S, Fukuda Y, Yoshimi H, et al. 1988. Calcitonin gene-related peptide receptor in cultured vascular smooth muscle and endothelial cells. Biochem Biophys Res Commun 151: 1113-1121.

Hoff AO, Catala-Lehnen P, Thomas PM, Priemel M, Rueger JM, et al. 2002. Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene. J Clin Invest 110: 1849-1857.

Hoffman PW, Ravindran A, Huganir RL. 1994. Role of phosphorylation in desensitization of acetylcholine receptors expressed in *Xenopus oocytes*. J Neurosci 14: 4185-4195.

Holman JJ, Craig RK, Marshall I. 1986. Human alpha- and beta-CGRP and rat alpha-CGRP are coronary vasodilators in the rat. Peptides 7: 231-235.

Hoppener JW, Ahren B, Lips CJ. 2000. Islet amyloid and type 2 diabetes mellitus. N Engl J Med 343: 411-419.

Horiuchi M, Hayashida W, Kambe T, Yamada T, Dzau VJ. 1997. Angiotensin type 2 receptor dephosphorylates Bcl-2 by activating mitogen-activated protein kinase phosphatase-1 and induces apoptosis. J Biol Chem 272: 19022-19026.

Hu XH, Markson LE, Lipton RB, Stewart WF, Berger ML. 1999. Burden of migraine in the United States: disability and economic costs. Arch Intern Med 159: 813-818.

Huganir RL, Greengard P. 1983. cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor. Proc Natl Acad Sci USA 80: 1130-1134.

Huganir RL, Greengard P. 1990. Regulation of neurotransmitter receptor desensitization by protein phosphorylation. Neuron 5: 555-567.

Hulme EC, Lu ZL, Ward SD, Allman K, Curtis CA. 1999. The conformational switch in 7-transmembrane receptors: the muscarinic receptor paradigm. Eur J Pharmacol 375: 247-260.

Imaizumi S, Shimizu H, Ahmad I, Kaminuma T, Tajima M, et al. 1996. Effect of calcitonin gene-related peptide on delayed cerebral vasospasm after experimental subarachnoid hemorrhage in rabbits. Surg Neurol 46: 263-270; discussion 270–261.

Inagaki S, Kito S, Kubota Y, Girgis S, Hillyard CJ, et al. 1986. Autoradiographic localization of calcitonin gene-related peptide binding sites in human and rat brains. Brain Res 374: 287-298.

Inoue T, Shimizu H, Kaminuma T, Tajima M, Watabe K, et al. 1996. Prevention of cerebral vasospasm by calcitonin gene-related peptide slow-release tablet after subarachnoid hemorrhage in monkeys. Neurosurgery 39: 984-990.

Inzerillo AM, Zaidi M, Huang CL. 2002. Calcitonin: the other thyroid hormone. Thyroid 12: 791-798.

Itier V, Bertrand D. 2001. Neuronal nicotinic receptors: from protein structure to function. FEBS Lett 504: 118-125.

Ittner LM, Luessi F, Koller D, Born W, Fischer JA, et al. 2004. Aspartate(69) of the calcitonin-like receptor is required for its functional expression together with receptor-activity-modifying proteins 1 and -2. Biochem Biophys Res Commun 319: 1203-1209.

Jansen-Olesen I, Mortensen A, Edvinsson L. 1996. Calcitonin gene-related peptide is released from capsaicin-sensitive nerve fibres and induces vasodilatation of human cerebral arteries concomitant with activation of adenylyl cyclase. Cephalalgia 16: 310-316.

Jennings CG, Mudge AW. 1989. Chick myotubes in culture express high-affinity receptors for calcitonin gene-related peptide. Brain Res 504: 199-205.

Johnson EC, Shafer OT, Trigg JS, Park J, Schooley DA, et al. 2005. A novel diuretic hormone receptor in Drosophila: evidence for conservation of CGRP signaling. J Exp Biol 208: 1239-1246.

Juaneda C, Dumont Y, Quirion R. 2000. The molecular pharmacology of CGRP and related peptide receptor subtypes. Trends Pharmacol Sci 21: 432-438.

Juang KD, Wang SJ, Fuh JL, Lu SR, Su TP. 2000. Comorbidity of depressive and anxiety disorders in chronic daily headache and its subtypes. Headache 40: 818-823.

Juul R, Aakhus S, Bjornstad K, Gisvold SE, Brubakk AO, et al. 1994. Calcitonin gene-related peptide (human alpha-CGRP) counteracts vasoconstriction in human subarachnoid haemorrhage. Neurosci Lett 170: 67-70.

Kamitani S, Asakawa M, Shimekake Y, Kuwasako K, Nakahara K, et al. 1999. The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. FEBS Lett 448: 111-114.

Kapas S, Clark AJ. 1995. Identification of an orphan receptor gene as a type 1 calcitonin gene-related peptide receptor. Biochem Biophys Res Commun 217: 832-838.

Kar S, Rees RG, Quirion R. 1994. Altered calcitonin gene-related peptide, substance P and enkephalin immunoreactivities and receptor binding sites in the dorsal spinal cord of the polyarthritic rat. Eur J Neurosci 6: 345-354.

Katafuchi T, Kikumoto K, Hamano K, Kangawa K, Matsuo H, et al. 2003. Calcitonin receptor-stimulating peptide, a new member of the calcitonin gene-related peptide family. Its isolation from porcine brain, structure, tissue distribution, and biological activity. J Biol Chem 278: 12046-12054.

Katafuchi T, Minamino N. 2004. Structure and biological properties of three calcitonin receptor-stimulating peptides, novel members of the calcitonin gene-related peptide family. Peptides 25: 2039-2045.

Katki KA, Zhao H, Dickerson IM, Di Pette DJ, Supowit SC. 2003. Upregulation of calcitonin gene-related peptide receptor component protein in subtotal nephrectomy-salt hypertension. Hypertension 42: 409 [Abstract].

Kato K, Yin H, Agata J, Yoshida H, Chao L, et al. 2003. Adrenomedullin gene delivery attenuates myocardial infarction and apoptosis after ischemia and reperfusion. Am J Physiol Heart Circ Physiol 285: H1506-H1514.

Keyse SM. 1995. An emerging family of dual specificity MAP kinase phosphatases. Biochim Biophys Acta 1265: 152-160.

Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. 1993. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 192(2): 553-560.

Klein SL, Strausberg RL, Wagner L, Pontius J, Clifton SW, Richardson P. 2002. Genetic and genomic tools for Xenopus research: The NIH Xenopus initiative. Dev Dyn 225(4): 384-391.

Koller D, Ittner LM, Muff R, Husmann K, Fischer JA, et al. 2004. Selective inactivation of adrenomedullin over calcitonin gene-related peptide receptor function by the deletion of amino acids 14–20 of the mouse calcitonin-like receptor. J Biol Chem 279: 20387-20391.

Krootila K, Oksala O, von Dickhoff K, Wimalawansa SJ, MacIntyre I, et al. 1992. Ocular irritative response to YAG laser capsulotomy in rabbits: release of calcitonin gene-related peptide and effects of methysergide. Curr Eye Res 11: 307-314.

Krootila K, Uusitalo H, Palkama A. 1988. Effect of neurogenic irritation and calcitonin gene-related peptide (CGRP) on ocular blood flow in the rabbit. Curr Eye Res 7: 695-703.

Kruger L, Mantyh PW, Sternini C, Brecha NC, Mantyh CR. 1988. Calcitonin gene-related peptide (CGRP) in the rat central nervous system: patterns of immunoreactivity and receptor binding sites. Brain Res 463: 223-244.

Kusari AB, Byon J, Bandyopadhyay D, Kenner KA, Kusari J. 1997. Insulin-induced mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) attenuates insulin-stimulated MAP kinase activity: a mechanism for the feedback inhibition of insulin signaling. Mol Endocrinol 11: 1532-1543.

Kuwasako K, Cao YN, Nagoshi Y, Tsuruda T, Kitamura K, et al. 2004. Characterization of the human calcitonin gene-related peptide receptor subtypes associated with receptor activity-modifying proteins. Mol Pharmacol 65: 207-213.

Kuwasako K, Kitamura K, Nagoshi Y, Cao YN, Eto T. 2003a. Identification of the human receptor activity-modifying protein 1 domains responsible for agonist binding specificity. J Biol Chem 278: 22623-22630.

Kuwasako K, Kitamura K, Nagoshi Y, Eto T. 2003b. Novel calcitonin-(8–32)-sensitive adrenomedullin receptors derived from co-expression of calcitonin receptor with receptor activity-modifying proteins. Biochem Biophys Res Commun 301: 460-464.

Kuwasako K, Shimekake Y, Masuda M, Nakahara K, Yoshida T, et al. 2000. Visualization of the calcitonin receptor-like receptor and its receptor activity-modifying proteins during internalization and recycling. J Biol Chem 275: 29602-29609.

Lanigan TM, De Raad SK, Russo AF. 1998. Requirement of the MASH-1 transcription factor for neuroendocrine differentiation of thyroid C cells. J Neurobiol 34: 126-134.

Lanigan TM, Russo AF. 1997. Binding of upstream stimulatory factor and a cell-specific activator to the calcitonin/calcitonin gene-related peptide enhancer. J Biol Chem 272: 18316-18324.

Lanigan TM, Tverberg LA, Russo AF. 1993. Retinoic acid repression of cell-specific helix–loop–helix-octamer activation of the calcitonin/calcitonin gene-related peptide enhancer. Mol Cell Biol 13: 6079-6088.

Lassen LH, Haderslev PA, Jacobsen VB, Iversen HK, Sperling B, et al. 2002. CGRP may play a causative role in migraine. Cephalalgia 22: 54-61.

Laufer R, Changeux JP. 1987. Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: possible neurotrophic role for a coexisting neuronal messenger. EMBO J 6: 901-906.

Laufer R, Changeux JP. 1989. Calcitonin gene-related peptide and cyclic AMP stimulate phosphoinositide turnover in

skeletal muscle cells. Interaction between two second messenger systems. J Biol Chem 264: 2683-2689.

Lechleitner P, Genser N, Mair J, Dienstl A, Haring C, et al. 1992. Calcitonin gene-related peptide in patients with and without early reperfusion after acute myocardial infarction. Am Heart J 124: 1433-1439.

Liberman MC. 1980. Efferent synapses in the inner hair cell area of the cat cochlea: an electron microscopic study of serial sections. Hear Res 3: 189-204.

Limmroth V, Katsarava Z, Fritsche G, Przywara S, Diener HC. 2002. Features of medication overuse headache following overuse of different acute headache drugs. Neurology 59: 1011-1014.

Limmroth V, Kazarawa Z, Fritsche G, Diener HC. 1999. Headache after frequent use of serotonin agonists zolmitriptan and naratriptan. Lancet 353: 378.

Lin HY, Harris TL, Flannery MS, Aruffo A, Kaji EH, et al. 1991. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. Science 254: 1022-1024.

Lind H, Edvinsson L. 2002. Enhanced vasodilator responses to calcitonin gene-related peptide (CGRP) in subcutaneous arteries in human hypertension. J Hum Hypertens 16: 53-59.

Lindsay RM, Harmar AJ. 1989. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. Nature 337: 362-364.

Linscheid P, Seboek D, Nylen ES, Langer I, Schlatter M, et al. 2003. In vitro and in vivo calcitonin I gene expression in parenchymal cells: a novel product of human adipose tissue. Endocrinology 144: 5578-5584.

Lipton JA, Ship JA, Larach-Robinson D. 1993. Estimated prevalence and distribution of reported orofacial pain in the United States. J Am Dent Assoc 124: 115-121.

Lipton RB, Stewart WF, Diamond S, Diamond ML, Reed M. 2001. Prevalence and burden of migraine in the United States: data from the American Migraine Study II. Headache 41: 646-657.

Liu D, Homan LL, Dillon JS. 2004. Genistein acutely stimulates nitric oxide synthesis in vascular endothelial cells by a cyclic adenosine 5′-monophosphate-dependent mechanism. Endocrinology 145: 5532-5539.

Longmore J, Hogg JE, Hutson PH, Hill RG. 1994. Effects of two truncated forms of human calcitonin-gene related peptide: implications for receptor classification. Eur J Pharmacol 265: 53-59.

Lou H, Cote GJ, Gagel RF. 1994. The calcitonin exon and its flanking intronic sequences are sufficient for the regulation of human calcitonin/calcitonin gene-related peptide alternative RNA splicing. Mol Endocrinol 8: 1618-1626.

Lou H, Gagel RF. 1998. Alternative RNA processing—its role in regulating expression of calcitonin/calcitonin gene-related peptide. J Endocrinol 156: 401-405.

Lou H, Gagel RF, Berget SM. 1996. An intron enhancer recognized by splicing factors activates polyadenylation. Genes Dev 10: 208-219.

Lou H, Yang Y, Cote GJ, Berget SM, Gagel RF. 1995. An intron enhancer containing a 5′ splice site sequence in the human calcitonin/calcitonin gene-related peptide gene. Mol Cell Biol 15: 7135-7142.

Lu B, Fu WM, Greengard P, Poo MM. 1993. Calcitonin gene-related peptide potentiates synaptic responses at developing neuromuscular junction. Nature 363: 76-79.

Lu JT, Son YJ, Lee J, Jetton TL, Shiota M, et al. 1999. Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. Mol Cell Neurosci 14: 99-120.

Luebke AE, Dickerson IM, 2002. Role of CGRP-Receptor Component Protein (RCP) in CGRP-mediated Signal Transduction. Assoc Res Otolaryngol Abs 309.

Luebke AE, Dahl GP, Roos BA, Dickerson IM. 1996. Identification of a protein that confers calcitonin gene-related peptide responsiveness to oocytes by using a cystic fibrosis transmembrane conductance regulator assay. Proc Natl Acad Sci USA 93: 3455-3460.

Lundberg JM. 1996. Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. Pharmacol Rev 48: 113-178.

Ma W, Chabot JG, Powell KJ, Jhamandas K, Dickerson IM, et al. 2003. Localization and modulation of calcitonin gene-related peptide-receptor component protein-immunoreactive cells in the rat central and peripheral nervous systems. Neuroscience 120: 677-694.

Ma W, Zheng WH, Kar S, Quirion R. 2000. Morphine treatment induced calcitonin gene-related peptide and substance P increases in cultured dorsal root ganglion neurons. Neuroscience 99: 529-539.

Mac Arthur L, Eiden L. 1996. Neuropeptide genes: targets of activity-dependent signal transduction. Peptides 17: 721-728.

Macdonald RL, Weir BK. 1994. Cerebral vasospasm and free radicals. Free Radic Biol Med 16: 633-643.

Mair J, Lechleitner P, Langle T, Wiedermann C, Dienstl F, et al. 1990. Plasma CGRP in acute myocardial infarction. Lancet 335: 168.

Maison SF, Adams JC, Liberman MC. 2003a. Olivocochlear innervation in the mouse: immunocytochemical maps, crossed versus uncrossed contributions, and transmitter colocalization. J Comp Neurol 455: 406-416.

Maison SF, Emeson RB, Adams JC, Luebke AE, Liberman MC. 2003b. Loss of alpha CGRP reduces sound-evoked activity in the cochlear nerve. J Neurophysiol 90: 2941-2949.

Mallee JJ, Salvatore CA, LeBourdelles B, Oliver KR, Longmore J, et al. 2002. Receptor activity-modifying

protein 1 determines the species selectivity of non-peptide CGRP receptor antagonists. J Biol Chem 277: 14294-14298.

Malminiemi OI, Malminiemi KH. 1992. [125I]calcitonin gene-related peptide binding in membranes of the ciliary body-iris block. Curr Eye Res 11: 1079-1085.

Martin CC, Svitek CA, Oeser JK, Henderson E, Stein R, et al. 2003. Upstream stimulatory factor (USF) and neurogenic differentiation/beta-cell E box transactivator 2 (NeuroD/BETA2) contribute to islet-specific glucose-6-phosphatase catalytic-subunit-related protein (IGRP) gene expression. Biochem J 371: 675-686.

Massari ME, Murre C. 2000. Helix–loop–helix proteins: regulators of transcription in eucaryotic organisms. Mol Cell Biol 20: 429-440.

Matteoli M, Haimann C, Torri-Tarelli F, Polak JM, Ceccarelli B, et al. 1988. Differential effect of alpha-latrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction. Proc Natl Acad Sci USA 85: 7366-7370.

McCulloch J, Uddman R, Kingman TA, Edvinsson L. 1986. Calcitonin gene-related peptide: functional role in cerebrovascular regulation. Proc Natl Acad Sci USA 83: 5731-5735.

McDermott MT, Kidd GS. 1987. The role of calcitonin in the development and treatment of osteoporosis. Endocr Rev 8: 377-390.

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, et al. 1998. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 393: 333-339.

Menard DP, van Rossum D, Kar S, St Pierre S, Sutak M, et al. 1996. A calcitonin gene-related peptide receptor antagonist prevents the development of tolerance to spinal morphine analgesia. J Neurosci 16: 2342-2351.

Merikangas KR, Angst J, Isler H. 1990. Migraine and psychopathology. Results of the Zurich cohort study of young adults. Arch Gen Psychiatry 47: 849-853.

Miles K, Greengard P, Huganir RL. 1989. Calcitonin gene-related peptide regulates phosphorylation of the nicotinic acetylcholine receptor in rat myotubes. Neuron 2: 1517-1524.

Miller RJ. 1997. Calcium channels prove to be a real headache. Trends Neurosci 20: 189-192.

Moncada S, Palmer RM, Higgs EA. 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43: 109-142.

Monia YT, Peleg S, Gagel RF, 1995. Cell type-specific regulation of transcription by cyclic adenosine 3, ′5′-monophosphate-responsive elements within the calcitonin promoter. Mol Endocrinol 9: 784-793.

Moreno MJ, Abounader R, Hebert E, Doods H, Hamel E. 2002. Efficacy of the non-peptide CGRP receptor antagonist BIBN4096BS in blocking CGRP-induced dilations in human and bovine cerebral arteries: potential implications in acute migraine treatment. Neuropharmacology 42: 568-576.

Muff R, Buhlmann N, Fischer JA, Born W. 1999. An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. Endocrinology 140: 2924-2927.

Muff R, Leuthauser K, Buhlmann N, Foord SM, Fischer JA, et al. 1998. Receptor activity modifying proteins regulate the activity of a calcitonin gene-related peptide receptor in rabbit aortic endothelial cells. FEBS Lett 441: 366-368.

Mukoyama M, Sugawara A, Nagae T, Mori K, Murabe H, et al. 2001. Role of adrenomedullin and its receptor system in renal pathophysiology. Peptides 22: 1925-1931.

Mulderry PK, Ghatei MA, Spokes RA, Jones PM, Pierson AM, et al. 1988. Differential expression of alpha-CGRP and beta-CGRP by primary sensory neurons and enteric autonomic neurons of the rat. Neuroscience 25: 195-205.

Mulle C, Benoit P, Pinset C, Roa M, Changeux JP. 1988. Calcitonin gene-related peptide enhances the rate of desensitization of the nicotinic acetylcholine receptor in cultured mouse muscle cells. Proc Natl Acad Sci USA 85: 5728-5732.

Muller B, White JC, Nylen ES, Snider RH, Becker KL, et al. 2001. Ubiquitous expression of the calcitonin-i gene in multiple tissues in response to sepsis. J Clin Endocrinol Metab 86: 396-404.

Naghashpour M, Dahl G. 2000. Sensitivity of myometrium to CGRP varies during mouse estrous cycle and in response to progesterone. Am J Physiol Cell Physiol 278: C561-C569.

Naghashpour M, Rosenblatt MI, Dickerson IM, Dahl GP. 1997. Inhibitory effect of calcitonin gene-related peptide on myometrial contractility is diminished at parturition. Endocrinology 138: 4207-4214.

Nakagawa T, Mabry M, de Bustros A, Ihle JN, Nelkin BD, et al. 1987. Introduction of v-Ha-ras oncogene induces differentiation of cultured human medullary thyroid carcinoma cells. Proc Natl Acad Sci USA 84: 5923-5927.

Nathan C, Xie QW. 1994. Regulation of biosynthesis of nitric oxide. J Biol Chem 269: 13725-13728.

Nelson MT, Huang Y, Brayden JE, Hescheler J, Standen NB. 1990. Arterial dilations in response to calcitonin gene-related peptide involve activation of K+ channels. Nature 344: 770-773.

New HV, Mudge AW. 1986. Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. Nature 323: 809-811.

Nikitenko LL, Brown NS, Smith DM, Mac Kenzie IZ, Bicknell R, et al. 2001. Differential and cell-specific expression of calcitonin receptor-like receptor and receptor activity modifying proteins in the human uterus. Mol Hum Reprod 7: 655-664.

Nikitenko LL, Smith DM, Bicknell R, Rees MC. 2003. Transcriptional regulation of the CRLR gene in human microvascular endothelial cells by hypoxia. FASEB J 17: 1499-1501.

Nishikimi T, Mori Y, Kobayashi N, Tadokoro K, Wang X, et al. 2002. Renoprotective effect of chronic adrenomedullin infusion in Dahl salt-sensitive rats. Hypertension 39: 1077-1082.

Nozaki K, Kikuchi H, Mizuno N. 1989. Changes of calcitonin-gene-related peptide-like immunoreactivity in cerebrovascular nerve fibers in the dog after experimentally produced subarachnoid hemorrhage. Neurosci Lett 102: 27-32.

O'Connor TP, van der Kooy D. 1986. Pattern of intracranial and extracranial projections of trigeminal ganglion cells. J Neurosci 6: 2200-2207.

O'Connor TP, van der Kooy D. 1988. Enrichment of a vasoactive neuropeptide (calcitonin gene related peptide) in the trigeminal sensory projection to the intracranial arteries. J Neurosci 8: 2468-2476.

Oh-Hashi Y, Shindo T, Kurihara Y, Imai T, Wang Y, et al. 2001. Elevated sympathetic nervous activity in mice deficient in alphaCGRP. Circ Res 89: 983-990.

Oksala O. 1988. Effects of calcitonin gene-related peptide and substance P on regional blood flow in the cat eye. Exp Eye Res 47: 283-289.

Olesen J, Diener HC, Husstedt IW, Goadsby PJ, Hall D, et al. 2004. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. N Engl J Med 350: 1104-1110.

Oliver KR, Kane SA, Salvatore CA, Mallee JJ, Kinsey AM, et al. 2001. Cloning, characterization and central nervous system distribution of receptor activity modifying proteins in the rat. Eur J Neurosci 14: 618-628.

Oliver KR, Wainwright A, Edvinsson L, Pickard JD, Hill RG. 2002. Immunohistochemical localization of calcitonin receptor-like receptor and receptor activity-modifying proteins in the human cerebral vasculature. J Cereb Blood Flow Metab 22: 620-629.

Oliver KR, Wainwright A, Heavens RP, Hill RG, Sirinathsinghji DJ. 1998. Distribution of novel CGRP1 receptor and adrenomedullin receptor mRNAs in the rat central nervous system. Brain Res Mol Brain Res 57: 149-154.

Oliver KR, Wainwright A, Kinsey AM, Heavens RP, Sirinathsinghji DJ, et al. 1999. Regional and cellular localization of calcitonin gene-related peptide-receptor component protein mRNA in the guinea-pig central nervous system. Brain Res Mol Brain Res 66: 205-210.

Ottosson A, Edvinsson L. 1997. Release of histamine from dural mast cells by substance P and calcitonin gene-related peptide. Cephalalgia 17: 166-174.

Parameswaran N, Disa J, Spielman WS, Brooks DP, Nambi P, et al. 2000. Activation of multiple mitogen-activated protein kinases by recombinant calcitonin gene-related peptide receptor. Eur J Pharmacol 389: 125-130.

Parsons AA, Strijbos PJ. 2003. The neuronal versus vascular hypothesis of migraine and cortical spreading depression. Curr Opin Pharmacol 3: 73-77.

Peleg S, Abruzzese RV, Cooper CW, Gagel RF. 1993. Down-regulation of calcitonin gene transcription by vitamin D requires two widely separated enhancer sequences. Mol Endocrinol 7: 999-1008.

Peleg S, Abruzzese RV, Cote GJ, Gagel RF. 1990. Transcription of the human calcitonin gene is mediated by a C cell-specific enhancer containing E-box-like elements. Mol Endocrinol 4: 1750-1757.

Peng HB, Chen QM, de Biasi S, Zhu DL. 1989. Development of calcitonin gene-related peptide (CGRP) immunoreactivity in relationship to the formation of neuromuscular junctions in Xenopus myotomal muscle. J Comp Neurol 290: 533-543.

Pietrobon D, Striessnig J. 2003. Neurobiology of migraine. Nat Rev Neurosci 4: 386-398.

Pokabla MJ, Dickerson IM, Papka RE. 2002. Calcitonin gene-related peptide-receptor component protein expression in the uterine cervix, lumbosacral spinal cord, and dorsal root ganglia. Peptides 23: 507-514.

Pondel M. 2000. Calcitonin and calcitonin receptors: bone and beyond. Int J Exp Pathol 81: 405-422.

Popper P, Micevych PE. 1989. Localization of calcitonin gene-related peptide and its receptors in a striated muscle. Brain Res 496: 180-186.

Poyner DR. 1992. Calcitonin gene-related peptide: multiple actions, multiple receptors. Pharmacol Ther 56: 23-51.

Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, et al. 2002. International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 54: 233-246.

Prado MA, Evans-Bain B, Dickerson IM. 2002. Receptor component protein (RCP): a member of a multi-protein complex required for G-protein-coupled signal transduction. Biochem Soc Trans 30: 460-464.

Prado MA, Evans-Bain B, Oliver KR, Dickerson IM. 2001. The role of the CGRP-receptor component protein (RCP) in adrenomedullin receptor signal transduction. Peptides 22: 1773-1781.

Preibisz JJ. 1993. Calcitonin gene-related peptide and regulation of human cardiovascular homeostasis. Am J Hypertens 6: 434-450.

Qin XP, Ye F, Hu CP, Liao DF, Deng HW, et al. 2004. Effect of calcitonin gene-related peptide on angiotensin II-induced proliferation of rat vascular smooth muscle cells. Eur J Pharmacol 488: 45-49.

Roa M, Changeux JP. 1991. Characterization and developmental evolution of a high-affinity binding site for

calcitonin gene-related peptide on chick skeletal muscle membrane. Neuroscience 41: 563-570.

Roh J, Chang CL, Bhalla A, Klein C, Hsu SY. 2004. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J Biol Chem 279: 7264-7274.

Rosen LB, Ginty DD, Weber MJ, Greenberg ME. 1994. Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 12: 1207-1221.

Rosenblatt MI, Dahl GP, Dickerson IM. 2000. Characterization and localization of the rabbit ocular calcitonin gene-related peptide (CGRP)-receptor component protein (RCP). Invest Ophthalmol Vis Sci 41: 1159-1167.

Rosenfeld MG, Mermod JJ, Amara SG, Swanson LW, Sawchenko PE, et al. 1983. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature 304: 129-135.

Ross R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362: 801-809.

Rossi SG, Dickerson IM, Rotundo RL. 2003. Localization of the calcitonin gene-related peptide receptor complex at the vertebrate neuromuscular junction and its role in regulating acetylcholinesterase expression. J Biol Chem 278: 24994-25000.

Roudenok V, Gutjar L, Antipova V, Rogov Y. 2001. Expression of vasoactive intestinal polypeptide and calcitonin gene-related peptide in human stellate ganglia after acute myocardial infarction. Ann Anat 183: 341-344.

Russell IJ. 1968. Influence of efferent fibres on a receptor. Nature 219: 177-178.

Russo AF, Nelson C, Roos BA, Rosenfeld MG. 1988. Differential regulation of the coexpressed calcitonin/alpha-CGRP and beta-CGRP neuroendocrine genes. J Biol Chem 263: 5-8.

Russwurm S, Stonans I, Stonane E, Wiederhold M, Luber A, et al. 2001. Procalcitonin and CGRP-1 mRNA expression in various human tissues. Shock 16: 109-112.

Sakaguchi M, Inaishi Y, Kashihara Y, Kuno M. 1991. Release of calcitonin gene-related peptide from nerve terminals in rat skeletal muscle. J Physiol 434: 257-270.

Salmon AM, Damaj MI, Marubio LM, Epping-Jordan MP, Merlo-Pich E, et al. 2001. Altered neuroadaptation in opiate dependence and neurogenic inflammatory nociception in alpha CGRP-deficient mice. Nat Neurosci 4: 357-358.

Salmon AM, Damaj MI, Sekine S, Picciotto MR, Marubio L, et al. 1999. Modulation of morphine analgesia in alphaCGRP mutant mice. Neuroreport 10: 849-854.

Samuelson UE, Dalsgaard CJ, Lundberg JM, Hokfelt T. 1985. Calcitonin gene-related peptide inhibits spontaneous contractions in human uterus and fallopian tube. Neurosci Lett 62: 225-230.

Schaeffer C, Vandroux D, Thomassin L, Athias P, Rochette L, et al. 2003. Calcitonin gene-related peptide partly protects cultured smooth muscle cells from apoptosis induced by an oxidative stress via activation of ERK1/2 MAPK. Biochim Biophys Acta 1643: 65-73.

Scimeca JC, Servant MJ, Dyer JO, Meloche S. 1997. Essential role of calcium in the regulation of MAP kinase phosphatase-1 expression. Oncogene 15: 717-725.

Seger R, Krebs EG. 1995. The MAPK signaling cascade. FASEB J 9: 726-735.

Sewell WF, Starr PA. 1991. Effects of calcitonin gene-related peptide and efferent nerve stimulation on afferent transmission in the lateral line organ. J Neurophysiol 65: 1158-1169.

Seybold VS, Galeazza MT, Garry MG, Hargreaves KM. 1995. Plasticity of calcitonin gene related peptide neurotransmission in the spinal cord during peripheral inflammation. Can J Physiol Pharmacol 73: 1007-1014.

Shew RL, Papka RE, McNeill DL. 1990. Calcitonin gene-related peptide in the rat uterus: presence in nerves and effects on uterine contraction. Peptides 11: 583-589.

Sobey CG, Heistad DD, Faraci FM. 1996. Effect of subarachnoid hemorrhage on dilatation of rat basilar artery in vivo. Am J Physiol 271: H126-H132.

Stachniak TJ, Krukoff TL. 2003. Receptor activity modifying protein 2 distribution in the rat central nervous system and regulation by changes in blood pressure. J Neuroendocrinol 15: 840-850.

Steenbergh PH, Hoppener JW, Zandberg J, Lips CJ, Jansz HS. 1985. A second human calcitonin/CGRP gene. FEBS Lett 183: 403-407.

Steiner S, Born W, Fischer JA, Muff R. 2003. The function of conserved cysteine residues in the extracellular domain of human receptor-activity-modifying protein. FEBS Lett 555: 285-290.

Steiner S, Muff R, Gujer R, Fischer JA, Born W. 2002. The transmembrane domain of receptor-activity-modifying protein 1 is essential for the functional expression of a calcitonin gene-related peptide receptor. Biochemistry (Mosc) 41: 11398-11404.

Stolarsky-Fredman L, Leff SE, Klein ES, Crenshaw EB III, Yeakley J, et al. 1990. A tissue-specific enhancer in the rat-calcitonin/CGRP gene is active in both neural and endocrine cell types. Mol Endocrinol 4: 497-504.

Supowit SC, Christensen MD, Westlund KN, Hallman DM, DiPette DJ. 1995a. Dexamethasone and activators of the protein kinase A and C signal transduction pathways regulate neuronal calcitonin gene-related peptide expression and release. Brain Res 686: 77-86.

Supowit SC, Gururaj A, Ramana CV, Westlund KN, DiPette DJ. 1995b. Enhanced neuronal expression of calcitonin

gene-related peptide in mineralocorticoid-salt hypertension. Hypertension 25: 1333-1338.

Supowit SC, Zhao H, Hallman DM, Di Pette DJ. 1997. Calcitonin gene-related peptide is a depressor of deoxycorticosterone-salt hypertension in the rat. Hypertension 29: 945-950.

Supowit SC, Zhao H, Hallman DM, Di Pette DJ. 1998. Calcitonin gene-related peptide is a depressor in subtotal nephrectomy hypertension. Hypertension 31: 391-396.

Supowit SC, Zhao H, Wang DH, Di Pette DJ. 2001. Omapatrilat in subtotal nephrectomy-salt hypertension: role of calcitonin gene-related peptide. Hypertension 38: 697-700.

Tabuchi A, Sakaya H, Kisukeda T, Fushiki H, Tsuda M. 2002. Involvement of an upstream stimulatory factor as well as cAMP-responsive element-binding protein in the activation of brain-derived neurotrophic factor gene promoter I. J Biol Chem 277: 35920-35931.

Terenghi G, Polak JM, Ghatei MA, Mulderry PK, Butler JM, et al. 1985. Distribution and origin of calcitonin gene-related peptide (CGRP) immunoreactivity in the sensory innervation of the mammalian eye. J Comp Neurol 233: 506-516.

Terenghi G, Zhang SQ, Unger WG, Polak JM. 1986. Morphological changes of sensory CGRP-immunoreactive and sympathetic nerves in peripheral tissues following chronic denervation. Histochemistry 86: 89-95.

Thiagalingam A, de Bustros A, Borges M, Jasti R, Compton D, et al. 1996. RREB-1, a novel zinc finger protein, is involved in the differentiation response to Ras in human medullary thyroid carcinomas. Mol Cell Biol 16: 5335-5345.

Thota C, Gangula PR, Dong YallampalliC YL, 2003. Changes in the expression of calcitonin receptor-like receptor, receptor activity-modifying protein (RAMP)1, RAMP2, and RAMP3 in rat uterus during pregnancy, labor, and by steroid hormone treatments. Biol Reprod 69: 1432-1437.

Tilakaratne N, Christopoulos G, Zumpe ET, Foord SM, Sexton PM. 2000. Amylin receptor phenotypes derived from human calcitonin receptor/RAMP coexpression exhibit pharmacological differences dependent on receptor isoform and host cell environment. J Pharmacol Exp Ther 294: 61-72.

Touyz RM, Schiffrin EL. 2000. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. Pharmacol Rev 52: 639-672.

Toyoda K, Chu Y, Heistad DD. 2003. Gene therapy for cerebral vascular disease: update 2003. Br J Pharmacol 139: 1-9.

Toyoda K, Faraci FM, Russo AF, Davidson BL, Heistad DD. 2000a. Gene transfer of calcitonin gene-related peptide to cerebral arteries. Am J Physiol Heart Circ Physiol 278: H586-H594.

Toyoda K, Faraci FM, Watanabe Y, Ueda T, Andresen JJ, et al. 2000b. Gene transfer of calcitonin gene-related peptide prevents vasoconstriction after subarachnoid hemorrhage. Circ Res 87: 818-824.

Tran Dinh YR, Debdi M, Couraud JY, Creminon C, Seylaz J, et al. 1994. Time course of variations in rabbit cerebrospinal fluid levels of calcitonin gene-related peptide- and substance P-like immunoreactivity in experimental subarachnoid hemorrhage. Stroke 25: 160-164.

Tritthart HA, Stark U, Stark G, Mahnert W, Stender CO, et al. 1992. Membrane actions of calcitonin gene-related peptide in cardiac and smooth muscle myocytes. Ann N Y Acad Sci 657: 216-227.

Tschopp FA, Henke H, Petermann JB, Tobler PH, Janzer R, et al. 1985. Calcitonin gene-related peptide and its binding sites in the human central nervous system and pituitary. Proc Natl Acad Sci USA 82: 248-252.

Tverberg LA, Russo AF. 1992. Cell-specific glucocorticoid repression of calcitonin/calcitonin gene-related peptide transcription. Localization to an 18-base pair basal enhancer element. J Biol Chem 267: 17567-17573.

Tverberg LA, Russo AF. 1993. Regulation of the calcitonin/calcitonin gene-related peptide gene by cell-specific synergy between helix–loop–helix and octamer-binding transcription factors. J Biol Chem 268: 15965-15973.

Uchida S, Yamamoto H, Iio S, Matsumoto N, Wang XB, et al. 1990. Release of calcitonin gene-related peptide-like immunoreactive substance from neuromuscular junction by nerve excitation and its action on striated muscle. J Neurochem 54: 1000-1003.

Uddman R, Edvinsson L, Ekblad E, Hakanson R, Sundler F. 1986. Calcitonin gene-related peptide (CGRP): perivascular distribution and vasodilatory effects. Regul Pept 15: 1-23.

Unger WG. 1989. Mediation of the ocular response to injury and irritation: peptides versus prostaglandins. Prog Clin Biol Res 312: 293-328.

Unger WG. 1990. Review: mediation of the ocular response to injury. J Ocul Pharmacol 6: 337-353.

Unger WG. 1992. Pharmacological and neural bases for eye irritation. Ann N Y Acad Sci 641: 176-186.

Unger WG, Terenghi G, Ghatei MA, Ennis KW, Butler JM, et al. 1985. Calcitonin gene-related polypeptide as a mediator of the neurogenic ocular injury response. J Ocul Pharmacol 1: 189-199.

Uusitalo H, Krootila K, Palkama A. 1989. Calcitonin gene-related peptide (CGRP) immunoreactive sensory nerves in the human and guinea pig uvea and cornea. Exp Eye Res 48: 467-475.

van den Maagdenberg AM, Pietrobon D, Pizzorusso T, Kaja S, Broos LA, et al. 2004. A cacna1a knockin migraine mouse model with increased susceptibility to cortical spreading depression. Neuron 41: 701-710.

van Oers CC, Adema GJ, Zandberg H, Moen TC, Baas PD. 1994. Two different sequence elements within exon 4 are necessary for calcitonin-specific splicing of the human calcitonin/calcitonin gene-related peptide I pre-mRNA. Mol Cell Biol 14: 951-960.

van Rossum D, Hanisch UK, Quirion R. 1997. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev 21: 649-678.

van Rossum D, Menard DP, Fournier A, St Pierre S, Quirion R. 1994. Autoradiographic distribution and receptor binding profile of [125I]Bolton Hunter-rat amylin binding sites in the rat brain. J Pharmacol Exp Ther 270: 779-787.

Van Valen F, Piechot G, Jurgens H. 1990. Calcitonin gene-related peptide (CGRP) receptors are linked to cyclic adenosine monophosphate production in SK–N–MC human neuroblastoma cells. Neurosci Lett 119: 195-198.

Vetter DE, Adams JC, Mugnaini E. 1991. Chemically distinct rat olivocochlear neurons. Synapse 7: 21-43.

Viney TJ, Schmidt TW, Gierasch W, Sattar AW, Yaggie RE, et al. 2004. Regulation of the cell-specific calcitonin/calcitonin gene-related peptide enhancer by USF and the Foxa2 forkhead protein. J Biol Chem 279: 49948-49955.

Watson A, Latchman D. 1995. The cyclic AMP response element in the calcitonin/calcitonin gene-related peptide gene promoter is necessary but not sufficient for its activation by nerve growth factor. J Biol Chem 270: 9655-9660.

Wellman GC, Quayle JM, Standen NB. 1998. ATP-sensitive K+ channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. J Physiol 507 (Pt 1): 117-129.

Westermark P, Wernstedt C, O'Brien TD, Hayden DW, Johnson KH. 1987. Islet amyloid in type 2 human diabetes mellitus and adult diabetic cats contains a novel putative polypeptide hormone. Am J Pathol 127: 414-417.

Wimalawansa SJ. 1996. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocr Rev 17: 533-585.

Yeakley JM, Hedjran F, Morfin JP, Merillat N, Rosenfeld MG, et al. 1993. Control of calcitonin/calcitonin gene-related peptide pre-mRNA processing by constitutive intron and exon elements. Mol Cell Biol 13: 5999-6011.

Zhang L, Bonev AD, Mawe GM, Nelson MT. 1994. Protein kinase A mediates activation of ATP-sensitive K+ currents by CGRP in gallbladder smooth muscle. Am J Physiol 267: G494-G499.

Zhang L, Hoff AO, Wimalawansa SJ, Cote GJ, Gagel RF, et al. 2001. Arthritic calcitonin/alpha calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity. Pain 89: 265-273.

Zigmond SH, Woodworth A, Daukas G. 1985. Effects of sodium on chemotactic peptide binding to polymorphonuclear leukocytes. J Immunol 135: 531-536.

Zumpe ET, Tilakaratne N, Fraser NJ, Christopoulos G, Foord SM, et al. 2000. Multiple ramp domains are required for generation of amylin receptor phenotype from the calcitonin receptor gene product. Biochem Biophys Res Commun 267: 368-372.

# 20 Substance P and the Tachykinins

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**Abstract:** The tachykinins are part of an important neurotransmitter pathway involving several neuropeptides and receptors, all of which are discussed in this chapter. The major pathway analyzed has been that of substance P (SP) and its high-affinity receptor, NK1. Tachykinins are widely distributed throughout the mammalian body in both the central nervous system (CNS) and the peripheral nervous system (PNS) with numerous functions being attributed to them in each of these systems. Tachykinins are predominantly synthesized in neurons of the CNS and the PNS and stored in large dense vesicles. Upon excitation of these neurons, tachykinins are released and act on their appropriate receptors on the target cells to evoke various responses. Although SP is predominantly thought of as a neurotransmitter, there is a growing appreciation of it as an inflammatory molecule acting analogous to a cytokine; its expression has been found in a variety of nonneuronal cells. We have also summarized this role, as expression of the tachykinins in the CNS in cells other than neurons may be important for its function in both normal physiology and pathological conditions. SP is implicated in a range of disorders ranging from itch and migraine through epilepsy, Parkinson's disease and psychiatric and cognitive disorders. In many of these disorders not only the level but also the duration and tissue specificity of expression contributes to overall function.

**List of Abbreviations:** AHR, airway hyperactivity; AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AP1, activator protein one; AVP, arginine vasopressin; BDNF, brain-derived neurotrophic factor; bHLH, basic helix–loop–helix; $Ca^{2+}$, calcium ion; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CNS, central nervous system; CRE, cAMP responsive element; CREB, cAMP response element binding; CGRP, calcitonin gene-related peptide; CVLM, caudal ventrolateral medulla; DAG, diacylglycerol; DRG, dorsal root ganglia; EC, extracellular region; EEG, electroencephalogram; EKA,B,C,D, endokininA, B, C, D; EL, extracellular loop; EPSP, excitatory postsynaptic potential; GABA, gamma-aminobutyric acid; GFR, growth factor receptor; GPCRs, G-protein-coupled receptors; HK1, hemokinin-1; 5-HT, serotonin; 5-HTT, serotonin transporter; IB-4, isolectin B-4; IL, interleukin; IC, intracellular region; IP3, inositol 1,4,5-triphosphate; $K^{+}$, potassium ion; kb, kilobase; K/O, knockout; $Mg^{2+}$, magnesium ion; μM, micromolar; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mRNA, messenger RNA; $Na^{+}$, sodium ion; NF-H, neurofilament H; NGF, nerve growth factor; NK1, neurokinin1; NKA, neurokinin A; NKB, neurokinin B; NMDA, *N*-methyl-D-aspartate; NMR, nuclear magnetic resonance; NO, nitric oxide; NOS, nitric oxide synthase; NPK, neuropeptide K; NPγ, neuropeptide γ; NRSE, neuronal restrictive silencer element; NRSF/REST, neuronal restrictive silencer factor; NT-3, neurotrophic factor; PAG, periaqueductal gray matter; PKG, protein kinase gamma; PLC, phospholipase C; PNS, peripheral nervous system; POU, octamer binding protein of the Pit/Oct/Unc family; PPS, perforant path stimulation; QPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; sGC, soluble guanylyl cyclase; SP, substance P; SSSE, self-sustaining status epilepticus; TAC1/PPT-A, tachykinin1/preprotachykinin-A; TAC3/PPT-B, tachykinin3/preprotachykinin-B; TAC4/PPT-C, tachykinin4/preprotachykinin-C; TACR1,2,3, tachykinin receptor1,2,3; TF, transcription factor; TLE, temporal lobe epilepsy; TNF, tumor necrosis factor; Trk, tyrosine kinase

## 1 Introduction

Ulf von Euler initially observed substance P (SP) while analyzing the distribution of acetylcholine in the rabbit gastrointestinal tract. A crude extract from horse brain and gut caused transient hypotension and contraction of the intestine. However, upon addition of atropine contractile activity was still observed, demonstrating that acetylcholine was not responsible. A paper soon followed describing an atropine-resistant "unidentified depressor substance" found in both brain and gut that stimulated smooth muscle and lowered blood pressure (von Euler and Gaddum, 1931). The term SP did not appear until Gaddum and Schild (1934) described the purification of a stable active proteinaceous powder previously termed "preparation P." In the 1950s, Bengt Pernow working with von Euler and Fred Lembeck made significant progress in determining the distribution of so-called preparation P in the brain and in the periphery, and subsequently, its association with Hirschsprung's disease (Ehrenpreis and Pernow, 1953; Pernow, 1953),

which led to the first suggestion that "preparation P" is indeed SP (see below) and could be a neurotransmitter (Lembeck, 1953).

Thirty years after the initial observation, Susan Leeman and Michael Chang purified a sialogogic peptide, which demonstrated properties and composition similar to that of the partially purified SP by Lembeck (Lembeck and Starke, 1968). Chang and Leeman called the unknown protein "substance P" and went on to sequence and synthesize the 11-amino-acid peptide as Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met from bovine hypothalamus (Chang et al., 1971; Tregear et al., 1971). The pharmacological properties of SP suggested it was part of a larger family of peptides. The amino acid sequence revealed homology with a group of nonmammalian molecules that were sequenced in the 1960s. Subsequently, in 1966 Erspamer coined the term "tachykinins" for the group of peptides that shared similar structural characteristics and exhibited activities such as rapid and potent smooth muscle contraction (Bernardi et al., 1966). The prefix "tachy" is from the Greek "Tachys" meaning quick, and kinin was defined as a "general name indicating a hypotensive polypeptide" that contracts most isolated smooth muscles, but relaxes duodenum. In addition, the name may be applied to any polypeptide that is related to bradykinin (quoted from Khawaja and Rogers, 1996). However, it is worthy to note that the tachykinins do not conform to the definition of kinin as they display little structural similarities to bradykinin and do not relax the duodenal smooth muscles.

The discovery of SP and its putative role as a neurotransmitter sparked the explosion of tachykinin research resulting in the discovery, albeit much later on, of other tachykinin family members, neurokinin A (NKA) (Kangawa et al., 1983; Nawa et al., 1984; Krause et al., 1987), neurokinin B (NKB) (Kanazawa et al., 1984; Kimura et al., 1984), neuropeptide γ (Kage et al., 1988), neuropeptide K (Tatemoto et al., 1985), and more recently hemokinin-1 (HK1) (Zhang et al., 2000).

Tachykinins are widely distributed throughout the mammalian body in both the central nervous system (CNS) and the peripheral nervous system (PNS), with numerous functions being attributed to them in each of these systems. Tachykinins are predominantly synthesized in neurons of the CNS and the PNS and stored in large dense vesicles. Upon excitation of these neurons, tachykinins are released and act on their appropriate receptors on the target cells to evoke various responses. In addition, tachykinins have been found to be expressed in a variety of nonneuronal cells (Quinn et al., 1995; Pennefather et al., 2004).

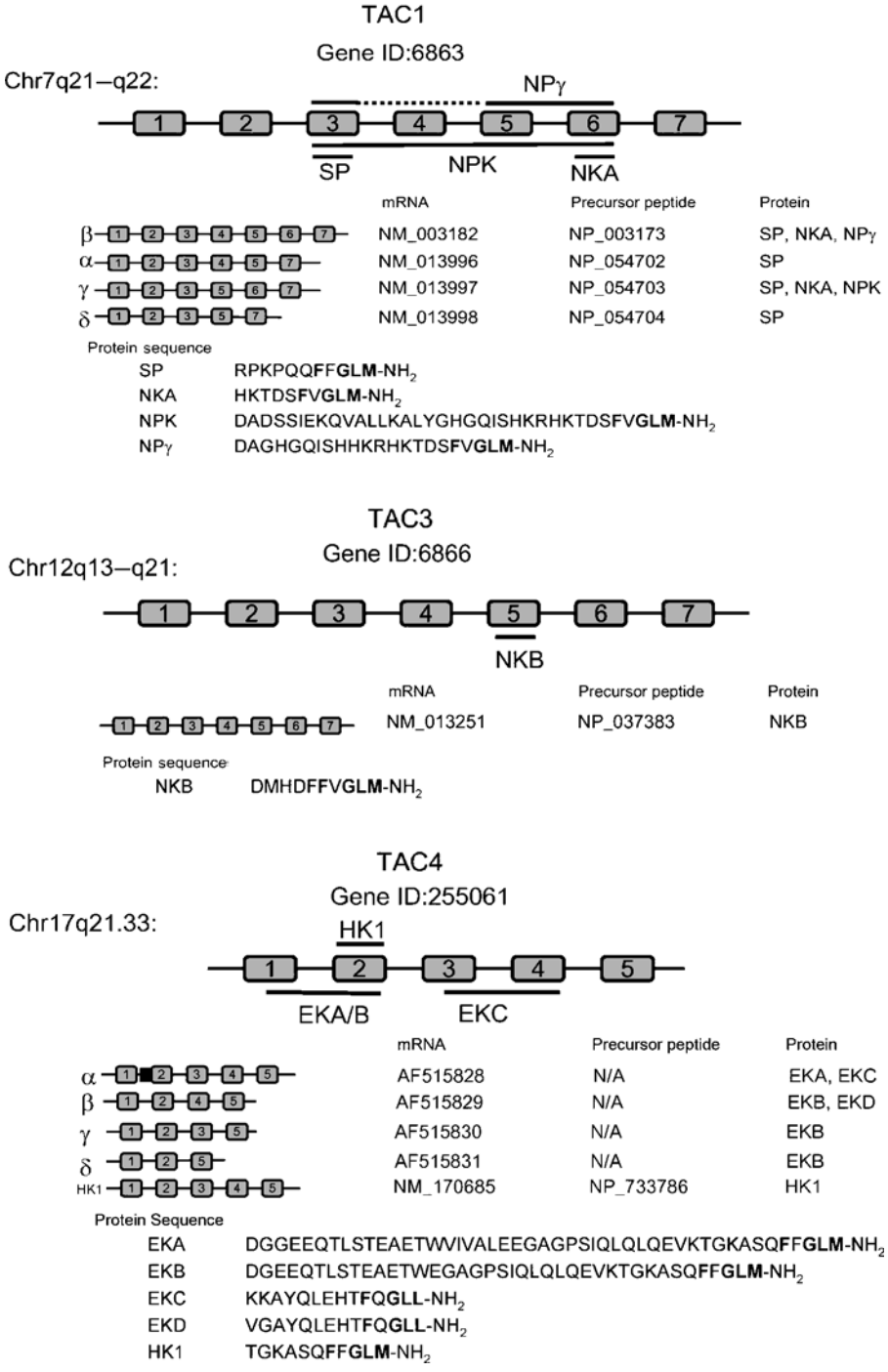
## 1.1 Gene Structure of Tachykinins and Their Receptors

### 1.1.1 Preprotachykinin-A

Mammalian SP is derived from the preprotachykinin-A (PPT-A) gene. The more recent discovery of the presence of two other related genes, preprotachykinin-B (PPT-B) and preprotachykinin-C (PPT-C), suggests that they along with PPT-A appear to originate from a common ancestral gene (Carter and Krause, 1990; Zhang et al., 2000). The human PPT-A gene is 8 kb long and consists of seven exons, which give rise to four alternatively spliced messenger (mRNA) transcripts termed α, β, γ, and δ (*Figure 20-1*). Each isoform differs only in its exon usage, the β isoform utilizing all seven exons of the PPT-A gene, α lacking the sixth exon, γ lacking the fourth, and the most recently discovered δ isoform lacking both the fourth and the sixth exon. Polypeptides produced from these splice variants confer cells with the ability to generate SP, being encoded by exon 3; however, NKA can only be produced by β and γ PPT-A mRNAs since they contain exon six that encodes NKA. Neuropeptide K (NPK) and neuropeptide γ (NPγ) are N-terminal extended forms of NKA produced from β and γ PPT-A mRNA, respectively (*Figure 20-1*). Nuclear magnetic resonance (NMR) analysis of SP suggests an α-helical core from Pro4 to Phe8 stabilized by two hydrogen bonds, one between Phe7-NH and Lys3-CO, and the other between Phe8-NH and Pro4-CO; an extended highly flexible $NH_2$ terminal Arg1-Pro2-Lys3; and a central turn on Gly9, thus bringing the COOH-terminal amide in contact with the γ-carbonyl oxygen atom of both glutamines (Lavielle et al., 1988; Regoli et al., 1994). SP is synthesized in the ribosomes as part of a larger protein and then enzymatically converted into an active peptide. SP is widely distributed in the CNS and the PNS of

**Figure 20-1**

**Schematic representation of the biosynthesis of the tachykinin genes. Genes TAC1, TAC3, and TAC4 are shown along with the transcription and translation products and relevant associated information, which can be accessed at http://www.ncbi.nlm.nih.gov/**



vertebrates. In the PNS, SP is expressed predominantly in the primary sensory neurons and the postganglionic neurons of the gut.

### 1.1.2 Preprotachykinin-B

NKB is the only tachykinin derived from the PPT-B gene. The human gene consists of seven exons and the sequence that encodes NKB is located on exon 5 (*Figure 20-1*). Human NKB mRNA expressed in the placenta appears to be encoded by seven exons interrupted by six introns spanning a region of 5.4 kb. Exons 2 to 6 encode the precursor, while exons 1 and 7 are untranslated regions. The human PPT-B gene generates only one mRNA that produces NKB, while in the bovine, PPT-B generates two mRNA transcripts, the difference being at the 5′ extremity of their untranslated regions (Kotani et al., 1986; Page et al., 2001).

Human placenta contains higher levels of the NKB mRNA transcript than those found individually in the human whole brain and spinal cord. However, it is undetectable in peripheral tissues from nonpregnant animals even though its endogenous receptor, NK3, has been found in a number of locations throughout the human body (Donaldson et al., 1996; Krause et al., 1997). Page et al. (2000, 2001) suggest that NKB has a possible role in placental physiology and preeclampsia. Most recently, NKB and NK3 mRNA have been found to be expressed in human airways and pulmonary arteries and veins, suggesting the involvement of NKB in lung physiopathology (Pinto et al., 2004).

### 1.1.3 Preprotachykinin-C (Hemokinin)

During the study of mouse B cell development an mRNA differential display screen revealed a predicted peptide sequence that contained the tachykinin signature motif (FXGLM) (Zhang et al., 2000). Subsequent characterization led to the discovery of a third PPT gene called PPT-C or TAC4; mouse TAC4 contains four exons while the rat homolog contains five, with both giving rise to a peptide called HK1 that displays a preference for the NK1 receptor in an SP-like manner. HK1 is expressed in mouse hematopoietic cells and so far has not been observed in neuronal tissue (Zhang et al., 2000; Pennefather et al., 2004). However, HK1 does display characteristics similar to SP in terms of its receptor binding, being an NK1, NK2, and NK3 agonist with the highest selectivity for NK1 (Kurtz et al., 2002). Furthermore, in humans, at least four other tachykinins have been found to be expressed (Page, 2004). Four alternatively spliced mRNAs, α, β, γ, and δ, give rise to four proteins named endokinin A (EKA), B, C, and D, respectively (Page, 2004; Patacchini et al., 2004). αTAC4 encodes both EKA and EKC; βTAC4 encodes EKC and EKD; both γTAC4 and δTAC4 encode EKB only (*Figure 20-1*). There is tissue-specific regulation of these transcripts; αTAC4 is expressed in the adrenal gland, liver, and spleen and βTAC4 in the heart, liver, bone marrow, prostate, adrenal gland, and testis. Both γ and δ isoforms showed similar expression patterns in the adrenal gland and the placenta (Page, 2004; Patacchini et al., 2004).

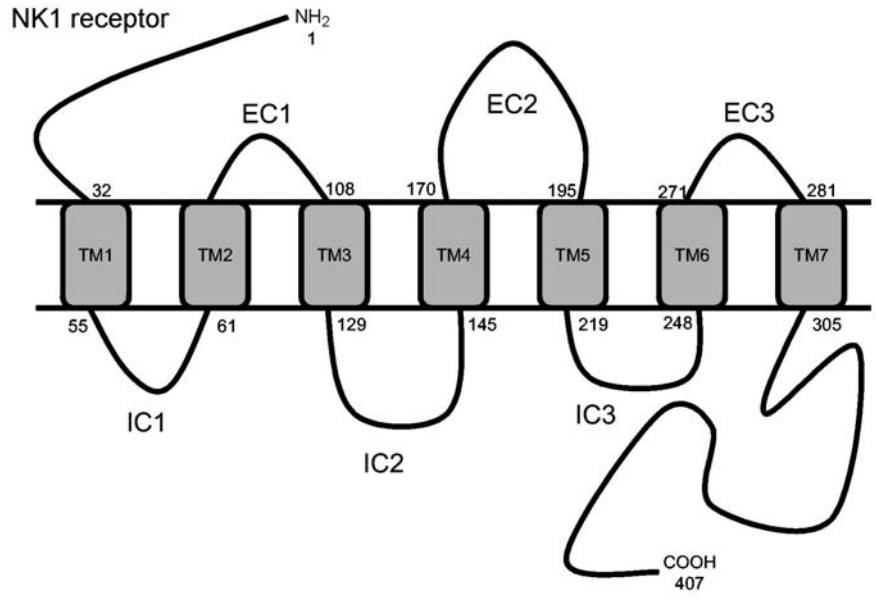
### 1.1.4 Tachykinin Receptors

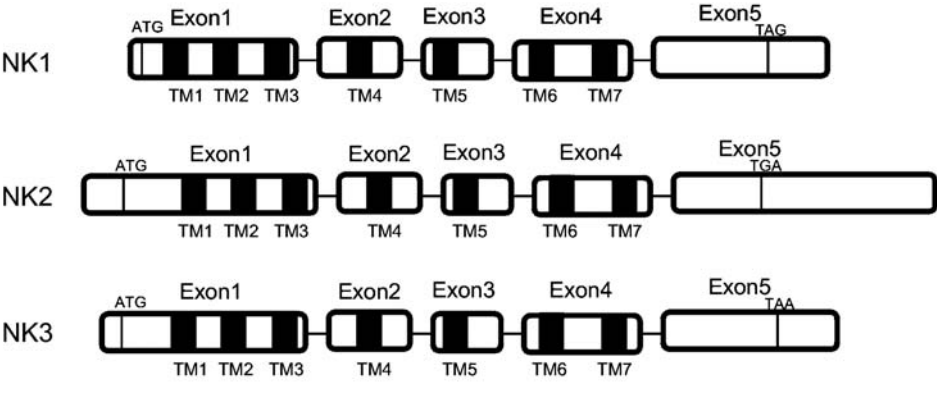
In parallel with the identification of a number of endogenous tachykinins, several classes of tachykinin receptors have been discovered. From an evolutionary perspective the vertebrate tachykinin receptors are highly conserved and have evolved from one common gene or one ancestral receptor (Pennefather et al., 2004). The tachykinin receptors belong to a family of G-protein-coupled receptors (GPCRs) and show a high incidence of homology between mammalian species (Gerard et al., 1993). Like all GPCRs they are composed of seven transmembrane domains (TMI–VII), three extracellular loops (EC1–3), three intracellular loops (IC1–3), an extracellular amino terminus, and an intracellular carboxy terminus (*Figure 20-2*) (Maggi, 1995).

Ligand binding and receptor chimera studies have identified three tachykinin receptors. These tachykinin receptors have also been designated as NK1, NK2, and NK3 and display preferential selectivity

**Figure 20-2**

**Schematic representation of the human NK1 receptor protein and the gene structure of NK1, NK2, and NK3. SP binds to extracellular (EC) regions 1 and 2, G proteins bind at transmembrane region (TM) 5, 6, and intracellular region (EC3). The human NK1, NK2, and NK3 genes contain five exons and four introns. The regions within exons encoding TMs are shown as *black boxes*. The *thin black lines* show the start and stop codons and highlight the difference in both 5′ and 3′ untranslated regions between the genes. Access numbers for each can be utilized for further information at http://www.ncbi.nlm.nih.gov/**




| | Gene | Gene ID | Location | mRNA | Peptide |
|---|---|---|---|---|---|
| NK1 | TACR1 | 6869 | Chr2p12 | NM_001058 | NP_001049 |
| NK2 | TACR2 | 6865 | Chr10q11-q21 | NM_001057 | NP_001048 |
| NK3 | TACR3 | 6870 | Chr4q25 | NM_001059 | NP_001050 |

to SP, NKA, and NKB, respectively. The term "neurokinin" is used to denote tachykinins expressed in the nervous system (Buck et al., 1984; Lee et al., 1986; Harada et al., 1987; Maggi, 1995). NK1 is also the preferred receptor for the recently identified tachykinins HK1, EKA, and EKB (Zhang et al., 2000). It has

been demonstrated that all tachykinins exhibit a limited selectivity for a particular receptor and that there is crosstalk between tachykinin ligands and the receptors (Mussap et al., 1993). NK1 function has been addressed not only by the interactions with SP and other tachykinins but also by specific receptor antagonists such as WIN-51, 708, L-733, 060, RPR100893 and GR205171, MK-0869 (L-754,030), L-758, 298, and MEN 11467 and MEN 11149 (Cirillo et al., 1998; Tattersall et al., 2000; Santarelli et al., 2002). The NK1 receptor is also the preferred target for HK1 and endokinins in the periphery, where the ligand binding produces effects analogous to SP binding (Morteau et al., 2001; Kurtz et al., 2002; Page et al., 2003). Although the receptor-binding model eloquently highlights the preferential affinities of the tachykinin ligands to their receptor species, tissue anomalies in this relationship have raised questions about the existence of more tachykinin receptors for SP, NKA, and NKB (reviewed in Maggi, 2000).

Tachykinin GPCRs may be regulated in a conformational manner and ligand binding, membrane lipid composition, and extracellular medium may all act to stabilize the receptor favoring one ligand binding/effector conformation over the other (Berthold and Bartfai, 1997). Ligand binding to the tachykinin receptors initiates internalization of the ligand–receptor complex, resulting in phospholipase $C_\beta$ ($PLC_\beta$) activation. PLC utilizes membrane lipids increasing the intracellular inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 induces intracellular calcium release from the endoplasmic reticulum, leading to the activation of a number of signal transduction cascades. DAG activates protein kinase C (PKC), nitric oxide synthase (NOS), and arachidonic acid production, thus stimulating further regulatory pathways resulting in upregulation of cyclic nucleotides (cyclic guanosine monophosphate, cGMP; cyclic adenosine monophosphate, cAMP) (Krause et al., 1992; Garcia et al., 1994; Radhakrishnan et al., 1995).

SP binding to NK1 receptor initiates signal transduction upon NK1-SP internalization. GPCR redistribution induced by agonist binding determines the subsequent responsiveness of the cell to particular agonists and plays a major role in the regulation of signal transduction pathways (Quartara and Maggi, 1997). Receptor endocytosis/internalization is responsible for both desensitization by reducing the number of cell surface receptors available to interact with agonists, and resensitization following receptor processing (i.e., dissociation of ligand, dephosphorylation) and recycling to the plasma membrane.

NK1-GPCR endocytosis pathway involves the formation of clathrin-coated pits and is mediated by a family of cytosolic proteins termed arrestins (McConalogue et al., 1998). Binding of SP to the NK1 receptor causes a rapid translocation of β-arrestins from the cytosol to the plasma membrane, followed by the redistribution of both NK1 and β-arrestins to endosomes, where they remain associated until gradually (after 4–6 h) NK1 is recycled to the plasma membrane and β-arrestins return to the cytosol (McConalogue et al., 1999).

The NK1 receptor has been studied more extensively than NK2 and NK3. NK1 has two isoforms, one with a shorter C-terminal tail denoted as the short isoform and the other, the long isoform. The longer isoform is found predominantly in the brain, while the shorter isoform is associated with peripheral tissues (Caberlotto et al., 2003). The existence of these isoforms may account for discrepancies arising in the potency and preferential binding of tachykinins to their receptors in different tissues. Additionally, the recent discovery of human NK2 receptor isoforms may also account for the differences reported in the tissue specificity of NKB (Naline et al., 1989; Croci et al., 1998; Candenas et al., 2002). The existence of multiple induced conformations of NK1 and NK2 has been confirmed, with each study showing distinct binding affinities for antagonists and agonists and in some cases the ability to activate different effector systems (Maggi and Schwartz, 1997; Palanche et al., 2001; Patacchini and Maggi, 2001; Lecat et al., 2002). With the discovery of additional tachykinins and tachykinin-related peptides such as hemokinins and endokinins, the debate on whether additional tachykinin receptors exist has been readdressed (Pennefather et al., 2004). NK1 is the most abundantly distributed neurokinin receptor in the CNS, and it is also present in a number of target organs that are innervated by SP-expressing small-diameter primary afferent neurons. Clinical and experimental animal model studies have suggested the involvement of NK1 in a number of functions such as nociception, cognition, and basal ganglia functions as well as psychiatric and neurological disorders (Quartara and Maggi, 1997; Kramer et al., 1998; Quartara and Maggi, 1998).

### 1.2 Tissue-Specific and Stimulus-Inducible Tachykinin Gene Expression

The expression of neuropeptide genes is extremely plastic in that the PPT-A gene can be induced by a number of different stimuli and this induction is dependent, in part, upon transcription factors binding to regulatory consensus sequence in the promoter of the gene. Induction of the PPT-A gene not only is crucial for normal physiological signaling but also accounts for modulation of gene expression in pathological states. A fragment of the PPT-A gene promoter spanning base pairs −865 to +445 has been extensively studied and contains a number of regulatory DNA elements upstream and downstream of the transcriptional start site and is capable of supporting marker/reporter gene expression in a number of cell types including dorsal root ganglion (DRG) and hippocampal neurons in culture (Harmar et al., 1993; Morrison et al., 1994; Mendelson and Quinn, 1995; Mendelson et al., 1995; Paterson et al., 1995a–c; Walker et al., 2000). Nerve growth factor (NGF) is a known endogenous inducer of the PPT-A gene in states of chronic pain and inflammation (Ma and Bisby, 1998). This promoter fragment also supports NGF regulation of marker gene, determining in part that the region could mediate differential PPT-A expression to this stimulus (Harrison et al., 1999). The PPT-A promoter contains many transcription factors that have been defined as mediating a response to NGF including binding sites for activator protein one (AP1), cAMP responsive element (CRE), basic helix–loop–helix (bHLH) proteins, and members of the POU domain family of proteins (Fiskerstrand and Quinn, 1996). Both AP1 and CRE can mediate inducible PPT-A expression in response to NGF. Members of the cAMP response element binding (CREB) TF family are activated via phosphorylation by a Ras-dependent protein kinase and are capable of upregulating gene expression following NGF stimulation (Hawley et al., 1992; Ginty et al., 1994). In addition, the cellular composition of AP1-binding transcription factors (TF such as fos and jun) is modulated in response to NGF stimulation (Quinn et al., 1989; Gizang-Ginsberg and Ziff, 1990) and may vary in a tissue-specific manner (Andrews et al., 1993). Octamer binding sites show functional inducibility to NGF with increases in the concentration of N-oct-2 binding protein in DRG following treatment with NGF (Wood et al., 1992; Mendelson et al., 1998).

In nonneuronal cells, neuronal genes are in part dominantly repressed by the binding of neuronal restrictive silencer factor (NRSF) at the neuronal restrictive silencer element (NRSE) located in the gene (Palm et al., 1998). NRSF binding at the transcriptional start site of the PPT-A gene results in dominant repression of transcription supported by the proximal promoter fragment and accounts for the lack of gene expression from this fragment in PC12 and HeLa cells (Mendelson et al., 1995). However, more recently it has been suggested that PPT-A expression could be enhanced by expression and function of REST isoforms (Quinn et al., 2002) as observed for other neuropeptide genes such as arginine vasopressin (AVP) (Coulson et al., 1999; Quinn et al., 2002). A role has also been demonstrated for the regulation of the PPT-A promoter by members of the bHLH family of transcription factors (Paterson et al., 1995a–c; MacKenzie et al., 2000). bHLH and REST isoforms are differentially regulated during a CNS challenge such as epilepsy (Palm et al., 1998). It will be interesting to directly correlate whether the modulation of specific transcription factors will affect PPT-A plasticity at the level of transcription in the CNS. It is likely that synergistic action of these TFs at a number of regulatory DNA sites mediate the modulation of the PPT-A promoter, in neurons, e.g., in response to NGF during states of chronic pain and inflammation and in CNS neurons.

## 2 Distribution of Tachykinins and Their Receptors

Tachykinins are widely distributed in both the nervous system and the other peripheral tissues, and numerous functions have been attributed to tachykinins in each of these tissues. A number of methods have been used to study the distribution of the tachykinin-encoding genes and their receptors in the CNS and peripheral tissues, including:

1. in situ hybridization, using probes directed against the mRNA for the receptor;
2. immunocytochemistry with antibodies raised against synthetic peptide sequences corresponding to different parts of the receptor. Initial studies of SP localization were described using antibodies

raised against the carboxyl terminus, which has now been shown to be common among all tachykinins; and

3. reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative PCR (QPCR).

SP and NKA are mostly synthesized and stored in large dense-core vesicles of primary afferents of PNS (McCarthy and Lawson, 1989) and CNS neurons (Pickel et al., 1983; Maley, 1996). In addition, the majority of SP-containing neurons also contain NKA, particularly the capsaicin-sensitive afferent neurons (Carter and Krause, 1990). Both peptides have been implicated centrally in numerous and diverse processes such as neurotransmission (Otsuka and Yoshioka, 1993; Patacchini et al., 1998), inflammation (Barnes, 1991, 1992; Donaldson et al., 1992; Leslie et al., 1995), neurological pain/neuralgia (Noguchi et al., 1988; Cao et al., 1998; Basbaum, 1999a, b; Honor et al., 1999), memory and learning, depression and anxiety (Kramer et al., 1998; Rupniak and Kramer, 1999), and epilepsy (Liu et al., 1999a, b; Liu et al., 2000; Fetissov et al., 2003). Peripheral SP functions are diverse and are involved in immune system stimulation (Iwamoto et al., 1993; Maggi, 1997), fibroblast and smooth muscle growth (Nilsson et al., 1985; Katayama and Nishioka, 1997), hypotension, smooth muscle contraction, and cellular proliferation (Lecci et al., 2000). NKB is present in the brain and the spinal cord (Patacchini et al., 2000) and has recently been detected in the reproductive system of both humans and rodents (Page et al., 2000; Pinto et al., 2001; Patak et al., 2003). The newly discovered PPT-C/TAC4 gene that encodes HK1 is widely expressed in nonneuronal tissue in humans and mice (Page et al., 2003). The function of HK1 and its isoforms are not yet clear but they are believed to function in a manner similar to SP via the activation of the NK1 receptor (Kurtz et al., 2002; Page et al., 2003; Patacchini et al., 2004).

## 2.1 Distribution of PPT-A and Tachykinin Receptors in the Primary Sensory Afferents

The bulk of the SP present in the PNS is synthesized in the primary sensory neurons, which are located within the DRG (Hokfelt et al., 1975a, b). SP along with other neuropeptides such as NKA is stored in dense-core vesicles within the DRG neurons (McCarthy and Lawson, 1989). Not all sensory afferent neurons express PPT-A mRNA. There are different subpopulations of DRG neurons and only a small proportion of these neurons support PPT-A expression and SP synthesis. DRG neurons consist of two main populations; the first group (15–20%) are large-diameter (>30 μm), light, nonpeptidergic neurons giving rise to myelinated Aδ fibers, and the second group (75–80%) are small-diameter (<30 μm), dark, mostly peptidergic neurons giving rise to unmyelinated C-fibers (Lawson et al., 1993, 1997).

The large-diameter DRG neurons can also be distinguished on the basis of protein expression profile such as the expression of high-molecular-weight neurofilament protein, NF-H (Averill et al., 1995; Molliver et al., 1995), neurotrophin receptors, and tyrosine kinase C (*trkC*) (Wright and Snider, 1995). These neurons are largely glutamatergic and do not express PPT-A under basal conditions. Upon low-frequency stimulation they release glutamate from their central terminals that bind to postsynaptic *N*-methyl-D-aspartate (NMDA) receptors on the second-order projection neurons and islet cell excitatory interneurons in lamina II of the dorsal horn and to a lesser extent in lamina V of the spinal cord. Under normal physiological conditions, myelinated Aδ fibers transmit impulses generated by nonnoxious stimuli such as pressure (mechanoceptors) and heat (proprioceptors) from the periphery.

The peptidergic small-diameter DRG neurons can be further subdivided into two groups: the capsaicin-sensitive peptidergic (majority) and the nonpeptidergic neurons. Peptidergic neurons can also be distinguished immunohistochemically based on the expression of the high-affinity NGF receptor, *trkA* (Basbaum and Woolf, 1999). The capsaicin-sensitive peptidergic neurons support PPT-A mRNA expression and thus express SP and NKA. Under normal physiological conditions, C-fibers transmit impulses generated by noxious (peptidergic neurons) and thermal stimuli (nonpeptidergic neurons) (Basbaum, 1999a, b). The C-fibers also release glutamate-like Aδ fibers in the dorsal horn of the spinal cord and, in addition, they release SP, calcitonin gene-related peptide (CGRP), brain-derived neurotrophic factor (BDNF), and other neuromodulators that bind to their appropriate receptors pre- and postsynaptically, thus potentiating

further glutamate release. The majority of C-fibers terminate in lamina I and the outer region of lamina II (substantia gelatinosa) of the dorsal horn and to a lesser extent in laminae III, VI, and X of the spinal cord and also as projection fibers in the trigeminal ganglion of the CNS, suggesting the role of SP in trigeminal neuralgia (Basbaum and Woolf, 1999).

The nonpeptidergic small-diameter neurons do not support PPT-A expression under basal conditions. These neurons can be distinguished from the peptidergic neurons as they do not express CGRP, and they exhibit surface proteins not found in the peptidergic population such as isolectin B-4 (IB-4) and the purinergic P2X3 receptor. In addition, these neurons express c-Ret growth factor receptor (GFR-α) for GDNF (Basbaum and Woolf, 1999; Caterina and Julius, 1999).

The three types of DRG neurons described above broadly define the DRG populations although some crossover in marker molecule expression is evident between them. This is particularly apparent in neurotrophin receptor expression; some populations express only one trk receptor (Mu et al., 1993; Kashiba et al., 1995; Wright and Snider, 1995), whereas the others express more than one trk receptor (McMahon et al., 1994; Karchewski et al., 1999), suggesting complex neurotrophin-dependent plasticity in DRG. Differential expression of growth factor receptors on sensory neurons has major implications for regulatory control and modulation of gene expression during pathological states.

Whether or not sensory fibers express functional NK1 receptors is a topic for debate. A number of studies propose the presence of the NK1 receptor on DRG neurons and their fibers (McCarson, 1999), while others do not (Andoh et al., 1996; von Banchet and Schaible, 1999). These studies suggest that the putative NK1 receptors present in DRG may be involved in a negative/positive feedback mechanism(s) in which SP acts in an autocrine or paracrine manner to modulate its own release. The controversies with respect to NK1 receptors' expression in DRG is due to the methods used for detection, with some showing more sensitivity than others, which could be due to, for example, the source of antibodies in case of immunocytochemistry and Western blotting. The expression of PPT-A/SP and its functions in DRG, described in this chapter, conform to the observations determined under basal conditions. It should be noted that PPT-A/SP expression, like the expression of all other neuropeptides, is highly dynamic and under certain physiological and pathological conditions the expression profiles can be modulated (discussed in Section 1.2). However, under pathological conditions SP has been demonstrated to increase its own release via presynaptic IP3-mediated release of calcium from the internal stores leading to depolarization and increased neuronal excitability (Xie et al., 1995).

## 2.2 Distribution of SP and NK1 in the Spinal Cord

The axons of primary afferents from the DRG terminate in the lamina of the dorsal horn of the spinal cord. These sensory neurons synthesize, store, and release a variety of neurotransmitters (mostly glutamate) and neuropeptides (SP, NKA, and CGRP) from their terminals. The anatomical location of the presynaptic release sites in relation to the postsynaptic receptor sites in the dorsal horn of the spinal cord is discussed in this section.

The NK1 receptor is highly expressed in lamina I (the marginal), lamina III, and to a lesser extent in laminae II, IV, and V of the dorsal horn of the spinal cord (Brown et al., 1995; Marvizon et al., 1999). SP is found in laminae I and II (Hokfelt et al., 1975a, b; Cuello and Kanazawa, 1978) and to a lesser extent in lamina V (Ruda et al., 1986).

NK1 is expressed by 80% of lamina I neurons that project to the thalamus, periaqueductal gray matter (PAG), parabrachial area, and caudal ventrolateral medulla (CVLM), as demonstrated by retrograde tracing combined with immunocytochemistry (Marshall et al., 1996; Todd et al., 2000). NK1 immunoreactivity has also been detected in lamina II, albeit at lower levels than in lamina I and also in moderate levels in laminae III–VI. This immunoreactivity is associated with the surface membranes of cell bodies and dendritic processes. The majority of NK1-expressing neurons in the dorsal horn do not contain either gamma-aminobutyric acid (GABA) or glycine. It is therefore likely that SP released in the superficial laminae of the dorsal horn acts almost exclusively on NK1 receptors of the excitatory neurons (Littlewood et al., 1995). Acute noxious peripheral stimulation causes excitation of dorsal horn neurons that express the NK1

receptor. In the rat, it has been observed that following acute noxious stimulation, the majority of NK1-immunoreactive neurons in lamina I show internalization of the NK1 receptor (upon SP binding) and upregulation of *c-fos* expression (Mantyh et al., 1995; Doyle and Hunt, 1999).

As mentioned previously, NK1 is the preferential target for SP released from primary afferents. The distribution of the NK1 receptor in relation to the release sites for SP is crucial for modulating impulse summation and filtering. This suggests the juxtaposition of SP-releasing sensory afferents and NK1 receptors in the dorsal horn of the spinal cord under normal physiological conditions. However, under certain pathological conditions such as nociception and inflammation, the SP/NK1 organization alters. In contrast to the PNS, SP-containing sensory afferent terminals in the CNS are mostly located in close proximity to NK1-expressing postsynaptic neurons (Ribeiro-Da-Silva and Hokfelt, 2000). However, mismatches can be seen in lamina III of the dorsal horn where NK1 expression is detected although SP release sites are absent. This suggests that SP released in the lamina I might diffuse to the deeper lamina of the dorsal horn to exert its action on NK1 receptors. This further highlights the importance of other signaling pathways in which the response of central neurons in the brain region to SP release at the level of the spinal cord would be both delayed and sustained, the mechanism of which is shown to be of particular importance in the modulation of pain signaling. In addition to the SP released from sensory afferents of the DRG, the intrinsic neurons of the dorsal horn of the spinal cord express SP and the terminals of the descending serotonergic pathways from higher centers in the brain also release SP (Hokfelt et al., 1978; Gilbert et al., 1982). Of the DRG neuron-derived SP, only 25% is released centrally and the rest is released from peripheral terminals to modulate the excitability of the contractile tissues and control endocrine function and the response to inflammatory agents (Harrison and Geppetti, 2001)

Immunostaining for *c-fos* and NK1 receptor was carried out to examine whether the nociceptive signals from specific peripheral tissues (e.g., skin, muscle, and knee joint) or activity generated by a particular insult (nerve injury or formalin-induced inflammation) was preferentially modulated by SP in the spinal cord (Doyle and Hunt, 1999). Although the number of *c-fos*/NK1-positive neurons was correlated with the intensity of the noxious stimulus in lamina I, no such correlation was observed in the deeper laminae (V–X). It was also noted that *c-fos*/NK1 immunoreactivity in the superficial laminae was unrelated to any particular peripheral target, which was not the case for deeper laminae. In the deeper layers of the dorsal horn, the greatest colocalization of *c-fos*/NK1 immunoreactivity was observed following stimulation of knee joint nociceptors and formalin-induced inflammation, suggesting the direct role for SP in the regulation of joint pain and inflammatory hyperalgesia (Doyle and Hunt, 1999). Therefore, NK1-expressing neurons in lamina I may be involved in discrimination of the intensity of pain-inducing stimuli, whereas NK1 receptors in deeper laminae are concerned with special localization or the detection of particular nociceptive stimuli.

In addition to being involved in the response of NK1 neurons in the dorsal horn to noxious stimulation, NK1 receptors may also be involved in the maintenance of hyperalgesia (Mantyh et al., 1997). The intrathecal administration of SP conjugated to cytotoxin saporin resulted in the ablation of NK1-immunoreactive neurons and was accompanied by reduction in the capsaicin-induced hyperalgesia in rats. Studies from NK1 K/O mice have also revealed that the mice were resistant to inflammatory hyperalgesia induced by injection of complete Freund's adjuvant in the ipsilateral paw (De Felipe et al., 1998a, b). Overall, these observations suggest that lamina I projection neurons expressing NK1 may control dorsal horn excitability via reciprocal projections with the brainstem. Ablation of lamina I NK1-expressing neurons disrupts the ascending pathway to the brainstem and the descending pathways that control spinal cord excitability, thus reducing behavioral hypersensitivity due to peripheral injury.

The blocking effect of analgesics such as opioids on SP release from primary afferents has been demonstrated both in vivo and in vitro (Trafton et al., 1999). To investigate the functional implications of opioid regulation of the tachykinin pathway, Trafton et al. (1999) used NK1 receptor internalization as a measure of the postsynaptic response to morphine administration. They reported a slight reduction in NK1 receptor signaling following morphine administration at a dosage that was sufficient to produce opioid analgesia in awake animals. However, a combination of morphine with a low (ineffective) dose of the NK1 antagonist (GR205171) was able to decrease NK1 receptor internalization to a degree that was greater than that of either drug on its own. The possible explanation for this is that the SP released from primary

afferents may either diffuse into the extracellular space (extracellular pool) or bind to NK1 receptors and become internalized (intracellular pool). Only the intracellular pool is directly measurable because it depends on the saturation of NK1 receptor binding. The extracellular SP is unable to mediate postsynaptic signaling due to lack of NK1 binding sites on the plasma membrane. Opioids may be able to reduce the total amount of SP released by primary afferents; however, this reduction has no effect on the already saturated NK1 binding sites. It is evident that a high proportion of tachykinin signaling remains intact following opioid administration.

Many NK1 neurons in lamina I receive high-density contacts from serotonergic fibers from the raphe nuclei of the medulla. Serotonergic function has been found to increase following pharmacological blockade or genetic disruption of the NK1 receptors in mice, suggesting crosstalk between tachykinin and 5-HT (Gross et al., 2000). This might also suggest the role of tachykinins and 5-HT in anxiety-related behaviors (Ranga and Krishnan, 2002; Adell, 2004; Blier et al., 2004) (see Section 4.2).

## 2.3 Distribution of SP and NK1 in the Brain

Many techniques have been utilized by numerous groups to determine the NK1 and SP content in mammalian brains, including the brains of humans and rats. These techniques include QPCR, immunohistochemistry, in situ hybridization, and radioimmunoassay. Most of these studies agree with each other concerning the distribution and density of SP or NK1 in specific regions of the brain, although slight variations in the intensity are reported between studies, which are most likely due to the sensitivity of the techniques used and the source of antibodies (*Table 20-1*).

In both humans and rats, increased SP content is observed in the caudate putamen of the forebrain, the nucleus accumbens, the globus pallidus, the medial amygdaloid nucleus, the medial habenular nucleus, the lateral habenular nucleus, the substantia nigra, the superior colliculus, the periaqueductal gray, the parabrachial nuclei, the locus coeruleus, the medullary raphe nuclei (project to the spinal cord), the lamina I and outer lamina II of the trigeminal subnucleus caudalis, and the dorsal motor nucleus of the vagus as compared with the content in the spinal cord) (Warden and Young, 1988; Ribeiro-Da-Silva and Hokfelt, 2000; Ribeiro-Da-Silva et al., 2000, for extensive review). In the rodent, moderate levels of SP are detected in the hypothalamus and low levels in the thalamic nuclei, the cortex, the hippocampal areas, and the cerebellum. In contrast, these areas show high levels of SP expression in humans (Mai et al., 1986; Pioro and Cuello, 1990).

In rodents (guinea pigs and rats), intense NK1 expression is detected in the caudate putamen and superior colliculus, while there is moderate to low concentration in the inferior colliculus, the olfactory bulb, the hypothalamus, the hippocampus, the substantia nigra, the cerebral cortex, the septum, the striatum, and in various regions of the mesencephalon (Shults et al., 1984; Dam and Quirion, 1986; Mantyh et al., 1989). In humans, NK1 expression is intense in the caudate putamen, the nucleus accumbens (ventral striatum), the superior colliculus, the cortex, the amygdala, and the locus coeruleus (very high level expression). Moderate NK1 expression is detected in the human superficial cortical regions, the visual cortex, the hippocampus (CA regions including dentate gyrus), and the hypothalamus, while low NK1 mRNA expression is detected in the thalamus (central medial, mammillary body), the globus pallidus, the cerebellum, and the dorsal raphe nucleus (Caberlotto et al., 2003).

The collective data from the above studies suggest that there is a mismatch between the concentration/innervation of SP and the density of NK1 receptors in a number of regions within the brain. This mismatch is particularly evident in the rodent hippocampus. NK1 occurs throughout the hippocampal formation and is strong in the hilus of the dentate gyrus, while there is little SP or NKA immunoreactivity in this region (Mantyh et al., 1989; Ribeiro-Da-Silva and Hokfelt, 2000). The mismatch between SP/NKA and NK1 in these areas may indicate that NKB or NK3 may play a major role in these regions; however, one should consider that the levels of mRNA are not static and that SP can also diffuse from other areas. It is interesting to note that the human hippocampus does not show the mismatch between SP and NK1. However, the above studies do not distinguish between SP and NKA. Overall, this may also indicate that despite the high sequence homology of the rat and human PPT-A and NK1 genes, the mechanisms involved in modulation of neurotransmission via tachykinergic signaling may differ considerably between humans and rats.

**Table 20-1**
**SP/PPT-A and the NK1 receptor distribution in rat and human brain**

| Brain Regions | Rats | | Humans | |
|---|---|---|---|---|
| | SP | NK1 | SP | NK1 |
| Cerebral cortex | | | | |
| Neocortex | + | | ++/+++ | ++/+++ |
| Cingulate cortex | ++ | | | ++/+++ |
| Hippocampal formation | | | | |
| CA layers | | | | |
| CA1 | + | | | |
| CA2 | + | | | |
| CA3 | + | +++ | +++ | ++ |
| Dentate gyrus | + | +++ | +++ | ++ |
| Basal ganglia | | | | |
| Caudate putamen | +++ | +/+++ | +++ | +++ |
| Nucleus accumbens | +++ | ++/+++ | +++ | +++ |
| Globus pallidus | +++ | | +++ | ++ |
| Amygdala | | | | |
| Medial amygdaloid nucleus | +++ | — | | ++ |
| Basolateral amygdaloid nucleus | | ++ | | ++ |
| Diencephelon | | | | |
| Hypothalamus | +/++ | +/+++ | | ++ |
| Thalamus | + | +/+++ | | + |
| Habenular nucleus | +++ | +/++ | +++ | |
| Mesencephelon | | | | |
| Substantia nigra | +++ | + | +++ | + |
| Interpeduncular nucleus | +++ | ++ | | |
| Superior colliculus | ++/+++ | +++ | | +++ |
| Inferior colliculus | ++ | +/++ | | |
| Periaquaductal gray | +++ | ++ | | |
| Raphe nuclei | + | + | | |
| Pons | | | | |
| Parabrachial nucleus | +++ | ++/+++ | | |
| Locus coeruleus | +++ | +++ | | +++ |
| Dorsal raphe nucleus | +++ | +++ | +++ | + |
| Trigeminal sensory neurones | + | | | |

Data are based on a range of studies and represent the SP or PPT-A mRNA and NK1 or NK1 mRNA expression based on in situ hybridization (Warden and Young, 1988; Hurd et al., 1999; Caberlotto et al., 2003), receptor-binding studies (Mantyh et al., 1989), and immunohistochemical studies. Intensity ranges from none (−) to high (+++). In some cases, detailed information is not available for NK1 in the rat brain or for SP/NK1 in the human brain. The detection methods used in the above studies did not distinguish between SP or NKA. There are some striking differences between rats and humans, and mismatches between SP concentration and NK1 receptor content in specific brain regions, for example, the rat hippocampus and basal amygdaloid nucleus. The type of staining observed for each region is not always disclosed and for this reason the table represents areas of the brain in which SP and NK1 can be seen, rather than their precise structural locations. Note that the NK1- or SP-expressing cells represent the complexity of tachykinergic signaling and the role of the tachykinins in a plethora of neuronal pathways

## 2.4 Peripheral Tachykinins

Although local nerves have been believed to be the major source of tachykinins in the peripheral tissues, recently PPT-A has been shown to be induced and expressed in other cell types such as monocytes, macrophages, pancreatic islet cells, and various tumors (McGregor et al., 1995; Ho et al., 1997; Germonpre et al., 1999; Lambrecht et al., 1999; Singh et al., 2000). This has led to the hypothesis that SP not only acts as a mediator of the neuroimmune system but is also involved in direct interaction between immune cells either in a paracrine or in an autocrine fashion independent of sensory nerves, i.e., "neurogenic inflammation" (Ho et al., 1997; Lai et al., 1998). Expression of NK1 receptors in nonneuronal tissues and cells, such as in osteoclasts and human mucosal mononuclear cells, is increasingly recognized (Ho et al., 1997; Lambrecht et al., 1999). Through these receptors, SP has been shown to regulate production of a number of cytokines including IL-1, IL-6, IL-8, and TNF-α to mediate inflammatory and cell proliferative responses (Lotz et al., 1988; Palma and Manzini, 1998). It has been suggested that SP is a key molecule in the neuroimmune axis. In addition to the classical peptides SP and NKA, the recently discovered HK1 and the EKs have been found in nonneuronal cells/tissues such as pulmonary, cardiovascular, and articular cartilages, and cells of the immune system. The role of tachykinins in respiration, joint function, and gut mobility is briefly discussed in this section when expressed both in neuronal and in nonneuronal cells. The interaction of PPT-A expression in the periphery may mimic regulation in the CNS under various stresses.

### 2.4.1 Respiration

TKs are synthesized and released in a subset of sensory neurons innervating the mammalian respiratory tract; the neuroeffector role of these neurons is attributed to the release of TKs from their peripheral nerve terminals. TKs induce smooth muscle contraction, glandular secretion, plasma protein extravasation, cough, and other effects.

NK1 receptors located on the endothelial cells lining the microvessels play a role in plasma protein extravasation elicited by a variety of irritant stimuli and by antigen challenge in the mammalian airways (Lagente and Advenier, 1998). The inhalation of irritants or hyperpneic conditions in normal animals or antigen administration in sensitized animals also induces acute episodes of bronchoconstriction leading to respiratory distress that are at least in part mediated by TKs (Yasumitsu et al., 1996; Yoshihara et al., 1996a, b; Tramontana et al., 1998; Lai and Lee, 1999; Lai et al., 1999). A similar mechanism of bronchoconstriction operates in asthmatic patients. Both NK1 and NK2 receptors located on smooth muscle cells lining the bronchioles are involved in the bronchoconstriction response that is predominantly induced by endogenous NK2 released from the DRG neurons (Lai and Lee, 1999). This also results in atropine-resistant bronchoconstriction-mediated (Yuan et al., 1996) acetylcholine release (Hey et al., 1996). Capsaicin, a derivative of capsicum, causes respiratory distress and lethality at a very high dose. Pretreatment of guinea pigs with NK1 and NK2 receptor antagonists offered complete protection against the lethal dose of capsaicin, whereas NK2 receptor antagonists alone protect ~80% of animals (Patacchini et al., 1999).

An acute inflammatory challenge, either allergic or nonallergic, induces both the early and the delayed airway hyperactivity (AHR) to bronchoconstrictor agents such as acetylcholine or histamine accompanied by infiltration of immune cells. The vagal stimulation releases acetylcholine and in turn leads to plasma protein extravasation in small bronchi and distal airways (Savoie et al., 1995). NK2 receptor antagonists block the AHR and reduce the infiltration of neutrophils and lymphocytes (Schuiling et al., 1999). The latter effect could be due to NK2 receptor antagonists binding on to the TK receptors, which reduces or prevents endogenous TK binding to NK1 receptor, thus blocking both the early and the delayed hyperresponsiveness to histamine induced by antigen in sensitized guinea pigs. NK1 K/O mice failed to recruit neutrophils upon challenge with allergens (Bozic et al., 1996). The proinflammatory effect induced by IL-17 or IL-1β in the rat airways is mediated by endogenous TKs acting through NK1 receptors (Hoshino et al., 1999).

It could be hypothesized that the inhibition of plasma protein extravasation by TK receptor antagonists accounts in part for a reduced infiltration of immune cells. However, if we take into account the profiles of the effects induced by NK1 or NK2 receptor antagonists on immune cell infiltration, it is clear that the

blockade of plasma extravasation cannot solely explain the reduction of inflammatory response by these antagonists. For example, NK2 receptor blockade does not affect eosinophil infiltration. In this respect, it is worth noting that TKs can activate resident immune cells such as alveolar macrophages (Brunelleschi et al., 1990) and that these cells can release chemokines that attract other immune cells (Newton and Vaddi, 1997). The clinical testing of NK2 antagonists in asthma and obstructive pulmonary diseases is of particular interest in view of the fact that the expression of NK2 receptor mRNA is greatly increased in these pathologies compared with that in controls (Bai et al., 1995). Saredutant (SR 48968), a nonpeptide NK2 receptor antagonist, has been found to reduce the bronchoconstriction induced by inhaled NKA in asthmatic subjects (Van Schoor et al., 1998), thus making this drug a likely candidate for clinical trials in asthma.

Another possible clinical application of TK receptor antagonists is as antitussive agents. Inhalation of NKA and SP induces cough in guinea pigs (Takahama et al., 1993). Both the NK1 and the NK2 receptor antagonists reduce capsaicin- and citric-acid-induced cough although in the latter case the role of NK2 predominates (Yasumitsu et al., 1996). Cough is a reflex due to the direct activation of sensory neurons by irritants; therefore it is difficult to explain an antitussive activity via the blockade of NK1 or NK2 receptors on endothelial, smooth muscle, or immune cells. This indicates the possibility of a central action for the antitussive effect of TK receptor antagonists (Bolser et al., 1997). However, peripheral NK2 receptors or central NK2 receptors located in areas where access via the blood–brain barrier is facilitated are also likely to be involved since peptide NK2 receptor antagonists also display antitussive activity (Yasumitsu et al., 1996).

### 2.4.2 Chondrocyte Mechanotransduction

SP has an osteogenic stimulating effect that is probably caused by stimulating stem cell mitosis, osteoprogenitor cell differentiation, or osteoblastic activity (Shih and Bernard, 1997) possibly via regulation of intracellular calcium levels (Mori et al., 1999). SP regulation of chondrocyte behavior is complex. It has been suggested that autocrine/paracrine signaling via SP and NK1 is important in the signaling pathway through which the chondrocytes respond to mechanical stimulation. Mechanical stimulation of human articular chondrocytes in monolayer culture results in the activation of an integrin-dependent IL-4 signaling loop (Millward-Sadler et al., 2000). This signaling is associated with cell membrane hyperpolarization and alteration in the relative levels of aggrecan and matrix metalloproteinase (Millward-Sadler et al., 2000). Further, PPT-A K/O mice and specific receptor antagonist studies confirmed that SP is necessary for both hyperpolarization and gene expression plasticity following mechanical stimulation (Millward-Sadler et al., 2003). Interestingly, the NK1 receptor antagonist had no effect on IL-4-induced hyperpolarization, whereas IL-4 receptor antibodies inhibited the hyperpolarization response of chondrocytes to SP. This suggests that SP activity is upstream of IL-4 release in the mechanotransduction pathway (Millward-Sadler et al., 2003). Blockade of the hyperpolarization response to SP but not IL-4 by inhibiting adenylate cyclase activity implies cAMP in NK1-mediated signaling and cytokine release (Laniyonu et al., 1988; Lacour et al., 1994).

Increased SP levels have been reported in synovial fluid and cerebrospinal fluid from patients with rheumatoid arthritis and osteoarthritis (Lindh et al., 1997). Immunohistochemical analysis of the joint capsules from patients with anterior knee pain syndrome revealed increased SP-immunoreactive nerve fibers (Witonski and Wagrowska-Danielewicz, 1999). Release of SP from chondrocytes, either by mechanical stimulation or by other means, influences the activity of a wide range of cell types in the joint and periarticular structures, including macrophages, osteocytes, and nociceptive fibers. These observations suggest that PPT-A gene expression plasticity plays a vital role in the pathophysiology of remodeling and regeneration of bone and cartilage in joint diseases.

### 2.4.3 Gut

In the gastrointestinal tract, SP-containing nerve fibers and their cell bodies are present along the entire length; they are least prominent in the esophagus and upper part of the stomach (Polak and Bloom, 1981).

According to Nilsson et al. (1975) the highest concentration of SP occurs in the duodenum. SP-positive postganglionic parasympathetic neurons are located in the myenteric plexuses, and the postganglionic nerve terminals of these neurons innervate chiefly the inner circular muscles, although the outer longitudinal muscles contain few SP-positive fibers. SP-containing nerve fibers are also in close contact with the blood vessels (Polak and Bloom, 1981).

In the human gastric mucosa, Ferri et al. (l984) have demonstrated SP immunoreactivity in the oxyntic (parietal) zone of the gastric glands. Fibers containing this peptide are numerous and interconnecting in the "antrum" 3 cm above the pyloric aperture, suggesting the role of SP in pyloric sphincter regulation. In the duodenum, SP-positive fibers were present in large numbers in the base and core of the villi as well as in the muscularis mucosae and around blood vessels, suggesting the role for SP in absorption of nutrients and peristalsis. SP was also present in nerve fibers in the submucosa, neuronal perikarya between the lobules of Brunner's glands, and in Meissner's plexus.

## 3 SP Function in Pain

### 3.1 Role of SP in the Perception and Transmission of Pain and Inflammation

Nociception is the detection of pain and can be divided into two distinct categories depending on the duration: acute and chronic pain. Acute pain is severe/sharp short-term and provides an important "warning system" that all is not well within the body/environment, e.g., pain felt when treading on a track. Chronic pain is pain persisting for the long term that may be associated with nonnoxious (non-painful) stimuli (allodynia) or may be due to increased sensitivity to noxious (painful) stimuli (hyperalgesia). Unlike acute pain, chronic pain serves no useful physiological function and is therefore the target of much pharmaceutical research.

Pain can be further subdivided into three more categories depending on its location or point of origin:

1. Viscerosomatic pain, or pain detected internally, e.g., abdominal pains. Viscerosomatic pains share many pathways, with spreadout of receptors leading to poor definition of the point of origin of the pain.
2. Inflammatory pain due to irritants or nonspecific stimuli, mediated by the immune system and usually associated with the release of chemical mediators, including histamine and SP.
3. Neurological pain or pain induced following prolonged peripheral nerve activation or peripheral/central nerve lesion (axotomy/nerve crush). Inflammatory and neurological pains are often chronic pain with associated allodynia (nonnoxious stimuli results in nociception) or hyperalgesia (noxious stimuli are perceived with a greater intensity). SP is thought to be a major modulator of the neurotransmission of pain, being in part, along with the glutamatergic activation of NMDA receptors, the major cause of allodynia and hyperalgesia.

Tachykinergic signaling, in particular NK1-SP-mediated, has been previously implicated as the neurotransmitter for pain. Thermal (Duggan et al., 1987), mechanical (Duggan et al., 1988), and chemical (capsaicin) (Duggan et al., 1988; Takano et al., 1993) stimulation of the skin all elicit SP release from sensory afferents, increasing the SP concentration in the dorsal horn. Furthermore, direct electrical stimulation of C-fibers (Brodin et al., 1987; Klein et al., 1992) also increases SP concentration in the dorsal horn. In addition, intrathecal injection of SP induces pain-like behavior in rodents (Piercey et al., 1981; Hylden and Wilcox, 1983; Matsumura et al., 1985). Most strikingly, NK1 antagonists block the response of dorsal horn neurons to noxious stimuli (Radhakrishnan and Henry, 1991; Snider et al., 1991), suggesting that NK1 receptors mediate the pain perception. Collectively, these data provide a strong argument for SP as a candidate neurotransmitter for pain signaling; however, more recent data suggest SP is not the neurotransmitter of noxious stimuli, rather it is the neuromodulator of painful stimuli as outlined below.

SP is synthesized by small-diameter nociceptive neurons whose central terminals release the peptide in the dorsal horn of the spinal cord following intense peripheral stimulation. This is thought to promote

central hyperexcitability and increased pain sensitivity (Fitzgerald and Gibson, 1984; De Felipe et al., 1998). However, the function of SP in pain and nociception remains unclear. Human clinical trials with NK1 antagonists have proved to be largely ineffective but there remains the possibility that the wrong pain conditions or time of administration could have been targeted. This possibility was raised again by the analysis of NK1 K/O mice where a marked reduction in various types of visceral nociception as well as a reduced response to noxious chemical stimulation from somatic tissues was recorded (Bester et al., 2001). Recent data suggest that many of the peripheral inflammations release SP within the spinal cord (Bueno and Fioramonti, 1999). The "silent" nociceptors are recruited into action following inflammatory lesions of the skin or deep tissues or following partial nerve ligation. The development of pain behaviors was unaffected, initially by NK1 receptor or PPT-A deletion (Cao et al., 1998; Basbaum, 1999a, b; Dery et al., 2001). This suggests a possible role for SP in chronic inflammatory diseases. This role for SP as a neuromodulator of painful stimuli is supported by the demonstration that short-duration noxious thermal stimuli do not cause SP release in the dorsal horn. Only intense and prolonged thermal stimuli result in SP release (Duggan et al., 1987, 1988, 1992). Iontophoretical application of SP to dorsal horn neurons produces a slow response characterized by delayed onset (20–40 s) but sustained (30–90 s) response was observed as a slow excitatory postsynaptic potential (EPSP) (Nowak and Macdonald, 1982; Urban and Randic, 1984). Further, SP-mediated slow EPSP in response to noxious stimuli is the generation of an initially fast response (glutamate-mediated) followed by a slow and delayed afterdischarge that lasts for the duration of the stimulus. This initial fast response is not abolished by application of NK1 antagonists, while the slow prolonged response is abolished either by NK1 receptor antagonists (Radhakrishnan and Henry, 1991) or by depletion of SP by capsaicin treatment (Hey et al., 1996).

## 3.2 Generation of the Pain Signal by Primary Sensory Afferents or "First-Order Neurons"

Primary sensory afferent receptors detect noxious and nonnoxious stimuli and relay impulses from the point of origin in the periphery to the thalamus via lamina I and interneurons in the dorsal horn of the spinal cord. As mentioned previously, primary sensory afferents consist of two fiber types, myelinated, nonpeptidergic Aδ fibers and unmyelinated, mostly peptidergic C-fibers, whose cell bodies are found in the DRG of the PNS and whose central axons terminate in the dorsal horn of the spinal cord and trigeminal ganglion of the CNS.

The majority of Aδ afferents (proprioceptive) terminate in the inner region of lamina II of the dorsal horn and to a lesser extent in lamina V. Conversely, the majority of C-fibers (nociceptors) terminate in lamina I and the outer region of lamina II (substantia gelatinosa) of the dorsal horn and to a lesser extent in laminae III, VI, and X and also in the trigeminal ganglion of the CNS (Basbaum, 1999a, b). Under normal physiological conditions, Aδ fibers transmit impulses generated by nonnoxious stimuli such as pressure and heat, while C-fibers transmit impulses generated by both noxious and thermal stimuli (peptidergic and nonpeptidergic, respectively) (Basbaum and Woolf, 1999).

## 3.3 Relaying the Pain Message from Sensory Neurons to the Dorsal Horn

Wide-dynamic-range neurons respond to nonnoxious stimuli of differing intensity (mediated initially mostly via Aδ fibers), while the nociceptive neurons respond to noxious stimuli (mediated initially mostly by C-fibers) (Guirimand and Le Bars, 1996). The Aδ fibers synapse directly with interneurons (islet cells) in lamina II of the dorsal horn. However, those of C-fibers not juxtaposed to the interneurons in lamina II form close connections with the projection neurons of lamina I (Marshall et al., 1996). On low-threshold mechanical or thermal (nonnoxious) stimulation of sensory afferents, glutamate is released from Aδ terminals where it binds to postsynaptic alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and NMDA receptors in the dorsal horn interneurons or projection neurons. Glutamate binding to AMPA receptors facilitates influx of $Na^+$ ions into the postsynaptic terminal, resulting in depolarization.

The NMDA receptor is a ligand-dependent voltage-gated $Ca^{2+}$ ion channel. On initial AMPA-mediated depolarization and binding of glutamate, $Mg^{2+}$ blockade of the NMDA channel pore is removed, allowing influx of $Ca^{2+}$ into the second-order neurons resulting in depolarization and propagation of the impulse.

Upon high-intensity noxious stimulation, both glutamate and SP are released from C-fibers. As mentioned above, the binding of glutamate to its receptors elicits a fast EPSP; however, the concomitant release of SP also elicits a second-phase slow EPSP. SP binding preferentially to the NK1 receptor on the postsynaptic membrane of projection neurons results in the activation of PLC$\gamma$, which in turn generates DAG and IP3, initiating calcium release from internal stores leading to further depolarization and propagation of impulse to the higher centers of the brain.

DAG activates PKC that indirectly modulates the response of the postsynaptic neuron to glutamate via phosphorylation of the NMDA receptor. Phosphorylation of the NMDA receptor increases the opening time of the channel leading to prolonged influx of $Ca^{2+}$ and $Na^{+}$, thereby prolonging depolarization of second-order neurons. DAG also contributes to the formation of arachidonic acid, an event in the formation of prostagladins, PGE2 and PI2 that sensitize the cell membrane to further depolarization. IP3 triggers intracellular calcium release from the internal stores (endoplasmic reticulum) resulting in a further increase in intracellular calcium levels, thus further prolonging depolarization of the second-order neurons.

In summary, the release of glutamate from A$\delta$ fibers following mechanical or thermal stimulation results in depolarization of the second-order interneurons in lamina II of the dorsal horn. However, the intensity of a noxious stimulus could result in the corelease of glutamate and SP from C-fiber terminals. SP diffuses from its release site into the interneurons (islet cells) of lamina II and projection neurons of laminae I and III, thus propagating the nociception. Following high-potency noxious stimulation or during inflammation, SP release is increased and NK1 receptor binding is extended beyond laminae I and III to the deeper layers of laminae IV and VI (Brown et al., 1995). This plasticity of SP release results in recruitment and prolonged activation of second-order neurons. It is this action of SP that may be responsible for the development of allodynia and hyperalgesia. The depolarization of second-order neurons is prolonged in response to the lower-intensity noxious stimuli (hyperalgesia) or mechanical and thermal stimuli (allodynia), resulting in the perception of intense pain (chronic pain) since the neuronal pathways have become hypersensitive. NK1 receptor internalization studies have supported the role of SP in hyperalgesia/allodynia. Ablation of the NK1 neurons of the dorsal horn using SP conjugated to a potent neurotoxin (saporin) results in the augmentation of hyperalgesia/allodynia in rodents. However, the behavior of NK1-ablated mice and control mice was unaltered in response to mild noxious stimuli, suggesting that SP plays a role in modulating the response to chronic intense pain and in supplementing persistent pain states, while acute pain is mediated by the NMDA cascade (Liu et al., 1997).

Both glutamate and SP mediate excitatory responses to sensory stimuli; however, it is also possible that both SP and glutamate can function as positive feedback loops directly or indirectly to modulate their own release. Increased intracellular $Ca^{2+}$ following NMDA receptor or NK1 receptor binding can activate the enzyme NOS in postsynaptic neurons. NOS catalyzes the production of a gaseous free radical NO from the substrate L-arginine. NO diffuses from the postsynaptic neuron and activates soluble guanylyl cyclase (sGC) in the presynaptic neuron to increase intracellular cyclic GMP (cGMP). Downstream of cGMP, PKG is activated, which may lead to modification of gene expression and modulation of transmitter release or may modulate ion channels. Furthermore, glutamate binding to presynaptic NMDA receptors (Liu et al., 1996) and kainate receptors (Chizh et al., 1997) on the central terminals of DRG neurons may enhance depolarization resulting in further release of both glutamate and SP. The existence of presynaptic NK1 receptors indicates that SP itself may directly enhance its own release by increasing depolarization of primary sensory afferents.

## 3.4 Neuronal Pathways of Pain Processing: Delivering the Message

The detection of painful stimuli, pressure, and temperature are coordinated by three main pain pathways: the spinoreticular, the spinothalamic, and the parabrachial pathways (Gauriau and Bernard, 2002).

This section describes these pathways in brief. The spinoreticular pathway, as its name suggests, is centered on the deep lamina of the dorsal horn of the spinal cord and the reticular system in the brainstem. The spinoreticular system is implicated in mediating somatic motor responses and the emotional behaviors associated with pain. Furthermore, this pathway offers a feedback regulation of nociception through a descending pathway, the reticulospinal loop. The second pathway, the spinothalamic pathway, originates in the superficial layers of lamina I of the dorsal horn and projects to thalamic areas and is most likely responsible for pain sensation of tactile origin. The spinobrachial pathway also originates in the superficial layer of lamina I of the dorsal horn but the axons terminate in the parabrachial area among others. This pathway is concerned with emotional, autonomic, and neuroendocrine aspects of the pain experience. In fact, the majority of nociceptive messages converge on the parabrachial area and are then connected to higher brain regions responsible for emotions (amygdala), emotional behavior (periaqueductal gray), and autonomic homeostatic adaptation (hypothalamus and ventrolateral medulla) in response to pain (Gauriau and Bernard, 2002). It is likely that neuromodulation of pain pathways by SP occurs not only at the levels of the spinal cord but also in the brain region. For example, NK1-expressing projection neurons of the dorsal horn relay nociceptive information, directly or indirectly, to brain areas such as the amygdala, the ventromedial nucleus of the hypothalamus, and PAG. These brain areas have been implicated in the mediation of antinociception caused by opioids, electrical brain stimulation, or stress-induced analgesia (Fields, 2000). It is possible that SP and the NK1 receptor play an important role in regulating the endogenous antinociception mediated by release of opioids at the synapses.

## 4 Neurodegenerative Diseases and Other CNS Disorders

PPT-A mRNA has been identified in the normal and the pathological state in various regions of the CNS in rats (Warden and Young, 1988; Harlan et al., 1989; Brene et al., 1990) and in humans (Hurd et al., 1999), suggesting a role for tachykinins in the pathophysiology of a variety of etiologies or diseases (Kramer et al., 1998; Maubach et al., 1998; Liu et al., 1999a, b). For example, SP can enhance neural or neurite growth in vitro (Iwasaki et al., 1989) and counteract the effects of neurotoxins administered to animals, and has mnemogenic and anxiolytic properties in vivo (Hasenohrl et al., 1989). SP/PPT-A expression has also been studied in several neurodegenerative disorders, including Parkinson's disease (Gresch and Walker, 1999), Alzheimer's disease (Bouras et al., 1990), and Huntington's disease (Richfield et al., 2002). All of these conditions are associated with a progressive loss of SP and PPT-A expression in the brain. Additionally, SP/NK1 antagonist MK-869 had antidepressant effects in patients with moderate to severe major depression, suggesting that SP may play an important role in psychiatric disorders (Kramer et al., 1998; Maubach et al., 1999). It is likely that inappropriate expression of the PPT-A gene is correlated with the disease profiles in which tachykinin gene products are implicated.

### 4.1 Epilepsy

Recent studies have shown a strong correlation between the incidence of epilepsy, SP, and the integrity of the dentate gyrus in a rodent model (Liu et al., 1999a, b). Epilepsy is a chronic medical condition produced by temporary and maladaptive alterations in the electrical function of the brain, causing seizures that affect awareness, movement, and/or sensation. Epilepsy affects more than 50 million people worldwide. Epilepsy is a biphasic disorder in which an initial seizure may lead to the generation of spontaneous, continuous, and long-lasting seizure activity. The process by which an initial seizure can lead to the development of epilepsy is termed epileptogenesis. The process of epileptogenesis itself is poorly understood, but it is believed that the initial imbalances in inhibitory and excitatory inputs during seizure lead to long-term plastic changes in glutamatergic and GABAergic signaling pathways, novel expression of neuropeptides in brain regions, and at a later stage synaptic reorganization and mossy fibers sprouting. Although many new antiepileptic drugs have been invented and tested in the last decade, about one-third of epileptic patients still suffer from

inadequately controlled seizures or significant side effects. Novel PPT-A expression has been widely implicated in both the initiation and maintenance phases of epilepsy.

### 4.1.1 Neuronal Cell Excitability Is Regulated via the Interplay Between Excitatory Glutamatergic and Inhibitory GABAergic Neurotransmission

There are many types of seizure showing a variety of symptoms and severity, whose presence can be determined by the pattern of burst firing activity as seen on an electroencephalogram (EEG). There are a number of animal models that show similarity to etiology and symptoms of human temporal lobe epilepsy (TLE), one of the most extensively studied forms of epilepsy. All seizures, however, have common features, including spontaneous, increased frequency and sustained, irregular neuronal firing patterns, i.e., all show signs of excessive excitability.

The control of cell excitability is important in maintaining normal physiological control of any neuronal cell. This control is manifested by the interplay between the excitatory and inhibitory inputs into this system. If oversimplified, it could be said that the balance between the glutamatergic signaling pathway (excitatory) and the GABAergic signaling pathway (inhibitory) controls neuronal excitability. Seizure-like activity and associated cell death has been attributed to increased excitability of neurons due to increased glutamate-mediated NMDA receptor activation (Wasterlain et al., 2000). In addition, the excessive release of presynaptic glutamate in vivo in kainic acid-induced seizures further confirms NMDA-receptor-mediated excitability during seizures (Bruhn et al., 1997).

### 4.1.2 The Biphasic Nature of Epilepsy: Initiation and Maintenance of Seizure

Epilepsy can be divided into two stages: the initiation phase, in which a single seizure may increase the propensity of a cell toward developing and maintaining continuous seizures, and the maintenance phase, in which a cell can be said to be epileptic due to the presence of spontaneous and continuous long-lasting firing activity.

Experimental initiation of epilepsy via perforant path stimulation (PPS) can be blocked by the application of agonists of inhibitory pathways ($GABA_A$ agonists) or antagonists of excitatory pathways (NMDA/AMPA/kainate), electrical stimulation of GABAergic pathways, NK1 receptor antagonists, galanin (an inhibitory neuropeptide) and its receptor agonists, opiate receptor agonists (delta) and antagonists (kappa), and finally by ionic imbalances across the neuronal membrane (elevated intracellular $Na^{2+}$ with low extracellular $K^+$, or high extracellular $Mg^{2+}$). Conversely, experimental initiation of epilepsy can be elicited by $GABA_A$ antagonists, glutamate receptor agonists (NMDA/AMPA/kainate), electrical stimulation of glutamatergic pathways, NK1 receptor agonists, SP or NKB (excitatory neuropeptides), galanin receptor antagonists, opiate receptor antagonists (delta), and agonists (kappa), and finally by ionic imbalances across the neuronal membrane (elevated extracellular $K^+$ with low extracellular $Na^{2+}$ or low extracellular $Mg^{2+}$) (Liu et al., 1999a, b; Wasterlain et al., 2002). So it would seem that the initiation of seizure has many entry points and is responsive to drug treatments, e.g., benzodiazepines are currently used in the treatment of seizure as agents that stimulate GABAergic pathways.

Experimental inhibition of the maintenance phase of self-sustaining status epilepticus (SSSE) is difficult to manage. Many of the above inhibitors of the initiation phase are ineffective in halting or preventing the sustained seizures associated with the maintenance phase. Pharmacological treatment with benzodiazepines is ineffective as are other drugs such as sodium channel blockers and non-NMDA receptor blockers (Wasterlain et al., 2002). Glutamatergic antagonists, NK1 antagonists, and inhibitory neuropeptides such as galanin, however, are effective in ameliorating SSSE, suggesting that the maintenance phase of epilepsy is strongly dependent on NMDA and tachykinin receptor activation. This implies that SP may play a modulatory role in sensitizing neuronal cells in glutamatergic pathways that is similar to that proposed for its role in modulating cell excitability in models of pain and inflammation.

### 4.1.3 Epileptic Pathways: the Hippocampus in the Limbic System Is a Major Brain Region Associated with Seizure

Metabolic studies using 2-deoxyglucose autoradiography have shown a number of regions of the brain with high metabolic activity during SSSE produced via perforant path stimulation (Wasterlain et al., 2002). These areas include the hippocampus, amygdala, the caudate putamen, the substantia nigra, the nucleus accumbens, and the medial thalamus. Interestingly, all of these areas express medium to high levels of SP.

Wasterlain et al. (2002) proposed a hippocampal model of initiation and maintenance of seizure/epilepsy based on these metabolic studies and those involving pharmacological agonists and antagonists of principal pathways. ❯ *Figure 20-3* depicts the excitatory and inhibitory pathways involved in the control of hippocampal excitability in nonepileptic brain models and their modification in epileptic brain models. PPS by electrodes generates SSSE by intense activation of GABAergic signaling in the dentate gyrus of the hippocampus, resulting in excessive GABA release. On binding of GABA to its receptors, they are internalized, thus effectively removing the GABA inhibitory control from the hippocampus. Removing inhibition of the GABAergic pathway in this manner indirectly increases neuronal excitability, increasing the likelihood of seizure. The pathological changes in neurotransmitter-mediated excitability lead to decreased anticonvulsant (galanin/dynorphin) and increased proconvulsant (SP/NKB) neuropeptide expression, producing long-lasting changes in excitability by further potentiating glutamate release. In particular, decreased galanin or dynorphin and increased SP mediate removal of inhibition and increase excitability on mossy fibers of the dentate gyrus, thus increasing glutamate release in the CA3 pyramidal layer. The CA3 neurons respond to glutamate release from the mossy fibers by increasing the firing activity. CA3 nerve terminals synapse with the frontal cortex, which is concerned with the regulation of motor activity. Additionally, excitatory connection to the cortex from the hippocampus usually results in negative feedback to the dentate gyrus to inhibit further glutamate release but on depletion of inhibitory neuropeptides (galanin/opioids), this negative feedback is lost; hence the glutamatergic input to cortical areas further increases seizure activity.

Seizures may last from anywhere between a few minutes to a few hours and in some cases recurrent episodic seizures may go on for days. Late synthesis and expression of galanin, depletion of excitatory neurotransmitters, and neuronal death all contribute to the cessation of seizure without pharmacological intervention. Unfortunately, massive cell death induced during seizure is not fully investigated and in some cases epilepsy could be fatal. However, recent research on adult neurogenesis suggests that the dentate gyrus granular zone is a promising source for the generation of new neurons as a replacement for dead neurons (Alvarez-Buylla and Lim, 2004).

### 4.1.4 SP is a Proconvulsant: the Modulation of the Transcriptional Regulation of PPT-A Gene Expression in Seizure and Epilepsy

Neuropeptide expression is modified during seizure, which in turn leads to increased excitability in the hippocampus and other brain regions. SP has been widely implicated in modifying excitability and mediating both the initiation and the maintenance phases of epilepsy (Liu et al., 1999a, b, 2000; Wasterlain et al., 2000, 2002). SP is detected at low levels in the rodent hippocampus. However, production of neuronal proteins is subject to "plasticity" at the level of gene regulation; thus the concentration of SP within tissues can be altered in response to specific stimuli, e.g., growth factors, stress, and drugs.

SP demonstrates highly proconvulsive properties. High levels of SP in hippocampal neurons can induce SSSE at electrical stimuli below the threshold stimulation normally required to induce such activity (Liu et al., 1999a, b). Furthermore, elevated levels of SP and PPT-A in the dentate gyrus subregions of the hippocampus (CA1 and CA3) have been implicated in neuronal damage following SSSE induced by PPS, or chemoconvulsant seizure induction with kainic acid, or lithium–pilocarpine induction (❯ *Figure 20-4*) (Brene et al., 1992; Liu et al., 1999a, b, 2000). NK1 receptor antagonists applied before or during the initiation and maintenance phases abolish SSSE (Liu et al., 1999a, b). Moreover, SP application to brain slices induces glutamate release (Liu et al., 1999a, b). In studies employing an in vivo kainic acid epilepsy

**Figure 20-3**

**Excitatory and inhibitory pathways in the rodent hippocampus. (a) shows the excitatory glutamatergic pathways stimulated in normal brain; normally inhibitory input from GABAergic interneurons in the hilus and from inhibitory neuropeptides (galanin, dynorphin, somatostatin, and NPγ) controls excitability of glutamatergic signaling, preventing the generation of seizure. However, in an epileptic model (b), excessive stimulation of inhibitory systems depletes inhibitory neurotransmitters and neuropeptides, allowing unbridled activation of glutamatergic pathways leading to the development of seizure. Additionally, novel excitatory neuropeptide expression (SP) further reinforces seizure by sensitizing glutamatergic pathways and increasing glutamate release. Late expression of galanin in the hilus restores some inhibitory control to dampen seizure**

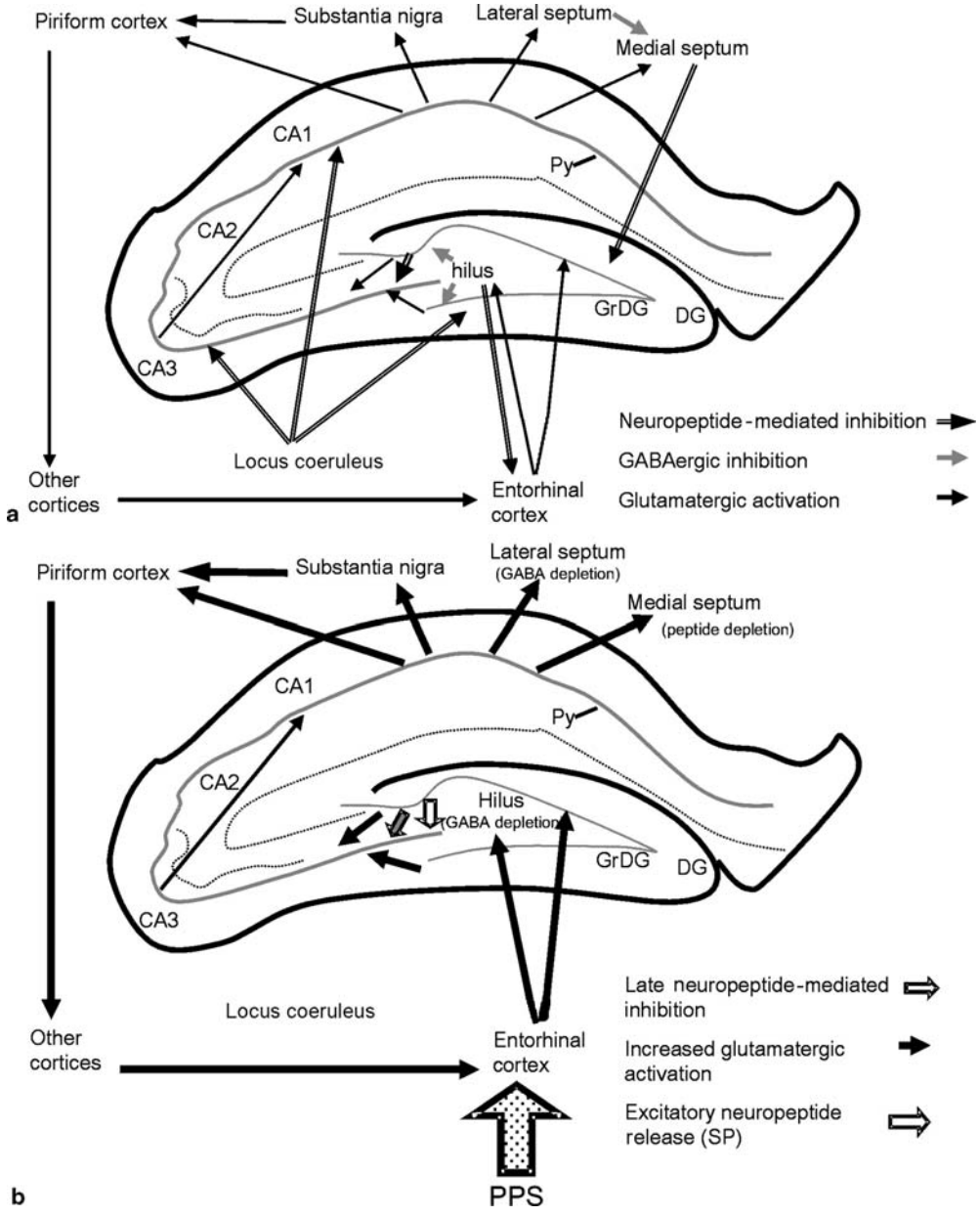
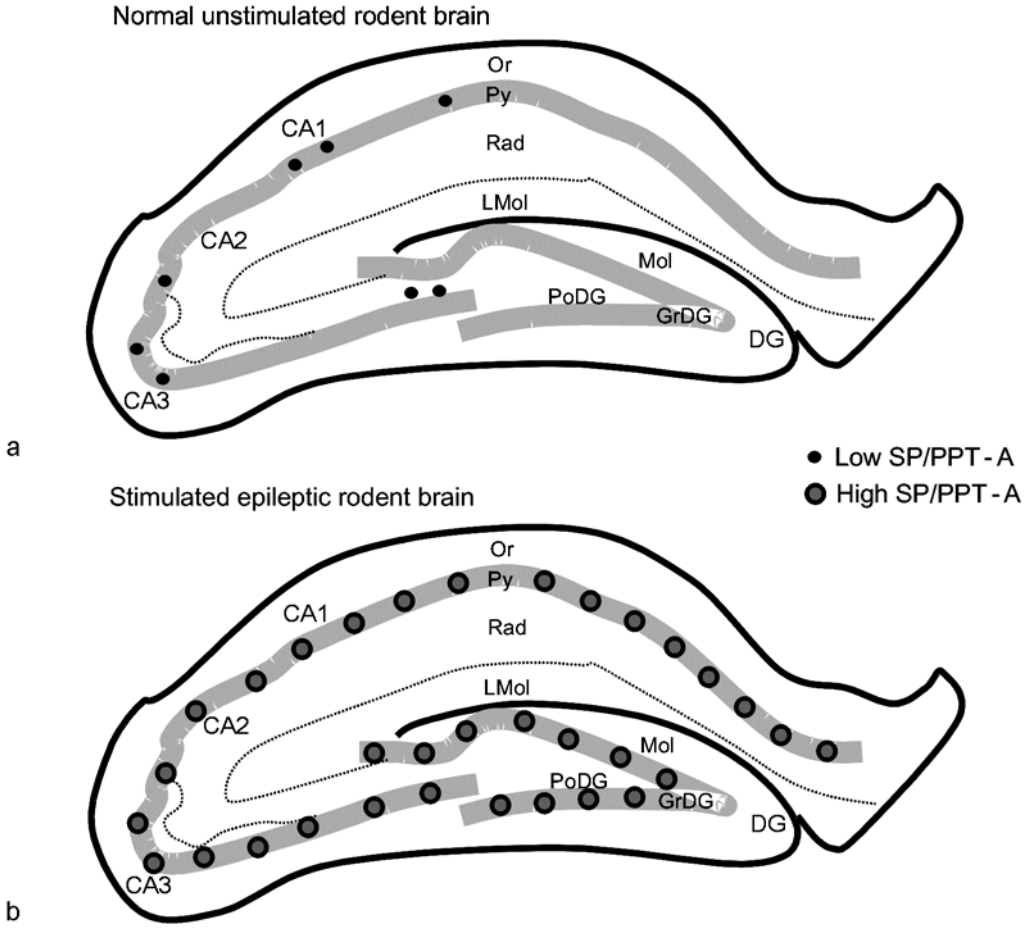


**Figure 20-4**

**Schematic representation of the inducibility of SP/PPT-A in the rodent hippocampus following experimental seizure induction. SP/PPT-A mRNA expression is very low in the normal unstimulated rodent hippocampus (a). Low levels of expression are detected by immunostaining and in situ hybridization in the CA1, CA2, and CA3 pyramidal cell layers (Py), and a few SP-positive cell bodies are found in the hilus (h) of the dentate gyrus (DG). In the epileptic mouse (b) massive induction and de novo synthesis of SP is observed. Marked upregulation of PPT-A and SP expression is seen in all areas that express in the unstimulated mouse. In addition, novel expression is seen in the granule cell layer (GrDG) of the dentate gyrus**




model, PPT-A gene K/O studies have correlated the lack of SP with the inability to induce seizures and associated damage within the hippocampus in mice (Liu et al., 1999a, b). Additionally, these K/O mice do not show induction of caspases and other genes associated with cell death that are normally expressed in seizure. It is not surprising that PPT-A mRNA is increased in the hippocampus during seizure, and de novo synthesis of SP in the dentate gyrus, an area that does not express SP during normal physiological functioning, is observed in a number of in vivo and in vitro epileptic models.

There are many unanswered questions as to how SP might be upregulated during seizure, e.g., which classes of regulatory transcription factors are induced during seizure and how do they affect the transcription of PPT-A? Defining the pathways regulating the PPT-A gene will help delineate general changes associated with seizure induction. Recent research suggests that bHLH factors are differentially regulated

following status epilepticus, with some increasing (Mash1, Id2), some decreasing (Hes5), and others remaining mostly unchanged (NeuroD/BETA2, NeuroD2/NDRF, Id3, Rath2/Nex1) (Elliott et al., 2001). The PPT-A gene contains a number of regulatory elements termed E boxes that bind bHLH factors to regulate transcription. It is possible that these bHLH factors are responsible, in part, for the modulation of the PPT-A gene expression during seizure and as such may provide a novel target for therapeutic intervention. Similarly, a major repressor of the proximal rat PPT-A promoter is the transcription factor NRSF, often also called REST (Bubb et al., 2002), which demonstrates differential expression during rodent epilepsy models (Palm, 1998). Interestingly, NRSF/REST has several isoforms, which have been suggested to act as both repressors and activators of transcription (Bubb et al., 2002); these isoforms are also differentially regulated during epilepsy (Palm, 1998).

## 4.2 Anxiety and Depression

Studies on the NK1 K/O mice and clinical studies suggest that SP is involved in emesis, stress responses, aggression, anxiety, depression, and reward (Cao et al., 1998; De Felipe et al., 1998a, b; Kramer et al., 1998; Rupniak and Kramer, 1999; Rupniak et al., 2000). These apparently diverse behavioral manifestations are potentially interlinked and SP could modulate these pathways that are important to the animals in the face of major environmental stressors (Culman and Unger, 1995; Culman et al., 1997; De Felipe et al., 1998a, b). The NK1 receptor is widely distributed within subcortical and brainstem regions and within the spinal cord, as described previously. The receptor is highly but heterogeneously expressed within the amygdala, and by the cholinergic neurons of the striatum, nucleus accumbens, and nucleus basalis. These areas of the brain have been linked to anxiety and reward behaviors. The NK1 receptor is also strongly expressed in the hypothalamus, PAG, and the superficial laminae of the spinal cord. These areas control pain processing and flight/fight responses (autonomic) following environmental challenges such as attack, injury, or invasion of territory (Lumb and Lovick, 1993; Bandler and Shipley, 1994; Lovick, 1996; De Felipe et al., 1998a, b).

Selective deletion of the NK1 receptor gene using homologous recombination in embryonic stem cells resulted in mice that bred and developed normally. However, close examination of these mice revealed a number of remarkable behavioral changes compared with wild-type litter mates; K/O mice were less aggressive as measured by the resident–intruder assay (De Felipe et al., 1998a, b) and had reduced levels of anxiety as judged by their response to brief maternal separation (Rupniak et al., 2000). These measures anticipated the current clinical trials of NK1 antagonists in human subjects suffering from anxiety and depression (Kramer et al., 1998).

The reduction in anxiety and aggressive behavior thought to be coupled with a number of other behavioral changes in NK1 K/O mice suggested that the NK1 receptor might be involved in orchestrating basic survival behaviors. For example, there was also a reduction in stress-induced analgesia that may be due to descending inhibitory control on the spinal processing of nociception. The behavioral monitoring suggested that the unaltered behavior of mutant mice was due to lack of change in the hot-plate threshold following a brief cold-water swim (De Felipe et al., 1998a, b). The behavioral changes were correlated with morphological changes using immunohistochemistry. For example, there was no change in the number of *c-fos*-positive neurons in the lumbar spinal cord following concurrent noxious stimulation of both fore- and hindpaws (Bester et al., 2001).

Remarkably, there was a failure of the NK1 K/O mice to develop a conditioned place preference to morphine (Murtra et al., 2000). The loss was specific to morphine as mice responded when cocaine or food were used as rewards. Moreover, the analgesic response to opiates was largely intact (De Felipe et al., 1998a, b). We conclude that SP plays an important and specific role in mediating the motivational aspects of opiates. While this may represent a major new pharmacological route for the control of drug abuse, there is also the possibility of dissecting away the analgesia from the euphoria-producing properties of the opiates. Morphine given exogenously binds to the μ opiate receptor and by this route it is thought to "hijack" the opiate reward pathways and other pathways that modulate nociception (Robbins and Everitt, 1999).

The data argue strongly that a key synapse in the opiate reward process lies within the area of ventral forebrain and occurs between collaterals of SP-releasing populations of striatal projection neurons and large

cholinergic neurons of the nucleus accumbens and nucleus basalis that express the NK1 receptor. These neurons have also been implicated in associative learning and respond to stimuli that serve to trigger a "learn and reward" task (Graybiel et al., 1994; Boix et al., 1995).

### 4.3 Parkinson's Disease

Parkinson's disease results in reduced levels of dopamine, which is due to loss of dopaminergic neurons (~50%) in the substantia nigra whose fibers have extensive synapses within the striatum. The decrease in levels of dopamine is reflected in the striatonigral neurons by lower levels of D1 receptors and SP, among others. It was demonstrated that the loss of SP led to further loss of dopaminergic neurons (Barker, 1986). SP striatal neurons also inhibit the neurons of the internal globus pallidus. In addition to dopamine neurotransmission, serotonin neurotransmission regulates striatal PPT-A mRNA levels. It has been suggested that the activation of 5-HT transmission could compensate for the loss of PPT-A in striatal neurons (Gresch and Walker, 1999a, b). Furthermore, it was demonstrated that serotonin 2A/2C receptors mediate PPT-A mRNA expression in the striatum after dopamine depletion produced with 6-hydroxydopamine in adult rats. However, higher levels of SP could be toxic. NK1 antagonists can block the toxic effects of methamphetamine administered to the striatum (Yu et al., 2002). A major role for PPT-A expression in Parkinson's disease was proposed from studies involving 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment of primates. MPTP induces Parkinson-like symptoms, which is due to the destruction of dopaminergic neurons in the substantia nigra. Animals with an acute or chronic parkinsonian phenotype had decreased levels of PPT-A in the striatum while in asymptomatic animals PPT-A levels were unaffected (Wade and Schneider, 2001). The authors suggest that PPT gene expression may be directly related to expression of parkinsonian motor symptomatology regardless of duration of MPTP exposure, duration of the parkinsonism, or extent of dopamine denervation. The plasticity of PPT-A expression in this CNS region is demonstrated in that ciproxifan, the histamine H3 receptor ligand, can reverse the effect of methamphetamine on the PPT-A gene (Pillot et al., 2003). Therefore intervention, which targets genes on the same pathway as PPT-A by increasing neuropeptide gene expression, could be useful therapy.

In addition to NK1 receptor activation by SP, there are reports of NK3 receptor involvement in the rat midbrain neurons in Parkinson's disease. Modulation of NK3 activity in the rat nigrostriatal dopamine neurons has been reported to affect dopamine levels and hence SP could be a potential therapeutic target for Parkinson's disease (Bannon and Whitty, 1995; Whitty et al., 1995).

## 5 Genetic Models Available for In Vivo Analysis

Gene disruption in order to study the role of a specific protein is commonly employed in the generation of preclinical animal models. In the case of the tachykinins, the study of their function(s) has significantly been enhanced by the generation of K/O mice with targeted disruptions of the PPT-A and TACR1 genes, which encode SP, NKA, and NK1, respectively (Cao et al., 1998; De Felipe et al., 1998a, b; Zimmer et al., 1998).

PPT-A K/O animals are deficient in SP and NKA but are able to develop normally, are fertile, and can take care of their offspring (Bilkei-Gorzo et al., 2002). Behavioral studies carried out in these animals have demonstrated a reduction in sensitivity to nociceptive stimulation in acute and chronic pain models, although the response to mildly painful stimuli is unaffected (Cao et al., 1998). Mutant animals do not experience neurogenic inflammation, which normally follows SP release. This is indicative of a role of SP/NKA in the production of moderate to intense pain.

The role of the tachykinins in stress-related behaviors has also been addressed in the PPT-A K/O animals. It has been shown that PPT-A(−/−) mice were more active in the forced-swimming test and tail suspension paradigm (both used as indicators of depression-related behaviors) and were less fearful in models of anxiety (such as the open-field arena and the elevated zero maze). Their behavior was comparable

with that of wild-type animals treated with antidepressant drugs, including tricyclic and selective serotonin re-uptake inhibitors. These observations support the view that the tachykinin system mediates the development of anxiety and depression disorders.

Mice lacking the PPT-A gene have been resistant to kainic acid-induced seizures that mimic hippocampal hyperexcitability seen in cases of status epilepticus. Neuronal cell death in the hippocampus caused by repetitive epileptic seizure activity could be compromised in the absence of SP release (Liu et al., 1999a, b).

Another approach to the study of the tachykinin pathway has been through the inactivation of the tachykinin NK1 receptor. Genetic disruption of the NK1 receptor does not affect the general health or fertility of the mutant mice but has a significant effect in the amplification ("wind up") and intensity coding of nociceptive reflexes, which appear to be absent in these animals (De Felipe et al., 1998a, b). The role of the NK1 receptor in pain and hyperalgesia has also been investigated using the NK1(−/−) model. It has been proposed that the response to noxious mechanical stimuli is modulated by NK1, which is also implicated in hyperalgesia pathway (Laird et al., 2000). Endogenous pain-control mechanisms, such as stress-induced analgesia, were found to be substantially impaired in the NK1(−/−) mice (Bester et al., 2001). In behavioral experiments, mice lacking the NK1 receptor were observed to be less aggressive than their wild-type counterparts in the "resident–intruder" test.

To further explore the function of the human tachykinins and understand their potential role in a variety of pathophysiological processes, the generation of genetic in vivo models of the human PPT-A and NK1 genes has been attempted. MacKenzie et al. (2000) have reported the production of a yeast artificial chromosome (YAC) transgenic model that comprises the human PPT-A gene (hPPT-A) and can drive appropriate expression of β-galactosidase within the adult mouse brain (MacKenzie and Quinn, 2002). YAC constructs have a large cloning capacity (within the range of several hundreds of kilobases) and may contain not only the coding region of a particular gene, but also the majority of regulatory sequences that are required for correct transcription. It has been demonstrated that the hPPT-A YAC transgenic mouse is able to express SP/NKA in appropriate areas of the developing mouse brain, and expression is observed in regions at significantly earlier time points than originally suggested from conventional analysis such in situ hybridization. By crossing the hPPT-A and hNK1 alleles onto the relevant ablated genetic backgrounds, PPT-A and NK1(−/−) are already available (De Felipe et al., 1998a, b; Zimmer et al., 1998), and as such it should be possible to construct an animal that expresses only the human genes. Such a "humanized" animal model would be a valuable tool for preclinical pharmacological and behavioral studies.

The use of transgenic in vivo models to complement biochemical analyses has proven extremely advantageous in the study of the role of the tachykinins in physiological conditions as well as in disease states. Further, exploiting the possibilities that these models can offer will undoubtedly assist future advances in our understanding of the function of the tachykinins. However, one should be careful to extrapolate too much from experiments done on only one mouse strain as dramatic differences have been observed in tachykinin function in the lung in the response to virus infection depending on the strain of mouse used (Payne et al., 2001).

## 6 Summary

The tachykinins are central to normal physiology and disease, both acting individually or in synergy with other neurotransmitters. They are involved in a plethora of disease states or conditions. The tachykinin receptor antagonists have been tested or are currently being tested in clinical trials for a number of conditions including depression, anxiety, pain, and cancer with limited success. Investigation of the molecular and cellular regulation and function of these neuropeptides and their receptors will lead to better strategies for clinical intervention. Further, analysis of emerging genomic information from clinical analysis of polymorphisms associated with disease may dictate those populations at risk that would be more amenable to therapeutic targeting of the tachykinins. We look forward to these research-defining mechanisms central to neural transmission in general and also for better therapeutic strategies.

## References


Adell A. 2004. Antidepressant properties of substance P antagonists: relationship to monoaminergic mechanisms? Curr Drug Targets CNS Neurol Disord 3: 113-121.

Alvarez-Buylla A, Lim DA. 2004. For the long run: maintaining germinal niches in the adult brain. Neuron 41: 683-686.

Andoh T, Nagasawa T, Kuraishi Y. 1996. Expression of tachykinin NK1 receptor mRNA in dorsal root ganglia of the mouse. Brain Res Mol Brain Res 35: 329-332.

Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, Orkin SH. 1993. Erythroid transcription factor NF-E2 is a haematopoietic-specific basic-leucine zipper protein. Nature 362: 722-728.

Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV. 1995. Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 7: 1484-1494.

Bai TR, Zhou D, Weir T, Walker B, Hegele R, et al. 1995. Substance P (NK1)- and neurokinin A (NK2)-receptor gene expression in inflammatory airway diseases. Am J Physiol 269: L309-L317.

Bandler R, Shipley MT. 1994. Columnar organization in the midbrain periaqueductal gray: modules for emotional expression? Trends Neurosci 17: 379-389.

Bannon MJ, Whitty CJ. 1995. Neurokinin receptor gene expression in substantia nigra: localization, regulation, and potential physiological significance. Can J Physiol Pharmacol 73: 866-870.

Barker R. 1986. Substance P and Parkinson's disease: a causal relationship? J Theor Biol 120: 353-362.

Barnes PJ. 1991. Neurogenic inflammation in airways. Int Arch Allergy Appl Immunol 94: 303-309.

Barnes PJ. 1992. Neurogenic inflammation and asthma. J Asthma 29: 165-180.

Basbaum AI. 1999a. Distinct neurochemical features of acute and persistent pain. Proc Natl Acad Sci USA 96: 7739-7743.

Basbaum AI. 1999b. Spinal mechanisms of acute and persistent pain. Reg Anesth Pain Med 24: 59-67.

Basbaum AI, Woolf CJ. 1999. Pain. Curr Biol 9: R429-R431.

Bernardi L, Bosisio G, Chillemi F, De Caro G, De Castiglione R, et al. 1966. Synthetic peptides related to eledoisin. Physalaemin-like peptides. Experientia 22: 29-31.

Berthold M, Bartfai T. 1997. Modes of peptide binding in G protein-coupled receptors. Neurochem Res 22: 1023-1031.

Bester H, De Felipe C, Hunt SP. 2001. The NK1 receptor is essential for the full expression of noxious inhibitory controls in the mouse. J Neurosci 21: 1039-1046.

Bilkei-Gorzo A, Racz I, Michel K, Zimmer A. 2002. Diminished anxiety- and depression-related behaviors in mice with selective deletion of the Tac1 gene. J Neurosci 22: 10046-10052.

Blier P, Gobbi G, Haddjeri N, Santarelli L, Mathew G, et al. 2004. Impact of substance P receptor antagonism on the serotonin and norepinephrine systems: relevance to the antidepressant/anxiolytic response. J Psychiatry Neurosci 29: 208-218.

Boix F, Sandor P, Nogueira PJ, Huston JP, Schwarting RK. 1995. Relationship between dopamine release in nucleus accumbens and place preference induced by substance P injected into the nucleus basalis magnocellularis region. Neuroscience 64: 1045-1055.

Bolser DC, DeGennaro FC, O'Reilly S, McLeod RL, Hey JA. 1997. Central antitussive activity of the NK1 and NK2 tachykinin receptor antagonists, CP-99,994 and SR 48968, in the guinea-pig and cat. Br J Pharmacol 121: 165-170.

Bouras C, Vallet PG, Hof PR, Charnay Y, Golaz J, et al. 1990. Substance P immunoreactivity in Alzheimer disease: a study in cases presenting symmetric or asymmetric cortical atrophy. Alzheimer Dis Assoc Disord 4: 24-34.

Bozic CR, Lu B, Hopken UE, Gerard C, Gerard NP. 1996. Neurogenic amplification of immune complex inflammation. Science 273: 1722-1725.

Brene S, Lindefors N, Ballarin M, Persson H. 1992. Kainic acid-mediated increase of preprotachykinin-A messenger RNA expression in the rat hippocampus and a region-selective attenuation by dexamethasone. Neuroscience 50: 611-618.

Brene S, Lindefors N, Friedman WJ, Persson H. 1990. Preprotachykinin A mRNA expression in the rat brain during development. Brain Res Dev Brain Res 57: 151-162.

Brodin E, Linderoth B, Gazelius B, Ungerstedt U. 1987. In vivo release of substance P in cat dorsal horn studied with microdialysis. Neurosci Lett 76: 357-362.

Brown JL, Liu H, Maggio JE, Vigna SR, Mantyh PW, et al. 1995. Morphological characterization of substance P receptor-immunoreactive neurons in the rat spinal cord and trigeminal nucleus caudalis. J Comp Neurol 356: 327-344.

Bruhn T, Christensen T, Diemer NH. 1997. Evidence for increased cellular uptake of glutamate and aspartate in the rat hippocampus during kainic acid seizures. A microdialysis study using the "indicator diffusion" method. Epilepsy Res 26: 363-371.

Brunelleschi S, Vanni L, Ledda F, Giotti A, Maggi CA, et al. 1990. Tachykinins activate guinea-pig alveolar macrophages: involvement of NK2 and NK1 receptors. Br J Pharmacol 100: 417-420.

Bubb VJ, Quinn JP, Marshall-Jones ZV, Coulson JM. 2002. Regul Pept 108(2): 135-141.

Buck SH, Maurin Y, Burks TF, Yamamura HI. 1984. High-affinity 3H-substance P binding to longitudinal muscle

membranes of the guinea pig small intestine. Life Sci 34: 497-507.

Bueno L, Fioramonti J. 1999. Effects of inflammatory mediators on gut sensitivity. Can J Gastroenterol 13 Suppl. A: 42A-46A.

Caberlotto L, Hurd YL, Murdock P, Wahlin JP, Melotto S, et al. 2003. Neurokinin 1 receptor and relative abundance of the short and long isoforms in the human brain. Eur J Neurosci 17: 1736-1746.

Candenas ML, Cintado CG, Pennefather JN, Pereda MT, Loizaga JM, et al. 2002. Identification of a tachykinin NK(2) receptor splice variant and its expression in human and rat tissues. Life Sci 72: 269-277.

Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, et al. 1998. Primary afferent tachykinins are required to experience moderate to intense pain. Nature 392: 390-394.

Carter MS, Krause JE. 1990. Structure, expression, and some regulatory mechanisms of the rat preprotachykinin gene encoding substance P, neurokinin A, neuropeptide K, and neuropeptide gamma. J Neurosci 10: 2203-2214.

Caterina MJ, Julius D. 1999. Sense and specificity: a molecular identity for nociceptors. Curr Opin Neurobiol 9: 525-530.

Chang MM, Leeman SE, Niall HD. 1971. Amino-acid sequence of substance P. Nat New Biol 232: 86-87.

Chizh BA, Cumberbatch MJ, Herrero JF, Stirk GC, Headley PM. 1997. Stimulus intensity, cell excitation and the *N*-methyl-D-aspartate receptor component of sensory responses in the rat spinal cord in vivo. Neuroscience 80: 251-265.

Cirillo R, Astolfi M, Conte B, Lopez G, Parlani M, et al. 1998. Pharmacology of the peptidomimetic, MEN 11149, a new potent, selective and orally effective tachykinin NK1 receptor antagonist. Eur J Pharmacol 341: 201-209.

Coulson JM, Fiskerstrand CE, Woll PJ, Quinn JP. 1999. Arginine vasopressin promoter regulation is mediated by a neuron-restrictive silencer element in small cell lung cancer [In Process Citation]. Cancer Res 59: 5123-5127.

Croci T, Aureggi G, Manara L, Emonds-Alt X, Le Fur G, et al. 1998. In vitro characterization of tachykinin NK2-receptors modulating motor responses of human colonic muscle strips. Br J Pharmacol 124: 1321-1327.

Cuello AC, Kanazawa I. 1978. The distribution of substance P immunoreactive fibers in the rat central nervous system. J Comp Neurol 178: 129-156.

Culman J, Klee S, Ohlendorf C, Unger T. 1997. Effect of tachykinin receptor inhibition in the brain on cardiovascular and behavioral responses to stress. J Pharmacol Exp Ther 280: 238-246.

Culman J, Unger T. 1995. Central tachykinins: mediators of defence reaction and stress reactions. Can J Physiol Pharmacol 73: 885-891.

Dam TV, Quirion R. 1986. Pharmacological characterization and autoradiographic localization of substance P receptors in guinea pig brain. Peptides 7: 855-864.

De Felipe C, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, et al. 1998a. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature 392: 394-397.

De Felipe C, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, et al. 1998b. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P [see comments]. Nature 392: 394-397.

Dery O, Defea KA, Bunnett NW. 2001. Protein kinase C-mediated desensitization of the neurokinin 1 receptor. Am J Physiol Cell Physiol 280: C1097-C1106.

Donaldson LF, Harmar AJ, McQueen DS, Seckl JR. 1992. Increased expression of preprotachykinin, calcitonin gene-related peptide, but not vasoactive intestinal peptide messenger RNA in dorsal root ganglia during the development of adjuvant monoarthritis in the rat. Brain Res Mol Brain Res 16: 143-149.

Donaldson LF, Haskell CA, Hanley MR. 1996. Functional characterization by heterologous expression of a novel cloned tachykinin peptide receptor. Biochem J 320 (Pt. 1): 1-5.

Doyle CA, Hunt SP. 1999. Substance P receptor (neurokinin-1)-expressing neurons in lamina I of the spinal cord encode for the intensity of noxious stimulation: a *c-fos* study in rat. Neuroscience 89: 17-28.

Duggan AW, Hendry IA, Morton CR, Hutchison WD, Zhao ZQ. 1988. Cutaneous stimuli releasing immunoreactive substance P in the dorsal horn of the cat. Brain Res 451: 261-273.

Duggan AW, Morton CR, Zhao ZQ, Hendry IA. 1987. Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. Brain Res 403: 345-349.

Duggan AW, Schaible HG, Hope PJ, Lang CW. 1992. Effect of peptidase inhibition on the pattern of intraspinally released immunoreactive substance P detected with antibody microprobes. Brain Res 579: 261-269.

Ehrenpreis T, Pernow B. 1953. On the occurrence of substance P in the rectosigmoid in Hirschsprung's disease. Acta Physiol Scand 27: 380-388.

Elliott RC, Khademi S, Pleasure SJ, Parent JM, Lowenstein DH. 2001. Differential regulation of basic helix–loop–helix mRNAs in the dentate gyrus following status epilepticus. Neuroscience 106: 79-88.

Fetissov SO, Jacoby AS, Brumovsky PR, Shine J, Iismaa TP, et al. 2003. Altered hippocampal expression of neuropeptides in seizure-prone GALR1 knockout mice. Epilepsia 44: 1022-1033.

Fields HL. 2000. Pain modulation: expectation, opioid analgesia and virtual pain. Prog Brain Res 122: 245-253.

Fiskerstrand C, Quinn JP. 1996. The molecular biology of preprotachykinin-A gene expression. Neuropeptides 30: 602-610.

Fitzgerald M, Gibson S. 1984. The postnatal physiological and neurochemical development of peripheral sensory C fibres. Neuroscience 13: 933-944.

Gaddum JH, Schild H. 1934. J Physiol 83: 1-14.

Garcia M, Sakamoto K, Shigekawa M, Nakanishi S, Ito S. 1994. Multiple mechanisms of arachidonic acid release in Chinese hamster ovary cells transfected with cDNA of substance P receptor. Biochem Pharmacol 48: 1735-1741.

Gauriau C, Bernard JF. 2002. Pain pathways and parabrachial circuits in the rat. Exp Physiol 87: 251-258.

Gerard NP, Bao L, Xiao-Ping H, Eddy RL Jr, Shows TB, et al. 1993. Human chemotaxis receptor genes cluster at 19q13.3–13.4. Characterization of the human C5a receptor gene. Biochemistry 32: 1243-1250.

Germonpre PR, Bullock GR, Lambrecht BN, Van De Velde V, Luyten WH, et al. 1999. Presence of substance P and neurokinin 1 receptors in human sputum macrophages and U-937 cells. Eur Respir J 14: 776-782.

Gilbert RF, Emson PC, Hunt SP, Bennett GW, Marsden CA, et al. 1982. The effects of monoamine neurotoxins on peptides in the rat spinal cord. Neuroscience 7: 69-87.

Ginty DD, Bonni A, Greenberg ME. 1994. Nerve growth factor activates a ras-dependent protein kinase that stimulates *c-fos* transcription via phosphorylation of CREB. Cell 77: 713-725.

Gizang-Ginsberg E, Ziff EB. 1990. Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains *c-fos*. Genes and Development 4: 477-491.

Graybiel AM, Aosaki T, Flaherty AW, Kimura M. 1994. The basal ganglia and adaptive motor control. Science 265: 1826-1831.

Gresch PJ, Walker PD. 1999a. Acute p-chloroamphetamine increases striatal preprotachykinin mRNA: role of the serotonin 2A/2C receptor. Brain Res Mol Brain Res 67: 190-193.

Gresch PJ, Walker PD. 1999b. Serotonin-2 receptor stimulation normalizes striatal preprotachykinin messenger RNA in an animal model of Parkinson's disease. Neuroscience 93: 831-841.

Gross C, Santarelli L, Brunner D, Zhuang X, Hen R. 2000. Altered fear circuits in 5-HT(1A) receptor KO mice. Biol Psychiatry 48: 1157-1163.

Guirimand F, Le Bars D. 1996. [Physiology of nociception]. Ann Fr Anesth Reanim 15: 1048-1079.

Harada Y, Takahashi T, Kuno M, Nakayama K, Masu Y, et al. 1987. Expression of two different tachykinin receptors in Xenopus oocytes by exogenous mRNAs. J Neurosci 7: 3265-3273.

Harlan RE, Garcia MM, Krause JE. 1989. Cellular localization of substance P- and neurokinin A-encoding preprotachykinin mRNA in the female rat brain. J Comp Neurol 287: 179-212.

Harmar AJ, Mulderry PK, Al-Shawi R, Lyons V, Sheward WJ, et al. 1993. 3.3 kb of 5′ flanking DNA from the rat preprotachykinin gene directs high level expression of a reporter gene in microinjected dorsal root ganglion neurons but not in transgenic mice. Regul Pept 46: 67-69.

Harrison PT, Dalziel RG, Ditchfield NA, Quinn JP. 1999. Neuronal-specific and nerve growth factor-inducible expression directed by the preprotachykinin-A promoter delivered by an adeno-associated virus vector. Neuroscience 94: 997-1003.

Harrison S, Geppetti P. 2001. Substance P. Int J Biochem Cell Biol 33: 555-576.

Hasenohrl RU, Oitzl MS, Huston JP. 1989. Conditioned place preference in the corral: a procedure for measuring reinforcing properties of drugs. J Neurosci Methods 30: 141-146.

Hawley RJ, Scheibe RJ, Wagner JA. 1992. NGF induces the expression of the VGF gene through a cAMP response element. J Neurosci 12: 2573-2581.

Hey JA, Danko G, del Prado M, Chapman RW. 1996. Augmentation of neurally evoked cholinergic bronchoconstrictor responses by prejunctional NK2 receptors in the guinea-pig. J Auton Pharmacol 16: 41-48.

Ho WZ, Lai JP, Zhu XH, Uvaydova M, Douglas SD. 1997. Human monocytes and macrophages express substance P and neurokinin-1 receptor. J Immunol 159: 5654-5660.

Hokfelt T, Kellerth JO, Nilsson G, Pernow B. 1975a. Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. Brain Res 100: 235-252.

Hokfelt T, Kellerth JO, Nilsson G, Pernow B. 1975b. Substance P: localization in the central nervous system and in some primary sensory neurons. Science 190: 889-890.

Hokfelt T, Ljungdahl A, Steinbusch H, Verhofstad A, Nilsson G, et al. 1978. Immunohistochemical evidence of substance P-like immunoreactivity in some 5-hydroxytryptamine-containing neurons in the rat central nervous system. Neuroscience 3: 517-538.

Honor P, Menning PM, Rogers SD, Nichols ML, Basbaum AI, et al. 1999. Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states. J Neurosci 19: 7670-7678.

Hoshino H, Lotvall J, Skoogh BE, Linden A. 1999. Neutrophil recruitment by interleukin-17 into rat airways in vivo. Role of tachykinins. Am J Respir Crit Care Med 159: 1423-1428.

Hurd YL, Keller E, Sotonyi P, Sedvall G. 1999. Preprotachykinin-A mRNA expression in the human and monkey brain: an in situ hybridization study. J Comp Neurol 411: 56-72.

Hylden JL, Wilcox GL. 1983. Antinociceptive action of intrathecal neurotensin in mice. Peptides 4: 517-520.

Iwamoto I, Nakagawa N, Yamazaki H, Kimura A, Tomioka H, et al. 1993. Mechanism for substance P-induced activation of human neutrophils and eosinophils. Regul Pept 46: 228-230.

Iwasaki Y, Kinoshita M, Ikeda K, Takamiya K, Shiojima T. 1989. Trophic effect of various neuropeptides on the cultured ventral spinal cord of rat embryo. Neurosci Lett 101: 316-320.

Kage R, McGregor GP, Thim L, Conlon JM. 1988. Neuropeptide-gamma: a peptide isolated from rabbit intestine that is derived from gamma-preprotachykinin. J Neurochem 50: 1412-1417.

Kanazawa I, Ogawa T, Kimura S, Munekata E. 1984. Regional distribution of substance P, neurokinin alpha and neurokinin beta in rat central nervous system. Neurosci Res 2: 111-120.

Kangawa K, Minamino N, Fukuda A, Matsuo H. 1983. Neuromedin K: a novel mammalian tachykinin identified in porcine spinal cord. Biochem Biophys Res Commun 114: 533-540.

Karchewski LA, Kim FA, Johnston J, McKnight RM, Verge VM. 1999. Anatomical evidence supporting the potential for modulation by multiple neurotrophins in the majority of adult lumbar sensory neurons. J Comp Neurol 413: 327-341.

Kashiba H, Noguchi K, Ueda Y, Senba E. 1995. Coexpression of trk family members and low-affinity neurotrophin receptors in rat dorsal root ganglion neurons. Brain Res Mol Brain Res 30: 158-164.

Katayama I, Nishioka K. 1997. Substance P augments fibrogenic cytokine-induced fibroblast proliferation: possible involvement of neuropeptide in tissue fibrosis. J Dermatol Sci 15: 201-206.

Khawaja AM, Rogers DF. 1996. Tachykinins: receptor to effector. Int J Biochem Cell Biol 28: 721-738.

Kimura S, Goto K, Ogawa T, Sugita Y, Kanazawa I. 1984. Pharmacological characterization of novel mammalian tachykinins, neurokinin alpha and neurokinin beta. Neurosci Res 2: 97-104.

Klein CM, Coggeshall RE, Carlton SM, Sorkin LS. 1992. The effects of A- and C-fiber stimulation on patterns of neuropeptide immunostaining in the rat superficial dorsal horn. Brain Res 580: 121-128.

Kotani H, Hoshimaru M, Nawa H, Nakanishi S. 1986. Structure and gene organization of bovine neuromedin K precursor. Proc Natl Acad Sci USA 83: 7074-7078.

Kramer MS, Cutler N, Feighner J, Shrivastava R, Carman J, et al. 1998. Distinct mechanism for antidepressant activity by blockade of central substance P receptors. Science 281: 1640-1645.

Krause JE, Chirgwin JM, Carter MS, Xu ZS, Hershey AD. 1987. Three rat preprotachykinin mRNAs encode the neuropeptides substance P and neurokinin A. Proc Natl Acad Sci USA 84: 881-885.

Krause JE, Staveteig PT, Mentzer JN, Schmidt SK, Tucker JB, et al. 1997. Functional expression of a novel human neurokinin-3 receptor homolog that binds [3H]senktide and [125I-MePhe7]neurokinin B, and is responsive to tachykinin peptide agonists. Proc Natl Acad Sci USA 94: 310-315.

Krause JE, Takeda Y, Hershey AD. 1992. Structure, functions, and mechanisms of substance P receptor action. J Invest Dermatol 98: 2S-7S.

Kurtz MM, Wang R, Clements MK, Cascieri MA, Austin CP, et al. 2002. Identification, localization and receptor characterization of novel mammalian substance P-like peptides. Gene 296: 205-212.

Lacour M, Arrighi JF, Muller KM, Carlberg C, Saurat JH, et al. 1994. cAMP up-regulates IL-4 and IL-5 production from activated CD4+ T cells while decreasing IL-2 release and NF-AT induction. Int Immunol 6: 1333-1343.

Lagente V, Advenier C. 1998. Tachykinins and airway function. Pulm Pharmacol Ther 11: 331-340.

Lai JP, Douglas SD, Rappaport E, Wu JM, Ho WZ. 1998. Identification of a delta isoform of preprotachykinin mRNA in human mononuclear phagocytes and lymphocytes. J Neuroimmunol 91: 121-128.

Lai YL, Chiou WY, Lu FJ, Chiang LY. 1999. Roles of oxygen radicals and elastase in citric acid-induced airway constriction of guinea-pigs. Br J Pharmacol 126: 778-784.

Lai YL, Lee SP. 1999. Mediators in hyperpnea-induced bronchoconstriction of guinea pigs. Naunyn Schmiedebergs Arch Pharmacol 360: 597-602.

Laird JM, Olivar T, Roza C, De Felipe C, Hunt SP, et al. 2000. Deficits in visceral pain and hyperalgesia of mice with a disruption of the tachykinin NK1 receptor gene. Neuroscience 98: 345-352.

Lambrecht BN, Germonpre PR, Everaert EG, Carro-Muino I, De Veerman M, et al. 1999. Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. Eur J Immunol 29: 3815-3825.

Laniyonu A, Sliwinski-Lis E, Fleming N. 1988. Different tachykinin receptor subtypes are coupled to the phosphoinositide or cyclic AMP signal transduction pathways in rat submandibular cells. FEBS Lett 240: 186-190.

Lavielle S, Chassaing G, Ploux O, Loeuillet D, Besseyre J, et al. 1988. Analysis of tachykinin binding site interactions using constrained analogues of tachykinins. Biochem Pharmacol 37: 41-49.

Lawson SN, Crepps BA, Perl ER. 1997. Relationship of substance P to afferent characteristics of dorsal root ganglion neurones in guinea-pig. J Physiol 505 (Pt. 1): 177-191.

Lawson SN, Perry MJ, Prabhakar E, McCarthy PW. 1993. Primary sensory neurones: neurofilament, neuropeptides, and conduction velocity. Brain Res Bull 30: 239-243.

Lecat S, Bucher B, Mely Y, Galzi JL. 2002. Mutations in the extracellular amino-terminal domain of the NK2 neurokinin receptor abolish cAMP signaling but preserve intracellular calcium responses. J Biol Chem 277: 42034-42048.

Lecci A, Giuliani S, Tramontana M, Carini F, Maggi CA. 2000. Peripheral actions of tachykinins. Neuropeptides 34: 303-313.

Lee CM, Campbell NJ, Williams BJ, Iversen LL. 1986. Multiple tachykinin binding sites in peripheral tissues and in brain. Eur J Pharmacol 130: 209-217.

Lembeck F. 1953. [Central transmission of afferent impulses. III. Incidence and significance of the substance P in the dorsal roots of the spinal cord.] Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 219: 197-213.

Lembeck F, Starke K. 1968. [Substance P and salivary secretion.] Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 259: 375-385.

Leslie TA, Emson PC, Dowd PM, Woolf CJ. 1995. Nerve growth factor contributes to the up-regulation of growth-associated protein 43 and preprotachykinin A messenger RNAs in primary sensory neurons following peripheral inflammation. Neuroscience 67: 753-761.

Lindh C, Liu Z, Lyrenas S, Ordeberg G, Nyberg F. 1997. Elevated cerebrospinal fluid substance P-like immunoreactivity in patients with painful osteoarthritis, but not in patients with rhizopatic pain from a herniated lumbar disc. Scand J Rheumatol 26: 468-472.

Littlewood NK, Todd AJ, Spike RC, Watt C, Shehab SA. 1995. The types of neuron in spinal dorsal horn which possess neurokinin-1 receptors. Neuroscience 66: 597-608.

Liu H, Cao Y, Basbaum AI, Mazarati AM, Sankar R, et al. 1999a. Resistance to excitotoxin-induced seizures and neuronal death in mice lacking the preprotachykinin A gene [In Process Citation]. Proc Natl Acad Sci USA 96: 12096-12101.

Liu H, Mantyh PW, Basbaum AI. 1997. NMDA-receptor regulation of substance P release from primary afferent nociceptors. Nature 386: 721-724.

Liu H, Mazarati AM, Katsumori H, Sankar R, Wasterlain CG. 1999b. Substance P is expressed in hippocampal principal neurons during status epilepticus and plays a critical role in the maintenance of status epilepticus. Proc Natl Acad Sci USA 96: 5286-5291.

Liu H, Sankar R, Shin DH, Mazarati AM, Wasterlain CG. 2000. Patterns of status epilepticus-induced substance P expression during development. Neuroscience 101: 297-304.

Liu YB, Lio PA, Pasternak JF, Trommer BL. 1996. Developmental changes in membrane properties and postsynaptic currents of granule cells in rat dentate gyrus. J Neurophysiol 76: 1074-1088.

Lotz M, Vaughan JH, Carson DA. 1988. Effect of neuropeptides on production of inflammatory cytokines by human monocytes. Science 241: 1218-1221.

Lovick TA. 1996. Midbrain and medullary regulation of defensive cardiovascular functions. Prog Brain Res 107: 301-313.

Lumb BM, Lovick TA. 1993. The rostral hypothalamus: an area for the integration of autonomic and sensory responsiveness. J Neurophysiol 70: 1570-1577.

Ma W, Bisby MA. 1998. Increase of preprotachykinin mRNA and substance P immunoreactivity in spared dorsal root ganglion neurons following partial sciatic nerve injury. Eur J Neurosci 10: 2388-2399.

MacKenzie A, Payne C, Boyle S, Clarke AR, Quinn JP. 2000. The human preprotachykinin-A gene promoter has been highly conserved and can drive human-like marker gene expression in the adult mouse CNS. Mol Cell Neurosci 16: 620-630.

MacKenzie A, Quinn J. 2002. A yeast artificial chromosome containing the human preprotachykinin-A gene expresses substance P in mice and drives appropriate marker-gene expression during early brain embryogenesis. Mol Cell Neurosci 19: 72-87.

Maggi CA. 1995. The mammalian tachykinin receptors. Gen Pharmacol 26: 911-944.

Maggi CA. 1997. The effects of tachykinins on inflammatory and immune cells. Regul Pept 70: 75-90.

Maggi CA. 2000. Principles of tachykininergic co-transmission in the peripheral and enteric nervous system. Regul Pept 93: 53-64.

Maggi CA, Schwartz TW. 1997. The dual nature of the tachykinin NK1 receptor. Trends Pharmacol Sci 18: 351-355.

Mai JK, Stephens PH, Hopf A, Cuello AC. 1986. Substance P in the human brain. Neuroscience 17: 709-739.

Maley BE. 1996. Immunohistochemical localization of neuropeptides and neurotransmitters in the nucleus solitarius. Chem Senses 21: 367-376.

Mantyh PW, Allen CJ, Ghilardi JR, Rogers SD, Mantyh CR, et al. 1995. Rapid endocytosis of a G protein-coupled receptor: substance P evoked internalization of its receptor in the rat striatum in vivo. Proc Natl Acad Sci USA 92: 2622-2626.

Mantyh PW, Gates T, Mantyh CR, Maggio JE. 1989. Autoradiographic localization and characterization of tachykinin receptor binding sites in the rat brain and peripheral tissues. J Neurosci 9: 258-279.

Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, et al. 1997. Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. Science 278: 275-279.

Marshall GE, Shehab SA, Spike RC, Todd AJ. 1996. Neurokinin-1 receptors on lumbar spinothalamic neurons in the rat. Neuroscience 72: 255-263.

Marvizon JC, Grady EF, Stefani E, Bunnett NW, Mayer EA. 1999. Substance P release in the dorsal horn assessed by receptor internalization: NMDA receptors counteract a tonic inhibition by GABA(B) receptors. Eur J Neurosci 11: 417-426.

Matsumura H, Sakurada T, Hara A, Sakurada S, Kisara K. 1985. Characterization of the hyperalgesic effect induced by intrathecal injection of substance P. Neuropharmacology 24: 421-426.

Maubach KA, Cody C, Jones RS. 1998. Tachykinins may modify spontaneous epileptiform activity in the rat entorhinal cortex in vitro by activating GABAergic inhibition. Neuroscience 83: 1047-1062.

Maubach KA, Rupniak NM, Kramer MS, Hill RG. 1999. Novel strategies for pharmacotherapy of depression. Curr Opin Chem Biol 3: 481-488.

McCarson KE. 1999. Central and peripheral expression of neurokinin-1 and neurokinin-3 receptor and substance P-encoding messenger RNAs: peripheral regulation during formalin-induced inflammation and lack of neurokinin receptor expression in primary afferent sensory neurons. Neuroscience 93: 361-370.

McCarthy PW, Lawson SN. 1989. Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. Neuroscience 28: 745-753.

McConalogue K, Corvera CU, Gamp PD, Grady EF, Bunnett NW. 1998. Desensitization of the neurokinin-1 receptor (NK1-R) in neurons: effects of substance P on the distribution of NK1-R, Galphaq/11, G-protein receptor kinase-2/3, and beta-arrestin-1/2. Mol Biol Cell 9: 2305-2324.

McConalogue K, Dery O, Lovett M, Wong H, Walsh JH, et al. 1999. Substance P-induced trafficking of beta-arrestins. The role of beta-arrestins in endocytosis of the neurokinin-1 receptor. J Biol Chem 274: 16257-16268.

McGregor GP, Hartel R, Haberberger R, Kummer W, Voigt K. 1995. Preprotachykinin-A gene expression occurs transiently in the developing rat endocrine pancreas and can be regulated in RINm5F cells. Endocrinology 136: 2538-2546.

McMahon SB, Armanini MP, Ling LH, Phillips HS. 1994. Expression and coexpression of Trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. Neuron 12: 1161-1171.

Mendelson SC, Fiskerstrand CE, Quinn JP. 1998. A role for the octamer-binding protein in preprotachykinin-A gene expression. Neuropeptides 32: 79-85.

Mendelson SC, Morrison CF, McAllister J, Paterson JM, Dobson SP, et al. 1995. Repression of preprotachykinin-A promoter activity is mediated by a proximal promoter element. Neuroscience 65: 837-847.

Mendelson SC, Quinn JP. 1995. Characterisation of potential regulatory elements within the rat preprotachykinin-A promoter. Neurosci Lett 184: 125-128.

Millward-Sadler SJ, Mackenzie A, Wright MO, Lee HS, Elliot K, et al. 2003. Tachykinin expression in cartilage and function in human articular chondrocyte mechanotransduction. Arthritis Rheum 48: 146-156.

Millward-Sadler SJ, Wright MO, Davies LW, Nuki G, Salter DM. 2000. Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. Arthritis Rheum 43: 2091-2099.

Molliver DC, Radeke MJ, Feinstein SC, Snider WD. 1995. Presence or absence of TrkA protein distinguishes subsets of small sensory neurons with unique cytochemical characteristics and dorsal horn projections. J Comp Neurol 361: 404-416.

Mori T, Ogata T, Okumura H, Shibata T, Nakamura Y, et al. 1999. Substance P regulates the function of rabbit cultured osteoclast; increase of intracellular free calcium concentration and enhancement of bone resorption. Biochem Biophys Res Commun 262: 418-422.

Morrison CF, McAllister J, Dobson SP, Mulderry PK, Quinn JP. 1994. An activator element within the preprotachykinin-A promoter. Mol Cell Neurosci 5: 165-175.

Morteau O, Lu B, Gerard C, Gerard NP. 2001. Hemokinin 1 is a full agonist at the substance P receptor. Nat Immunol 2: 1088.

Mu X, Silos-Santiago I, Carroll SL, Snider WD. 1993. Neurotrophin receptor genes are expressed in distinct patterns in developing dorsal root ganglia. J Neurosci 13: 4029-4041.

Murtra P, Sheasby AM, Hunt SP, De Felipe C. 2000. Rewarding effects of opiates are absent in mice lacking the receptor for substance P. Nature 405: 180-183.

Mussap CJ, Geraghty DP, Burcher E. 1993. Tachykinin receptors: a radioligand binding perspective. J Neurochem 60: 1987-2009.

Naline E, Devillier P, Drapeau G, Toty L, Bakdach H, et al. 1989. Characterization of neurokinin effects and receptor selectivity in human isolated bronchi. Am Rev Respir Dis 140: 679-686.

Nawa H, Doteuchi M, Igano K, Inouye K, Nakanishi S. 1984. Substance K: a novel mammalian tachykinin that differs from substance P in its pharmacological profile. Life Sci 34: 1153-1160.

Newton RC, Vaddi K. 1997. Biological responses to CC chemokines. Methods Enzymol 287: 174-186.

Nilsson G, Larsson LI, Hakanson R, Brodin E, Pernow B, et al. 1975. Localization of substance P-like immunoreactivity in mouse gut. Histochemistry 43: 97-99.

Nilsson J, von Euler AM, Dalsgaard CJ. 1985. Stimulation of connective tissue cell growth by substance P and substance K. Nature 315: 61-63.

Noguchi K, Morita Y, Kiyama H, Ono K, Tohyama M. 1988. A noxious stimulus induces the preprotachykinin-A gene expression in the rat dorsal root ganglion: a quantitative study using in situ hybridization histochemistry. Brain Res 464: 31-35.

Nowak LM, Macdonald RL. 1982. Substance P: ionic basis for depolarizing responses of mouse spinal cord neurons in cell culture. J Neurosci 2: 1119-1128.

Otsuka M, Yoshioka K. 1993. Neurotransmitter functions of mammalian tachykinins. Physiol Rev 73: 229-308.

Page NM. 2004. Hemokinins and endokinins. Cell Mol Life Sci 61: 1652-1663.

Page NM, Bell NJ, Gardiner SM, Manyonda IT, Brayley KJ, et al. 2003. Characterization of the endokinins: human tachykinins with cardiovascular activity. Proc Natl Acad Sci USA 100: 6245-6250.

Page NM, Woods RJ, Gardiner SM, Lomthaisong K, Gladwell RT, et al. 2000. Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. Nature 405: 797-800.

Page NM, Woods RJ, Lowry PJ. 2001. A regulatory role for neurokinin B in placental physiology and pre-eclampsia. Regul Pept 98: 97-104.

Palanche T, Ilien B, Zoffmann S, Reck MP, Bucher B, et al. 2001. The neurokinin A receptor activates calcium and cAMP responses through distinct conformational states. J Biol Chem 276: 34853-34861.

Palm K, Belluardo N, Metsis M, Timmusk T. 1998. Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. J Neurosci 18: 1280-1296.

Palma C, Manzini S. 1998. Substance P induces secretion of immunomodulatory cytokines by human astrocytoma cells. J Neuroimmunol 81: 127-137.

Patacchini R, Bartho L, De Giorgio R, Lenard L Jr, Stanghellini V, et al. 1999. Involvement of endogenous tachykinins and CGRP in the motor responses produced by capsaicin in the guinea-pig common bile duct. Naunyn Schmiedebergs Arch Pharmacol 360: 344-353.

Patacchini R, De Giorgio R, Bartho L, Barbara G, Corinaldesi R, et al. 1998. Evidence that tachykinins are the main NANC excitatory neurotransmitters in the guinea-pig common bile duct. Br J Pharmacol 124: 1703-1711.

Patacchini R, Giuliani S, Turini A, Navarra G, Maggi CA. 2000. Effect of nepadutant at tachykinin NK(2) receptors in human intestine and urinary bladder. Eur J Pharmacol 398: 389-397.

Patacchini R, Lecci A, Holzer P, Maggi CA. 2004. Newly discovered tachykinins raise new questions about their peripheral roles and the tachykinin nomenclature. Trends Pharmacol Sci 25: 1-3.

Patacchini R, Maggi CA. 2001. Peripheral tachykinin receptors as targets for new drugs. Eur J Pharmacol 429: 13-21.

Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, et al. 2003. Tachykinins and tachykinin receptors in human uterus. Br J Pharmacol 139: 523-532.

Paterson JM, Mendelson SC, McAllister J, Morrison CF, Dobson S, et al. 1995a. Three immediate early gene response elements in the proximal preprotachykinin-A promoter in two functionally distinct domains. Neuroscience 66: 921-932.

Paterson JM, Morrison CF, Dobson SP, McAllister J, Quinn JP. 1995b. Characterisation of a functional E box motif in the proximal rat preprotachykinin-A promoter. Neurosci Lett 191: 185-188.

Paterson JM, Morrison CF, Mendelson SC, McAllister J, Quinn JP. 1995c. An upstream stimulatory factor (USF) binding motif is critical for rat preprotachykinin-A promoter activity in PC12 cells. Biochem J 310 (Pt. 2): 401-406.

Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, et al. 2004. Tachykinins and tachykinin receptors: a growing family. Life Sci 74: 1445-1463.

Pernow B. 1953. Distribution of substance P in the central and peripheral nervous system. Nature 171: 746.

Pickel VM, Miller R, Chan J, Sumal KK. 1983. Substance P and enkephalin in transected axons of medulla and spinal cord. Regul Pept 6: 121-135.

Piercey MF, Schroeder LA, Folkers K, Xu JC, Horig J. 1981. Sensory and motor functions of spinal cord substance P. Science 214: 1361-1363.

Pillot C, Heron A, Schwartz JC, Arrang JM. 2003. Ciproxifan, a histamine H3-receptor antagonist/inverse agonist, modulates the effects of methamphetamine on neuropeptide mRNA expression in rat striatum. Eur J Neurosci 17: 307-314.

Pinto FM, Almeida TA, Hernandez M, Devillier P, Advenier C, et al. 2004. mRNA expression of tachykinins and tachykinin receptors in different human tissues. Eur J Pharmacol 494: 233-239.

Pinto FM, Cintado CG, Devillier P, Candenas ML. 2001. Expression of preprotachykinin-B, the gene that encodes neurokinin B, in the rat uterus. Eur J Pharmacol 425: R1-R2.

Pioro EP, Cuello AC. 1990. Distribution of nerve growth factor receptor-like immunoreactivity in the adult rat central nervous system. Effect of colchicine and correlation with the cholinergic system. II. Brainstem, cerebellum and spinal cord. Neuroscience 34: 89-110.

Polak JM, Bloom SR. 1981. The peripheral substance P-ergic system. Peptides 2 Suppl. 2: 133-148.

Quartara L, Maggi CA. 1997. The tachykinin NK1 receptor. Part I: ligands and mechanisms of cellular activation. Neuropeptides 31: 537-563.

Quartara L, Maggi CA. 1998. The tachykinin NK1 receptor. Part II: distribution and pathophysiological roles. Neuropeptides 32: 1-49.

Quinn JP, Bubb VJ, Marshall-Jones ZV, Coulson JM. 2002. Neuron restrictive silencer factor as a modulator of neuropeptide gene expression. Regul Pept 108: 135-141.

Quinn JP, Mendelson SC, Paterson JM, McAllister J, Morrison CF. 1995. Transcriptional control of neuropeptide gene expression in sensory neurons, using the preprotachykinin-A gene as a model. Can J Physiol Pharmacol 73: 957-962.

Quinn JP, Takimoto M, Iadarola M, Holbrook N, Levens D. 1989. Distinct factors bind the AP1 consensus sites in Gibbon Ape leukemia virus and simian virus 40 enhancers. J Virol 633: 1737-1742.

Radhakrishnan V, Henry JL. 1991. Novel substance P antagonist, CP-96,345, blocks responses of cat spinal dorsal horn neurons to noxious cutaneous stimulation and to substance P. Neurosci Lett 132: 39-43.

Radhakrishnan V, Yashpal K, Hui-Chan CW, Henry JL. 1995. Implication of a nitric oxide synthase mechanism in the action of substance P: L-NAME blocks thermal hyperalgesia induced by endogenous and exogenous substance P in the rat. Eur J Neurosci 7: 1920-1925.

Ranga K, Krishnan R. 2002. Clinical experience with substance P receptor (NK1) antagonists in depression. J Clin Psychiatry 63 Suppl. 11: 25-29.

Regoli D, Boudon A, Fauchere JL. 1994. Receptors and antagonists for substance P and related peptides. Pharmacol Rev 46: 551-599.

Ribeiro-Da-Silva A, Hokfelt T. 2000. Neuroanatomical localisation of substance P in the CNS and sensory neurons. Neuropeptides 34: 256-271.

Ribeiro-Da-Silva A, Ceullo AC, Henry JL. 2000. Neuropeptides 34(5): 281-291.

Richfield EK, Vonsattel JP, MacDonald ME, Sun Z, Deng YP, et al. 2002. Selective loss of striatal preprotachykinin neurons in a phenocopy of Huntington's disease. Mov Disord 17: 327-332.

Robbins TW, Everitt BJ. 1999. Drug addiction: bad habits add up. Nature 398: 567-570.

Ruda MA, Bennett GJ, Dubner R. 1986. Neurochemistry and neural circuitry in the dorsal horn. Prog Brain Res 66: 219-268.

Rupniak NM, Carlson EC, Harrison T, Oates B, Seward E, et al. 2000. Pharmacological blockade or genetic deletion of substance P (NK(1)) receptors attenuates neonatal vocalisation in guinea-pigs and mice. Neuropharmacology 39: 1413-1421.

Rupniak NM, Kramer MS. 1999. Discovery of the antidepressant and anti-emetic efficacy of substance P receptor (NK1) antagonists. Trends Pharmacol Sci 20: 485-490.

Santarelli L, Gobbi G, Blier P, Hen R. 2002. Behavioral and physiologic effects of genetic or pharmacologic inactivation of the substance P receptor (NK1). J Clin Psychiatry 63 Suppl. 11: 11-17.

Savoie C, Tousignant C, Rodger IW, Chan CC. 1995. Involvement of NK1 and NK2 receptors in pulmonary responses elicited by non-adrenergic, non-cholinergic vagal stimulation in guinea-pigs. J Pharm Pharmacol 47: 914-920.

Schuiling M, Zuidhof AB, Meurs H, Zaagsma J. 1999. Role of tachykinin NK2-receptor activation in the allergen-induced late asthmatic reaction, airway hyperreactivity and airway inflammatory cell influx in conscious, unrestrained guinea-pigs. Br J Pharmacol 127: 1030-1038.

Shih C, Bernard GW. 1997. Neurogenic substance P stimulates osteogenesis in vitro. Peptides 18: 323-326.

Shults CW, Quirion R, Chronwall B, Chase TN, O'Donohue TL. 1984. A comparison of the anatomical distribution of substance P and substance P receptors in the rat central nervous system. Peptides 5: 1097-1128.

Singh D, Joshi DD, Hameed M, Qian J, Gascon P, et al. 2000. Increased expression of preprotachykinin-I and neurokinin receptors in human breast cancer cells: implications for bone marrow metastasis. Proc Natl Acad Sci USA 97: 388-393.

Snider RM, Constantine JW, Lowe JA III, Longo KP, Lebel WS, et al. 1991. A potent nonpeptide antagonist of the substance P (NK1) receptor. Science 251: 435-437.

Takahama K, Fuchikami J, Isohama Y, Kai H, Miyata T. 1993. Neurokinin A, but not neurokinin B and substance P, induces codeine-resistant coughs in awake guinea-pigs. Regul Pept 46: 236-237.

Takano M, Takano Y, Yaksh TL. 1993. Release of calcitonin gene-related peptide (CGRP), substance P (SP), and vasoactive intestinal polypeptide (VIP) from rat spinal cord: modulation by alpha 2 agonists. Peptides 14: 371-378.

Tatemoto K, Lundberg JM, Jornvall H, Mutt V. 1985. Neuropeptide K: isolation, structure and biological activities of a novel brain tachykinin. Biochem Biophys Res Commun 128: 947-953.

Tattersall FD, Rycroft W, Cumberbatch M, Mason G, Tye S, et al. 2000. The novel NK1 receptor antagonist MK-0869 (L-754,030) and its water soluble phosphoryl prodrug, L-758, 298, inhibit acute and delayed cisplatin-induced emesis in ferrets. Neuropharmacology 39: 652-663.

Todd AJ, McGill MM, Shehab SA. 2000. Neurokinin 1 receptor expression by neurons in laminae I, III and IV of the rat spinal dorsal horn that project to the brainstem. Eur J Neurosci 12: 689-700.

Trafton JA, Abbadie C, Marchand S, Mantyh PW, Basbaum AI. 1999. Spinal opioid analgesia: how critical is the regulation of substance P signaling? J Neurosci 19: 9642-9653.

Tramontana M, Patacchini R, Giuliani S, Lippi A, Lecci A, et al. 1998. Characterization of the antibronchoconstrictor activity of MEN 11420, a tachykinin NK2 receptor antagonist, in guinea-pigs. Eur J Pharmacol 352: 279-288.

Tregear GW, Niall HD, Potts JT Jr, Leeman SE, Chang MM. 1971. Synthesis of substance P. Nat New Biol 232: 87-89.

Urban L, Randic M. 1984. Slow excitatory transmission in rat dorsal horn: possible mediation by peptides. Brain Res 290: 336-341.

Van Schoor J, Joos GF, Chasson BL, Brouard RJ, Pauwels RA. 1998. The effect of the NK2 tachykinin receptor antagonist SR 48968 (saredutant) on neurokinin A-induced bronchoconstriction in asthmatics. Eur Respir J 12: 17-23.

Von Banchet GS, Schaible HG. 1999. Localization of the neurokinin 1 receptor on a subset of substance P-positive and isolectin B4-negative dorsal root ganglion neurons of the rat. Neurosci Lett 274: 175-178.

von Euler US, Gaddum JH. 1931. An unidentified depressor substance in certain tissue extracts. J Physiol 72: 74-87.

Wade TV, Schneider JS. 2001. Expression of striatal preprotachykinin mRNA in symptomatic and asymptomatic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-exposed monkeys is related to parkinsonian motor signs. J Neurosci 21: 4901-4907.

Walker PD, Andrade R, Quinn JP, Bannon MJ. 2000. Real-time analysis of preprotachykinin promoter activity in single cortical neurons. J Neurochem 75: 882-885.

Warden MK, Young WS III. 1988. Distribution of cells containing mRNAs encoding substance P and neurokinin B in the rat central nervous system. J Comp Neurol 272: 90-113.

Wasterlain CG, Liu H, Mazarati AM, Baldwin RA, Shirasaka Y, et al. 2000. Self-sustaining status epilepticus: a condition maintained by potentiation of glutamate receptors and by plastic changes in substance P and other peptide neuromodulators. Epilepsia 41 Suppl. 6: S134-S143.

Wasterlain CG, Mazarati AM, Naylor D, Niquet J, Liu H, et al. 2002. Short-term plasticity of hippocampal neuropeptides and neuronal circuitry in experimental status epilepticus. Epilepsia 43 Suppl. 5: 20-29.

Whitty CJ, Walker PD, Goebel DJ, Poosch MS, Bannon MJ. 1995. Quantitation, cellular localization and regulation of neurokinin receptor gene expression within the rat substantia nigra. Neuroscience 64: 419-425.

Witonski D, Wagrowska-Danielewicz M. 1999. Distribution of substance-P nerve fibers in the knee joint in patients with anterior knee pain syndrome. A preliminary report. Knee Surg Sports Traumatol Arthrosc 7: 177-183.

Wood JN, Lillycrop KA, Dent CL, Ninkina NN, Beech MM, et al. 1992. Regulation of expression of the neuronal POU protein Oct-2 by nerve growth factor. J Biol Chem 267: 17787-17791.

Wright DE, Snider WD. 1995. Neurotrophin receptor mRNA expression defines distinct populations of neurons in rat dorsal root ganglia. J Comp Neurol 351: 329-338.

Xie R, Li L, Goshima Y, Strittmatter SM. 1995. An activated mutant of the alpha subunit of G(o) increases neurite outgrowth via protein kinase C. Brain Res Dev Brain Res 87: 77-86.

Yasumitsu R, Hirayama Y, Imai T, Miyayasu K, Hiroi J. 1996. Effects of specific tachykinin receptor antagonists on citric acid-induced cough and bronchoconstriction in unanesthetized guinea pigs. Eur J Pharmacol 300: 215-219.

Yoshihara S, Geppetti P, Hara M, Linden A, Ricciardolo FL, et al. 1996a. Cold air-induced bronchoconstriction is mediated by tachykinin and kinin release in guinea pigs. Eur J Pharmacol 296: 291-296.

Yoshihara S, Geppetti P, Linden A, Hara M, Chan B, et al. 1996b. Tachykinins mediate the potentiation of antigen-induced bronchoconstriction by cold air in guinea pigs. J Allergy Clin Immunol 97: 756-760.

Yu J, Cadet JL, Angulo JA. 2002. Neurokinin-1 (NK-1) receptor antagonists abrogate methamphetamine-induced striatal dopaminergic neurotoxicity in the murine brain. J Neurochem 83: 613-622.

Yuan L, Burcher E, Nail B. 1996. Tachykinin receptors and non-cholinergic bronchoconstriction in the anaesthetized guinea-pig. Clin Exp Pharmacol Physiol 23: 119-124.

Zhang Y, Lu L, Furlonger C, Wu GE, Paige CJ. 2000. Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. Nat Immunol 1: 392-397.

Zimmer A, Zimmer AM, Baffi J, Usdin T, Reynolds K, et al. 1998. Hypoalgesia in mice with a targeted deletion of the tachykinin 1 gene. Proc Natl Acad Sci USA 95: 2630-2635.

# 21 Secretin Superfamily: PACAP, VIP, and Related Neuropeptides

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**Abstract:** Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP) are important members of the secretin superfamily, specialized to function in the central and peripheral nervous systems. While the initial discovery of VIP as a vasoactive peptide revealed much if not all about its physiological role as a slow transmitter neuropeptide, PACAP's discovery as a putative hypophysiotropic hormone was initially misleading in terms of its far more ubiquitous and more physiologically important role as a neuropeptide cotransmitter similar to VIP. VIP and PACAP also function at "neuroimmunological synapses" upon release from nerve terminals, and perhaps also upon production in immunocytes. Thus, these two peptides have been implicated genetically, physiologically, and pharmacologically, in endocrine regulation, pain modulation, spatial memory, glucohomeostasis, arthritic inflammation, sepsis defense responses, respiratory control, circadian regulation, intermediary metabolism, and coordination of hormonal and cardiovascular control. Finally, along with their cognate receptors PAC1, VPAC1, and VPAC2, VIP and PACAP form an autoregulatory network that may function in cell differentiation, proliferation, and transformation.

**List of Abbreviations:** AC, adenylate cyclase; ACh, acetylcholine; ACTH, adrenocorticotropic hormone; BALT, bronchus-associated lymphoid tissue; CPE/H, carboxypeptidase E/H; FSH, follicle-stimulating hormone; GALT, gut-associated lymphoid tissue; GH, growth hormone; GIP, glucose-dependent insulinotropic polypeptide; GLUC, glucagon; GLP-1,-2, glucagon-like peptide-1,-2; GPCR, G-protein-coupled receptor; GRF, growth hormone-releasing factor; LDCVs, large dense core vesicles; LH, luteinizing hormone; LH-RH, luteinizing hormone-releasing hormone; MALT, mucosa-associated lymphoid tissue; PACAP, pituitary adenylate cyclase activating polypeptide; PAM, peptide glycine alpha-amidating monooxygenase; PC, prohormone convertase; PHM/PHI, peptide histidine methionine/peptide histidine isoleucine; PRL, prolactin; PRP, PACAP-related peptide; SCN, suprachiasmatic nucleus; SEC, secretin; SOM, somatostatin; SSVs, small synaptic vesicles; TSH, thyroid-stimulating hormone; TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal polypeptide; VSCC, voltage-sensitive calcium channel

## 1 Introduction

VIP and PACAP are two members of a neuropeptide superfamily that also includes secretin, growth hormone releasing factor glucagon, and glucagon-like peptides (Sherwood et al., 2000). Secretin was the first member of this family to be isolated and characterized and in fact holds the distinction of being the first hormone ever discovered, confirming Starling's hypothesis that factors released from neuroendocrine cells into the bloodstream might act at a distance (hence hormone) in organismic homeostasis (Henriksen and de Muckadell, 2000). The structure of secretin was not established until 1960. In the thirty years following, the other members of the family, their receptors, and functions were sequentially established. The discovery of PACAP in 1989 by Akira Arimura and coworkers, was a key event (Arimura, 1992) in completing a picture not only of the superfamily, but also of a special relationship, biochemical, anatomical, and functional, between PACAP and VIP (Waschek, 1995; Arimura, 1998; Vaudry et al., 2000).

This chapter focuses on VIP and PACAP and their receptors, and the special challenges of discerning neuropeptide function, when two ligands share overlapping specificity for three receptors, in the regulation of cell proliferation, differentiation, endocrine function, and synaptic signaling. The roles of PACAP and VIP highlight the features of neuropeptides as both neuroendocrine hormones and slow transmitters in the central and peripheral nervous systems, and the special role of neuropeptides and their G-protein-coupled receptors (GPCRs) in the metazoan genome, mediating paraphysiological homeostasis, and therefore adaptation to new environmental opportunities for speciation. The functional diversity brought about by alternatively spliced receptor variants and alternatively processed neuropeptide congeners contributes critically to the evolutionary and organismic flexibility of these signaling dyads, as illustrated for VIP and PACAP and their receptors.

## 2 The Secretin Superfamily: Paradigm for Neuropeptide Expression and Diversity; Processing, Secretion, and Receptor Signaling

### 2.1 Evolution and Divergence of the Secretin Superfamily

The secretin superfamily consists of nine functionally active peptides, encoded on six separate genes in mammals (*Figure 21-1*). PACAP, GRF, GIP, and SEC are singly encoded on four separate mammalian genes. VIP and PHM/PHI (C-terminal amino acid is methionine in humans; isoleucine in other mammalian species) are encoded on separate exons of the same gene. In addition, an extended form of PHM (PHV-42; HADGVFTSDFSKLLGQLSAKKYLESLMGKRVSSNISEDPVPV) has been identified in human serum and tissue (Yiangou et al., 1987a, b), and a putative PHI/PHV receptor characterized in the goldfish (Tse et al., 2002). Glucagon (GLUC) and glucagon-like peptide-1, and -2 (GLP-1 and GLP-2) are encoded on separate exons of a single gene (Sherwood et al., 2000). It is hypothesized that the entire family arose from multiple duplications of a single primordial PACAP/GRF gene. The modern mammalian PACAP gene also contains an exon encoding a peptide called PACAP-related peptide (PRP) processed like other family members, and with a homologous sequence, but PRP has no known biological activities or receptor (*Figure 21-1*). This peptide is found, mainly in fully processed form, at levels as high as that of PACAP-27 in PACAPergic hypothalamic neurons (Hannibal et al., 1995).

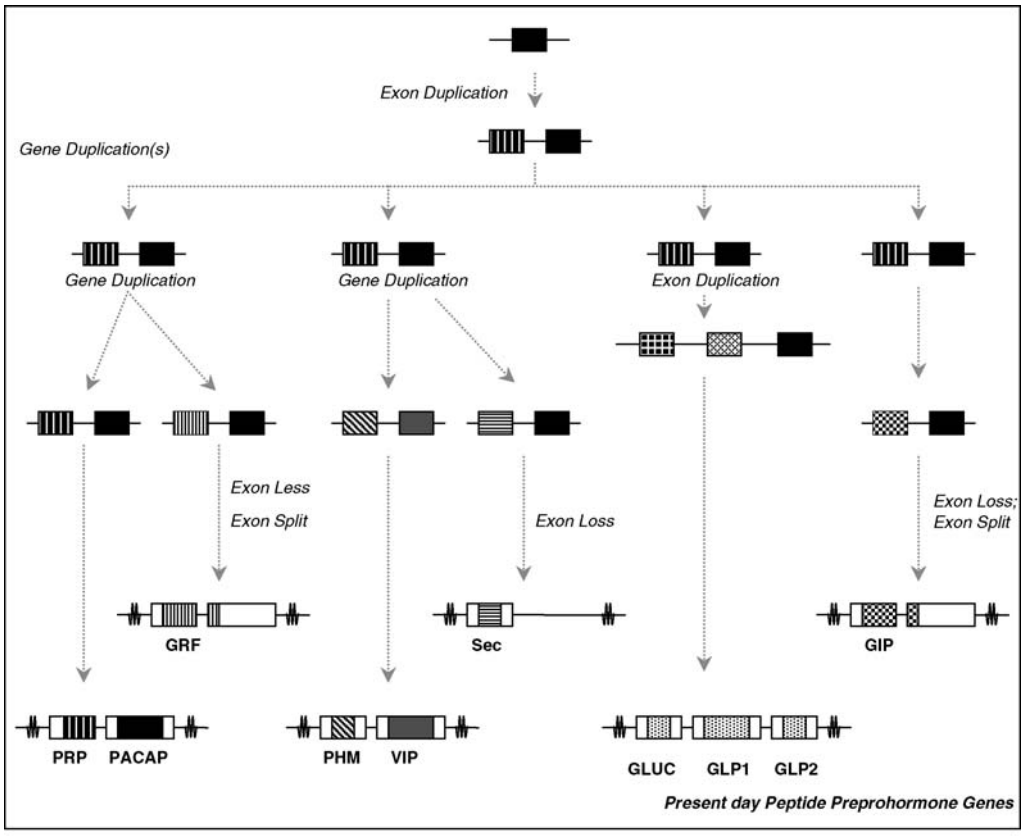
The nine secretin superfamily peptides vary in length from 27 to 44 amino acids. Receptor specificity and affinity of all nine peptides is found within the N-terminal 27 amino acids or so. This is illustrated clearly for PACAP, existing in two forms differing only in an 11-amino-acid C-terminal extension; there are no critical bioactivities or receptor subspecificities that distinguish PACAP-27 from PACAP-38, though there are some potency differences between them with PACAP-38 generally being more potent at all PACAP receptors than PACAP-27.

### 2.2 Prohormone Processing in the Secretin Superfamily

As indicated in *Figure 21-2* for PACAP and VIP processing, neuropeptides are synthesized from preprohormones. These in general have a structure that consists of an N-terminal signal sequence cleaved in the rough endoplasmic reticulum during translation, and the remaining prohormone, from which the biologically active peptide is freed by proteolytic cleavage (processing) at single and paired basic amino acid residues. Prohormone processing occurs through the sequential action of furin-like endoproteases of the prohormone convertase (PC) family, acting at C-terminal peptide bonds at basic residues. Processing to mature neuropeptides is completed by carboxypeptidase cleavage of single basic residues from the carboxy terminus of the nascent protein (Eiden, 1987; Seidah et al., 1999). In most cases neuropeptide synthesis concludes with the generation of a mature C-terminal amidated peptide via cleavage of a C-terminal glycine, with donation of the glycyl peptide bond amine to the residue which becomes amidated and forms the C-terminal residue of the active peptide under the action of the enzyme complex peptide glycine alpha-amidating monooxygenase (PAM) (Eipper et al., 1983).

The importance of secretory peptidomics—accounting for the precise complement of posttranslationally modified secretory products of a given cell type and their presence in the circulation at a given time—is underscored by the array of cell-specific processing products that exist for a given neuropeptide precursor, as well as their differential biological activities. This is most dramatically illustrated for chromogranin A, the most abundant secretory product of mammalian neuroendocrine cells, which exists in tissue and in the circulation in myriad bioactive and precursor forms (Feldman and Eiden, 2003). VIP is likewise secreted from pheochromocytomas, from which its cDNA was originally cloned, in both processed and unprocessed forms and at different secretory rates, which closely correlates with gastrointestinal symptomatology (Bloom et al., 1983, 1988). Differential processing of PACAP also occurs; PACAP exists in neuronal tissue in both a 27-and 38-amino-acid processed form, with PACAP-38 in about a tenfold greater abundance in most but not all tissues (Arimura et al., 1991).

**Figure 21-1**
**Hypothesized evolution of the secretin superfamily from an ancestral gene**

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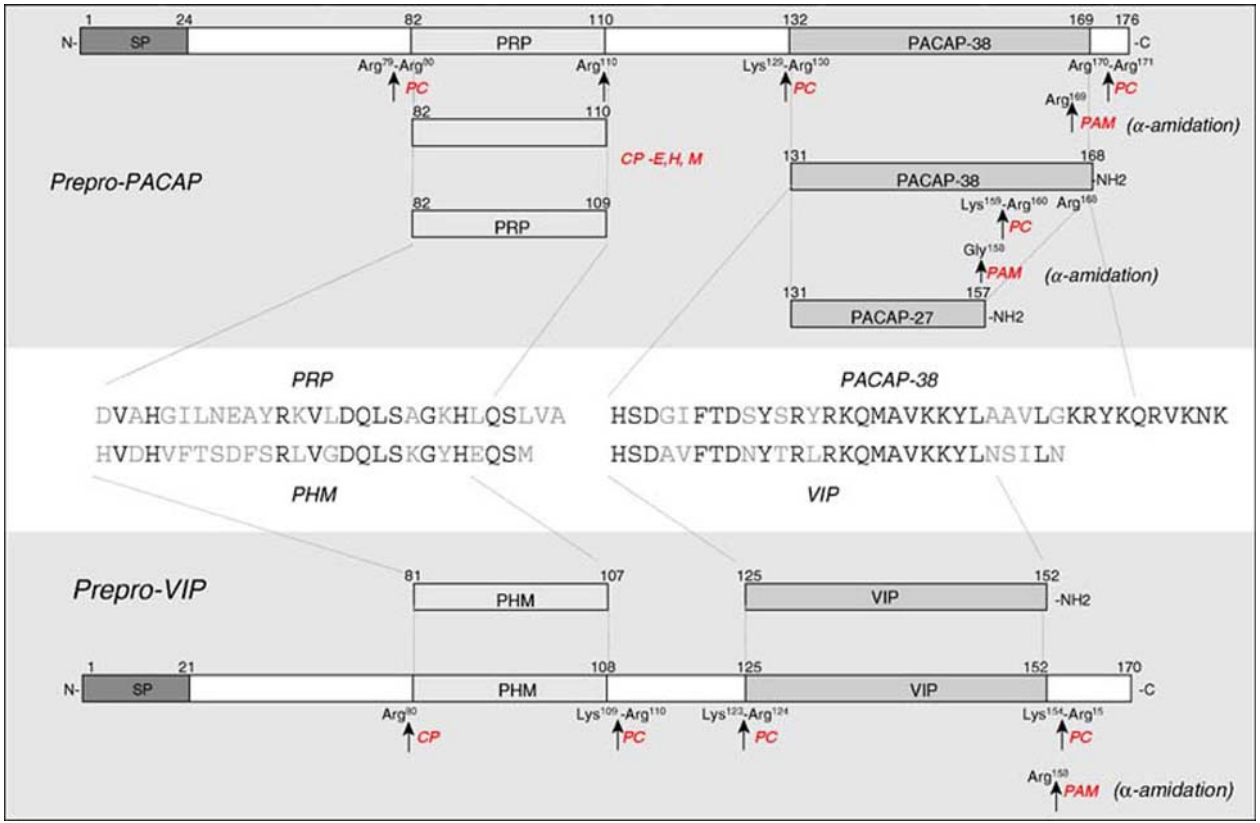
PACAP-38          HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK
PACAP-27          HSDGIFTDSYSRYRKQMAVKKYLAAVLG
PRP             DVAHGILNEAYRKVLDQLSAGKHLQSLVARGVGGSLGGGAGDDAEPLS
VIP               HSDAVFTDNYTRLRKQMAVKKYLNSILN
PHM               HADGVFTSDFSKLLGQLSAKKYLESLM
Secretin          HSDGTFTSELSRLREGARLQRLLQGLV
GRF               YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL
Glucagon          HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
GLP-1       HDEFERHAEGTFTSDVSSYLEGQAAKEFIAWLVKRG
GLP-2             HADGSFSDEMNTILDNLAARDFINWLIQTKITD
GIP               YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ
```

## 2.3 Slow Transmission at VIPergic and PACAPergic Synapses

Neuropeptides are biologically active oligo- or polypeptides processed from larger prohormone precursors, which are contained in and released from neurons and in some cases also from neuroendocrine cells. Neuropeptides including VIP and PACAP are stored in and released from large dense core vesicles (LDCVs) in neuronal and neuroendocrine cells. This is in contrast to release of classical neurotransmitters such as

**Figure 21-2**

**Neuropeptide preprohormone processing of pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP). Processing of preproPACAP and preproVIP during secretory granule maturation. Mature peptides shown as *shaded boxes*. Specific processing sites and the enzymes acting at them are depicted as *PC*, prohormone convertases and *CP*, carboxypeptidases**



glutamate and acetylcholine (ACh) from small synaptic vesicles (SSVs). Secretion from LDCVs and SSVs while having much in common, are regulated with distinctly different kinetics and using different types of regulatory proteins (see Eiden, 2003). Postsynaptically, neuropeptides in general interact with type II GPCRs (Gudermann et al., 1997) that are coupled to an array of downstream enzymes including adenylate cyclase (AC) and phospolipases C and D, to produce so called metabotropic effects.

These pre- and postsynaptic features mean that neuropeptides do not mediate fast neurotransmission, responsible for simple point-to-point information transfer across the synapse as typified by ionotropic receptor signaling, but rather complex time-delayed signaling affecting long-term cellular programs and encoding of cellular and synaptic experience—so-called slow neurotransmission (Greengard, 2001). This aspect of neuropeptide signaling accounts in large part for the ability of neuropeptides such as PACAP to support sustained functioning of synapses whose primary functions are carried by fast neurotransmitters such as ACh and glutamate. It should also be noted that even neuropeptide effects on ion channels, described for PACAP in this chapter, are metabotropic in that they are carried out not by direct ligand gating of calcium and general cation channels, but indirectly, presumably through G protein coupling and protein phosphorylation (Neves et al., 2002), and possibly other mechanisms not yet elucidated.

## 3 VIP and PACAP: Closely Related Species Within the Secretin Superfamily

### 3.1 VIP-28 and PACAP-27 and -38: Discovery, Distribution, and Function

VIP and PACAP are the most closely related members of the secretin superfamily with respect to primary amino acid sequence. By virtue of their complementary distributions in the central and peripheral nervous systems and their sharing of at least two separate GPCRs, VIP and PACAP and their receptors represent a unique "redundant network" for neuronal, neuroendocrine, and neuroimmune signaling.

VIP was isolated from intestinal extracts in 1970 (Said and Mutt, 1970), and as its name implies is a potent vasodilator. VIP is found peripherally in the enteric nervous system, in postganglionic cholinergic neurons of the parasympathetic nervous system and in the specialized cholinergic sympathetic innervation of sweat glands (Tyrrell and Landis, 1994). VIP is also reported to be present in sympathetic innervation of the periosteum (Asmus et al., 2000; Cherruau et al., 2003). The highest concentrations of VIP in the central nervous system are found in cerebral cortex, hippocampus, amygdala, and hypothalamus (Loren et al., 1979). Cortical VIPergic neurons are intrinsic and also express ACh (Eckenstein and Baughman, 1984). Hippocampal VIPergic neurons are neither granule nor pyramidal cells—they are intrinsic neurons of heterogeneous morphology (Loren et al., 1979). A major pepidergic pathway, which contains neurotensin as well as VIP (Uhl and Snyder, 1979) projects from amygdala to hypothalamus via the stria terminalis (Sims et al., 1980). The dorsal bed nucleus of the stria terminalis contains the highest levels of VIP found anywhere in the brain and appears to integrate input from a long ascending pathway from the mesencephalon, a ventral amygdalofugal pathway, and a central amygdalofugal pathway via the stria terminalis (Eiden et al., 1985). Finally, the suprachiasmatic nucleus is richly and intrinsically innervated by VIPergic cells (Sims et al., 1980), and VIP as well as PACAP release play a major role in circadian regulation at this site (vide infra).

In a classic study, Lundberg and coworkers demonstrated that VIP costored with ACh in parasympathetic nerves innervating the pancreas potentiates the effects of ACh on pancreatic zymogen secretion by increasing vasodilation in conjunction with the primary secretory effects of ACh (Lundberg et al., 1980). VIP was found to act on a GPCR to activate adenylate cyclase not only in liver and pancreas but also in the brain (Deschodt-Lanckman et al., 1977), to be present at high levels in both brain and gut (Bryant et al., 1976; Said and Rosenberg, 1976) and to be released from brain synaptosomes upon depolarization (Giachetti et al., 1977). These observations, along with isolation and demonstration of VIP action upon release in intestine, peripheral nerves, and brain (see Fahrenkrug, 1979 and references therein), served to illustrate vividly that VIP should be considered not only a gut hormone, as previously, but also a neurotransmitter, i.e. a neuropeptide.

VIP may be released from the hypothalamus into the portal circulation to affect prolactin (PRL) secretion, but sufficient evidence for VIP to play a major role in pituitary hormone secretion and for its concentration in nerve terminals of the median eminence for it to be classified as a hypophysiotropic hormone such as LHRH, TRH, SOM, GRF, or CRH, is lacking in rodents (Samson et al., 1979). VIP may be more prominent in hypothalamohypophyseal regulation in primates (Samson et al., 1978). VIP is present in the pituitary as well, but once again is unlikely to play a hormonal role upon release into the general circulation, as GH, LH, FSH, TSH, PRL and ACTH do, but rather has a paracrine regulatory role within the pituitary itself (Rostene, 1984).

VIP is typical of a subset of neuropeptides, including PACAP, that appear to lack a true physiological hormone function in addition to their roles as paracrine and autocrine factors and as neurotransmitters. Even in the gut, VIP is released mainly from a widely distributed nerve terminal network rather than from neuroendocrine cells and appears to act locally rather than being carried in the circulation to distant sites of action, as are true gut hormones. Although VIP is expressed in at least one major endocrine organ, the adrenal medulla (Hökfelt et al., 1981; Holzwarth, 1984; Waschek et al., 1987), and released from chromaffin cells (Eiden et al., 1983), it appears to act locally on steroidogenesis in the adrenal cortex (Edwards and Jones, 1993; Bornstein et al., 1996; Bodnar et al., 1997) rather than hormonally, like adrenomedullary catecholamines. An exception is during the pathophysiological condition of watery diarrhea syndrome, characterized by VIP release from pheochromocytoma tissue into the circulation to the gut, where electrolyte, water, and bicarbonate secretion stimulated by VIP can cause electrolyte imbalance (Bloom et al., 1983; Bloom et al., 1988).

Perhaps the most physiologically robust role of VIP in the nervous system is as a cotransmitter with ACh in the postganglionic neurons of the parasympathetic nervous system, where VIP is an important vasodilatory substance in cerebrovascular regulation (Duckles and Said, 1982) and at various target organs including salivary glands (Uddman et al., 1980), male genitourinary tract (Willis et al., 1983), and exocrine pancreas, at which ACh and VIP potentiate each other's actions as primary secretagogue and local vasodilator, respectively (Lundberg et al., 1980).

PACAP, as its name implies, was discovered as part of a comprehensive screen of hypothalamic extracts for peptides capable of stimulating AC activity in anterior pituitary cells, and thus was likely to be a hypophysiotropic hormone (Miyata et al., 1989). Indeed PACAP potently releases four of the six pituitary hormones in perifused pituitary experiments, albeit its role in endocrine regulation at the level of the pituitary is clearly less dominant than those of ACTH, LHRH, TRH, CRF, dopamine, or SOM (Arimura, 1998; Sherwood et al., 2000). PACAP-27 and PACAP-38 do not appear to have unique signaling properties apart from one another at the mamalian PAC1, VPAC1 and VPAC2 receptors (see ❯ *Table 21-2*, and references therein). Differences may be present in nonmammalian species, including those in which PACAP-27 rather than PACAP-38 is the dominant transmitter (Somogyvari-Vigh et al., 2000).

The distribution of VIP and PACAP differ in important respects in both the central and peripheral nervous systems, and in some cases are neuroanatomically and functionally complementary. Neither VIP nor PACAP are prominent neuropeptides of the basal ganglia, with the exception that the substantia nigra seems to be richly innervated by PACAPergic but not VIPergic terminals (Ghatei et al., 1993). PACAP levels in cerebral cortex are relatively low compared with VIP, while PACAP levels (nerve terminals) are high in cerebellar cortex, in which VIP is found only at very low concentrations (Arimura et al., 1991; Masuo et al., 1994). PACAP levels are high in thalamus and nucleus accumbens, while VIP levels are not (Arimura et al., 1991; Masuo et al., 1994). Common sites of VIP and PACAP expression include the suprachiasmatic nucleus (SCN) of the hypothalamus (VIP in intrinsic neurons, PACAPergic fibers innervating the nucleus) (Sims et al., 1980; Kim et al., 2000) and retina (VIP in amacrine and PACAP in retinal ganglion cells) (Koves et al., 2000 and references therein). PACAP levels appear to be higher than VIP levels in hypothalamus, except in SCN. However, caution about drawing conclusions about the roles of either peptide, for example, as a hypophysiotropic hormone in hypothalamus based on immunochemical results must be exercised for two reasons: first, earlier studies of VIP distribution may have inadvertently reported PACAP-cross-reacting immunoreactivity and second, there are sharp species differences in the abundance

and distribution of neuropeptides in general (Elde et al., 1980) and PACAP/VIP in particular (Samson et al., 1978), in the median eminence of the hypothalamus.

In the autonomic nervous system, VIP and PACAP distributions are again complementary. There seems to be a consensus that PACAP is contained in sensory neurons and preganglionic neurons of the sympathetic and parasympathetic nervous systems (Moller et al., 1993; Mulder et al., 1994, 1999; Sundler et al., 1996; Nielsen et al., 1997, 1998; Beaudet et al., 1998; Hamelink et al., 2002b), while VIP, but not PACAP, is a neurotransmitter of both sympathetic (e.g. sudomotor) and parasympathetic postganglionic cholinergic neurons (Fahrenkrug, 1979; Goyal et al., 1980; Holzwarth, 1984; Habecker et al., 1997; Klimaschewski, 1997). The fact that PACAP is strongly upregulated in postganglionic sympathetic neurons by denervation and inflammation may explain discrepancies concerning its presence in, or absence from, postganglionic sympathetic neurons in vivo (Moller et al., 1997; Beaudet et al., 1998, 2000; Mohney and Zigmond, 1998; Zhang et al., 1998; Mulder et al., 1999; Zhou et al., 1999b; DiCicco-Bloom et al., 2000; Zigmond, 2000; Girard et al., 2002). Sentence on VIP in sensory neurons.

A mention of VIPergic and PACAPergic innervation of the gut is necessary, since VIP is the "index" gut neuropeptide and exemplifies in this tissue the importance of species-specific neuropeptide neuroanatomy. First discovered as a gut paracrine factor (Said and Mutt, 1970), VIP is released from postganglionic parasympathetics (also containing ACh) and from the so-called NANC (nonadrenergic/noncholinergic) innervation of the enteric nervous system (Burnstock et al., 1979; Goyal et al., 1980; Schemann et al., 1995; Bennett, 1997). PACAP is likely contained only in efferent sensory neurons in gut. The extent of NANC innervation in gut may differ among species. In human, it appears that very few VIPergic fibers are actually noncholinergic (Anlauf et al., 2003), although in rodent the percentage of VIPergic NANC transmission (i.e. VIPergic neurons lacking ACh as a transmitter) may be considerably higher. As far as neuropeptides and NANC transmission are concerned, the emphasis on whether or not a classical neurotransmitter is stored in some, most, or all of these neurons may be less important than the fact that VIP is a unique inhibitory neurotransmitter/paracrine factor in the gut regardless of its corelease with ACh or other classical neurotransmitters. VIP and ACh are released from LDCVs and SSVs, within the same neurons, preferentially by high- and low-frequency stimulation, respectively (Agoston et al., 1988). The differential regulation of secretion of classical and neuropeptide transmitters underscores that whether released from the same or different neurons in the gut and elsewhere, VIP and PACAP are unique slow neurotransmitters throughout the nervous and neuroendocrine systems (see Sect. 1.4).

## 3.2 PAC1, VPAC1, and VPAC2: General Features of a VIP/PACAP Receptor Family

The pharmacology, structure, and properties of the three receptors recognizing PACAP and VIP are described in Sect. 4. We note some overall features of the receptors here. PAC1, VPAC1, and VPAC2 are a family of type II (secretin-type) GPCRs, which are thought to have arisen through a primordial gene duplication followed by a second duplication yielding the PAC1 and VPAC1 genes, with VPAC2 arising initially from the first duplication of the primordial gene (Chow et al., 2003). Since VIP and PACAP are structurally very similar and have overlapping receptor specificities, it can be difficult to predict the physiological or pharmacological effects of these peptides, without a detailed knowledge of their receptors expressed in a given tissue. Macrophages, SCN, cerebellum, hippocampus, and other target nuclei and tissues contain combinations of PAC1, VPAC1, and VPAC2 receptors, often in a species-specific pattern, as described in detail in other sections of this chapter (and see Vaudry et al., 2000 for general review of PACAP/VIP receptor distributions and functions). Splice variants of the receptors, PAC1 in particular, can alter both ligand specificity with respect to VIP and PACAP, and the second messenger systems to which the receptor is coupled. Finally, receptor coupling to second messenger systems can be cell-type specific (Hamelink et al., 2002a) and altered by costimulation with other peptide neurotrophins (Lelievre et al., 2002), and VIP and PACAP can modulate each other's expression and the expression of their receptors (Lelièvre et al., 1996; Waschek, 1996; Waschek et al., 1997).

### 3.3 Overview of Functional Interrelationships of VIP and PACAP

VIP and PACAP are interdependent neuropeptides. Their distributions are unique, but highly overlapping. In the SCN, adrenal medulla, sympathetic and parasympathetic nervous systems, PACAP is usually "presynaptic" to VIP, i.e. acts to regulate VIP expression and secretion in a postsynaptic neuron or neuroendocrine cell. VIP and PACAP can also be coexpressed in cell types that generally express only one or the other of the two peptides, under certain conditions. Thus, coexpression of VIP and PACAP occurs in pineal innervation from the trigeminal ganglion (Moller and Baeres, 2003), although generally PACAP and not VIP is expressed in subpopulations of sensory ganglia and nerve terminals (Moller et al., 1993; Mulder et al., 1994; Sundler et al., 1996) unless after inflammation or nerve damage (Nielsch and Keen, 1989; Nahin et al., 1994). PACAP and VIP are expressed in principal sympathetic ganglion cells after axotomy and during development (Sun et al., 1994; Moller et al., 1997; Zigmond and Sun, 1997), although generally only VIP is expressed in specific (cholinergic) subpopulations of principal ganglion cells and nerve terminals in adult mammals (Morales et al., 1995). PACAP is expressed in motor neurons, but only after neuronal injury (Zhou et al., 1999b). VIP but not PACAP is expressed in adrenomedullary chromaffin cells, although some cells express PACAP following splanchnic denervation (Holgert et al., 1996).

### 3.4 PACAP-like Peptides in Nonmammalian Species: Amnesiac and Maxadilan

An interesting feature of VIP/PACAP receptor recognition is the apparently independent evolution of peptides in sand flies and fruit flies that, while having little homology to PACAP, recognize its receptor. Maxadilan (CDATCQFRKAIDDCQKQAHHSNVLQTSVQTTATFTSMDTSQLPGNSVFKECMKQKKEFKAGK) is a potent vasodilator secreted by the sand fly. Although its primary amino acid sequence has little homology to PACAP, it is a potent agonist at the mammalian PAC1 receptor (Tatsuno et al., 2001). The Drosophila amnesiac mutant exhibits memory deficits and ras- and cAMP-signaling deficits. Mammalian PACAP can act on a GPCR on fly muscle cells at which the putative neuropeptide encoded at the *amnesiac* locus is hypothesized to act (Zhong, 1995). The presumed amnesiac prohormone is deduced to be MLWRCTAYYCFTLFFLLFRASALRRRVVSGSKGSAALALCRQFEQLSASRRERAEECRTTQLRYHYHRNGAQSRSLCAAVLCCKRSYIPRPNFSCFSLVFPVGQRFAAARTRFGPTLVASWPLCNDSETKVLTKWPSCSLIGRRSVPRGQPKFSRENPRALSPSLLGEMR, with the putative processed peptide E-29-C (underlined above) the most homologous to PACAP (Feany and Quinn, 1995).

## 4 Physiology and Paraphysiology of PACAP and VIP

### 4.1 Overview of Determining Peptide Function from Knockouts and Pharmacology: The Role of VIP and PACAP in Development and Differentiation

Perhaps no neuropeptide functional studies are more fraught with conceptual and methodological difficulties than those in which peptide involvement in organismic development and cellular differentiation are concerned. Whether the endogenous peptide is implicated through pharmacological studies, including passive immunization, or through loss-of-function in ligand or receptor knockout mice or other organisms, it can be difficult to know what aspects of development cell culture models are actually addressing, whether the peptide is acting as an autocrine or paracrine factor, hormone, or neurotransmitter, or whether its action in an isolated system is predictive of a discrete loss-of-function in vivo, or merely an alteration in the statistical probability that a particular developmental step will proceed successfully or go awry.

VIP was one of the first neuropeptides demonstrated to have neurotrophic, or neuron survival enhancing properties on central nervous system neurons during development. Early suggestions that VIP is important in development include the demonstration that there is an electrical activity-dependent influence of VIP on the survival of developing spinal cord neurons in culture (Brenneman and Eiden,

1986). This culminated in the discovery of the glial-derived activity-dependent neurotrophic factor released from glial cells by neuronally released VIP (Brenneman et al., 1987). Numerous subsequent experiments in cultured central and peripheral neuronal preparations (see Waschek, 1995 and references therein) have lead to the proposal that VIP may be a critical regulator of neuronal development, albeit definitive proof of this, reflected in developmental impairments of VIP-deficient mice, is lacking. One of the most dramatic experiments implicating VIP in development was the observation by Gressens et al. that the growth of postimplantation mouse embryos in culture is retarded by treatment with VIP antagonist and accelerated by provision of VIP in culture medium (Gressens et al., 1993). However, it has proven difficult to find standard conditions in which the effects of VIP in this assay can be easily observed and assessed (Sheward et al., 1998).

PACAP and VIP have been implicated in both central (cortical) and peripheral (sympathetic) neurogenesis, and in neuronal survival, based on pharmacological, physiological, and cell culture studies (DiCicco-Bloom et al., 1998, 2000). PACAP receptors are expressed early in development, consistent with involvement in neural tube closure (Waschek et al., 1998; Zhou et al., 1999a). PACAP and PAC1 receptors are expressed in the developing cerebellum and have a pronounced effect on neurogenesis in the inner granule layer of the cerebellum (Basille et al., 1993; Gonzalez et al., 1996; Vaudry et al., 1999). There is strong circumstantial evidence of a role for PACAP in the development of the nervous system. However, it remains problematic that severe developmental impairment consistent with an important role in central and peripheral neurogenesis, survival, and differentiation has not been reported in either PACAP- or VIP-deficient mice (Hashimoto et al., 2001; Gray et al., 2002; Hamelink et al., 2002b; Colwell et al., 2003; Vaudry et al., 2005).

## 4.2 Adrenomedullary Transmission and Glucohomeostasis

The adrenomedullary synapse is a physiologically and conceptually important model synapse for the study of neuropeptide slow transmission in the nervous system. In particular, it illustrates several key caveats in understanding the neuroanatomy, physiology, and neurochemistry of neuropeptide signaling carried out in an interrelated way by neuropeptide "family members." Assignment of synaptic function to individual neuropeptides requires not only that they exert a postsynaptic effect when administered exogenously but also that they are present neuroanatomically at the synapse of interest. This can be experimentally difficult, as it requires identifying immunological reagents that reliably distinguish between closely related family members. The evidence that PACAP is an adrenomedullary neurotransmitter has been recently reviewed (Hamelink et al., 2003). Here, the coordinate roles of PACAP and VIP in adrenal regulation are emphasized.

It was initially reported that VIP caused the pharmacological stimulation of catecholamine release from the adrenal medulla and that VIP was present in preganglionic nerve terminals of the adrenal medulla (Holzwarth, 1984; Edwards and Jones, 1993). Later, the discovery of PACAP prompted the examination of its effects on adrenomedullary secretion, and PACAP was found to be a more potent secretagogue than VIP (Watanabe et al., 1992; Edwards and Jones, 1994; Hahm et al., 1998), consistent with the presence of PAC1 in greater abundance than VPAC1 or VPAC2 on chromaffin cells of most species (Shivers et al., 1991; Babinski et al., 1996; Przywara et al., 1996; Tanaka et al., 1996b; Hahm et al., 1998; Mazzocchi et al., 2002). Initial immunohistochemical evidence suggested that PACAP innervation of the adrenal medulla was primarily sensory (Dun et al., 1996). However, antibodies against PACAP-38 validated for specificity by immunohistochemical analysis in wild-type versus PACAP knockout mice have established that virtually all of the PACAPergic innervation of the adrenal medulla is also cholinergic (Hamelink et al., 2002b), and thus ACh and PACAP are cotransmitters at the adrenomedullary synapse. Although some details remain to be filled in, the current neuroanatomical picture throughout the autonomic nervous system with respect to the expression of PACAP and VIP is that VIP is expressed in postganglionic parasympathetic neurons as well as the small population of postganglionic sympathetic neurons that are also cholinergic. VIP is absent, contrary to initial reports, from autonomic preganglionic neurons under most circumstances. PACAP is expressed in essentially all preganglionic autonomic neurons, in a subpopulation of sensory neurons, and might also be expressed in some postganglionic parasympathetic neurons and, in more specialized circumstances, in postganglionic sympathetic neurons as well, but these instances are likely to be dependent on para- or pathophysiological events and may also be species dependent (Zigmond, 2000).

The presence of a noncholinergic component to adrenomedullary transmission, the functional role of PACAP in maintaining catecholamine stores in the adrenal medulla by activation of tyrosine hydroxylase, and the lethal effect of insulin challenge on PACAP-deficient mice, rescuable with glucose, PACAP, or catecholamines, establishes that PACAP is a neuropeptide neurotransmitter at the adrenomedullary synapse (Wakade, 1988; Hamelink et al., 2002b, 2003). A recent report suggests that PC12 rat pheochromocytoma cells contain in addition to PAC1 receptors, receptors for secretin that can regulate chromogranin A gene transcription (Mahapatra et al., 2003). Characterization of secretin receptors on chromaffin cells, or visualization of endogenous secretin in splanchnic innervation of the adrenal medulla in any mammalian species, however, has not yet been reported, although the secretion preferring VPAC1 is expressed in rat adrenal medulla (Usdin et al., 1994).

The postsynaptic role of PACAP as an adrenomedullary slow transmitter appears to be threefold. First, PACAP affects the secretion of both catecholamines and neuropeptides present in the adrenal medulla of mouse, rat, and cow in culture and in vivo (Watanabe et al., 1992; Isobe et al., 1993; Edwards and Jones, 1994; Babinski et al., 1996; Geng et al., 1997; Hahm et al., 1998; Lamouche et al., 1999; Inoue et al., 2000; Hamelink et al., 2002b). Second, PACAP upregulates catecholamine biosynthesis both in culture and in vivo (Haycock, 1996; Hamelink et al., 2002b) by phosphorylation-dependent activation of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis, and upregulates neuropeptide biosynthesis both in culture and in vivo (Babinski et al., 1996; Hahm et al., 1998; C Hamelink, personal communication). By stimulating the biosynthesis of stored secretory materials that already exist in chromaffin cells, PACAP signaling assures that secretion is not exhausted by long-term secretory episodes during prolonged metabolic, psychogenic, or environmental stress, thus contributing to so-called stimulus-secretion-synthesis coupling at this synapse (Eiden et al., 1984). Finally, PACAP transmission at the adrenomedullary synapse stimulates the expression and release of neuropeptides that are present usually at only very low levels in chromaffin cells, i.e., under "nonemergency" conditions. Two examples in the mouse adrenal gland are galanin and VIP. Upregulation of mRNA encoding these neuropeptides, both very low under normal physiological conditions, occurs during hypoglycemia induced by insulin administration which causes prolonged splanchnic nerve firing and ACh and PACAP release, and is dependent on the presence of PACAP at this synapse (C. Hamelink, personal communication). VIP biosynthesis is also controlled by PACAP in bovine chromaffin cells in primary culture and depends on both calcium and cAMP (Hahm et al., 1998; Hamelink et al., 2002a), as does PACAP-induced catecholamine release from perfused rat adrenal medulla (Przywara et al., 1996), also mediated through PAC1 receptors.

The regulation of VIP biosynthesis in the adrenal medulla by presynaptic PACAP release appears to be physiologically specific. Thus, VIP is released as a hormone from chromaffin cells only during the pathophysiological events of pheochromocytoma, when chromaffin cells are not under synaptic control. Under normal physiological circumstances, the cellular plasticity driven by PACAP to control VIP biosynthesis probably functions mainly to allow VIP to act as a neurotransmitter or paracrine regulator of glucocorticoid biosynthesis in the adrenal cortex (Bornstein et al., 1996). Thus, glucohomeostasis is regulated by both PACAP and VIP, in series, in this important endocrine gland.

## 4.3 Light Sensing and Circadian Regulation

Besides the cerebral cortex, the highest levels of VIP in the brain are contained in the SCN, the location of the mammalian brain circadian pacemaker. Similar to their innervation in the adrenal gland, PACAPergic terminals innervate VIP-containing cells of the SCN. These terminals constitute a subpopulation of the glutamate-containing retinohypothalamic ganglion cells that are afferent to the SCN from the retina, and contain the photopigment melanopsin (Hannibal et al., 2002). The role of PACAP in this circuit is to enhance the ability of glutamate neurotransmission to relay a light-induced phase delay in early night, and block the ability of glutamate neurotransmission to relay a light-induced phase advance in late night, to the circadian clock of the SCN (Chen et al., 1999). These results imply that PACAP exists in this circuit to provide a more highly adaptive coupling between environmental changes in light and circadian function. VIP is contained within the SCN pacemaker cells themselves (Koves et al., 2000) and appears to coordinate

communication between these cells to synchronize the SCN as a circadian pacemaker nucleus that responds as a unit to altered input from the retinohypothalamic tract, and conveys output to the pineal and other targets (Colwell et al., 2003; Aton et al., 2005).

The ability of VIP- and PACAP-deficient mice, as well as those deficient in PAC1, VPAC1, or VPAC2 receptors, to phenocopy the effects of specific PAC1, VPAC1, and VPAC2 receptor blockade in circadian rhythmicity, has been examined by several laboratories. VPAC2 knockout mice are impaired in maintenance of SCN circadian rhythmicity and in response to exogenous VIP in slices of the SCN in culture (Harmar et al., 2002; Cutler et al., 2003). The inability of VPAC2 knockout mice and VIP-deficient mice (Colwell et al., 2003; Itri et al., 2004; Aton et al., 2005) to exhibit circadian rhythmicity in the SCN is consistent with a critical role for VIP within the SCN, and its action on SCN cells, for maintenance of a coherent rhythm within this cell group. There is likewise a relatively strong correspondence between PAC1- and PACAP-deficient mice in failing to exhibit the full scale of adaptive responses to changes in photic input through the retinohypothalamic tract to the SCN (Colwell and Waschek, 2001; Hannibal et al., 2001; Colwell et al., 2004; Lindberg et al., 2004).

## 4.4 Inflammatory and Immune Function

Receptors for a variety of neuropeptides are present on lymphocytes and macrophages; and lymph nodes, thymus, spleen, and lymphoid tissues are all richly endowed with neuropeptide-positive nerve fibers (Weihe et al., 1991). Pharmacologically, neuropeptides can affect adherence, mobility, activation, proliferation, and apoptosis of immune cells ex vivo, and their roles in specific aspects of innate and acquired immunity in vivo are still not completely clear (Jessop, 2002). One reason for this is the inherent complexity of the cellular and humoral immune responses that occur in mobile rather than sessile cells and involve the rapid formation and dissolution of "immune synapses", and that the actual neuroanatomical depots from which neuropeptides are released to affect immune-inflammatory function are still anatomically undefined. Some neuropeptides may be elaborated within immunocytes themselves. Endogenous neuronal neuropeptide secretion at discrete sites such as thymus, spleen, bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT), bone marrow, and tissue sites of injury and inflammation to which inflammatory and immune cells migrate, may also mediate VIP/PACAP signaling to macrophages and lymphocytes during immune and inflammation responses.

VIP and PACAP are both functionally implicated in lymphocyte maturation, antiinflammatory action in sepsis, and balance between proliferation of type 1 and type 2 helper T cells during infection (Voice et al., 2002; Delgado et al., 2003). The relevance of VIP/PACAP immune signaling in these conditions is evident in the protection from lethality afforded by treatment with either peptide during LPS-induced septic shock in both rats and mice; decreased erosion of bone and cartilage upon VIP/PACAP treatment in collagen-induced arthritis in rodents; protection against intestinal inflammation by VIP in early, acute, and chronic TNBS-induced colitis; and in boosting of $T_H2$ memory cell survival in vivo (Delgado et al., 2003, 2004). Complete characterization of the various effects of PACAP and VIP on distinct compartments within the immune/inflammatory response system and on production of endogenous VIP/PACAP themselves at sites of immune activation may ultimately lead to the development of receptor-specific therapeutic VIP/PACAPergic ligands as treatments for septic shock, rheumatoid arthritis, and Crohn's disease. VIP has been suggested as a treatment for bronchial asthma due to its beneficial effects on vasodilation via stimulation of VPAC receptors in the lung (Berisha et al., 2002).

As depicted in ❯ *Table 21-2*, VIP and PACAP act in an overlapping fashion at PAC1, VPAC1, and VPAC2 receptors. They can in addition affect each other's biosynthesis (Lelièvre et al., 1996; Waschek et al., 1997; Armstrong et al., 2003). A clear picture of the site of release of these two neuropeptides from the nervous system for communicating with immunoinflammatory cells in target tissues (i.e. the neuroimmune synapses at which VIP and PACAP act during early innate immune response leading to inflammation, adaptive immunity in responses to bacterial and viral infection and stress, thymic development, and T-cell education), does not yet exist. Although primary and secondary lymphoid tissue is predominantly innervated by the sympathetic nervous system (Elenkov et al., 2000), mucosal lymphoid tissue (MALT), including BALT and GALT, is heavily innervated by the parasympathetic nervous system, which expresses

VIP, and by sensory efferents which contain PACAP. Thus, according to current neuroanatomical information, VIP and PACAP most likely exert their effects upon release from parasympathetic and sensory neurons, respectively, in lymphoid organs and tissues. Rigorous reexamination of the peripheral neuroanatomy of VIP and PACAP in the mouse has not yet been carried out using antibodies validated for cross-reactivity in VIP- and PACAP-deficient mice.

Delgado and Ganea have reported that VIP or PACAP treatment ameliorates LPS-induced lethality (septic shock) in rodents at nanomolar concentrations (Delgado et al., 1999a). VIP has similarly potent effects on $T_H2$ differentiation in vivo, as do VIP and PACAP on macrophage cytokine secretion in vitro. All of these data suggest action through a VPAC1 or VPAC2 receptor, although there are no truly specific antagonists to rule out the involvement of a PAC1 receptor variant with affinity for VIP in these processes. Thus, it is unclear whether endogenous PACAP or VIP acts, and how, in any given immune response. Recently, PAC1 receptor knockout mice have been employed to show that the PAC1 receptor is required for some but not all of the effects of PACAP in protection from septic shock (Martinez et al., 2002; Martinez, 2005). Our own preliminary data involving LPS challenge of PACAP-deficient mice (C. Hamelink and Eiden, unpublished observations) suggest that endogenous PACAP is unlikely to act alone, i.e. in the absence of VIP, to provide protection from sepsis, since the magnitude of exacerbation of lethality after LPS challenge in PACAP-deficient mice is less than the corresponding protection from LPS lethality provided by treatment with either PACAP or VIP in vivo. Pharmacological analysis likewise suggests that the antiarthritic effects of VIP/PACAP are mediated through VPAC2 receptors (Delgado et al., 2001). VPAC2 receptors on T cells probably also mediate the shift toward $T_H2$ activation during inflammation and infection mediated by PACAP/VIP, since VPAC2-deficient mice show defects in $T_H2$ responses associated with immediate-type hypersensitivity (Goetzl et al., 2001), and specifically, impaired upregulation of the $T_H2$-type transcription factors c-Maf and JunB by VIP (Voice et al., 2004).

## 4.5 Neuroprotection in Stroke and other Ischemic Insult

PACAP was shown to have neuroprotective effects in stroke by Reglodi et al. in the rat middle cerebral artery occlusion (MCAO) model (Reglodi et al., 2000). Chen et al. have extended this to the mouse, showing that PACAP is active whether administered intracerebroventricularly or intravenously, and demonstrated using PACAP-deficient mice that neuroprotection is not only pharmacological but is a property of endogenous PACAP as well (Chen et al., 2002). Neuroprotection by PACAP has also been documented after brain injury, and circulatory impairment, and in reperfusion injury after brain and lung ischemia (Uchida et al., 1996; Delgado et al., 1999b; Mizushima et al., 1999). PACAP prevents the ischemic death of rat CA1 neurons when given either intracerebroventricularly or intravenously in a model of transient global ischemia, even if administration is delayed for 24 h after the ischemic event (Uchida et al., 1996). Significantly, systemic administration of PACAP effectively reduced infarct volume in a rat model of focal ischemia when administration was initiated 4 h after MCAO (Reglodi et al., 2000). The pharmacotherapeutic potential of PACAP for treatment of so-called "secondary neuronal injury" triggered by spreading depression, glutamate excitotoxicity, and cytokine/inflammatory mediator-induced neuronal injury in stroke, brain trauma, and spinal cord injury is under consideration by several laboratories (Chen et al., 2005). The neuroprotective effects of PACAP have been documented in several contexts including stroke and ethanol toxicity (Vaudry et al., 2002a; Chen et al., 2004). Both BDNF and IL-6 have been implicated as mediators of PACAP-induced neuroprotection in the central nervous system (Shioda et al., 1998; Frechilla et al., 2001). PACAP has also been proposed to act indirectly through IL-6 release from folliculostellate cells in the pituitary to effect pituitary hormone synthesis and secretion (Tatsuno et al., 1991).

## 4.6 Learning, Memory, and Locomotion

As summarized in ❯ *Table 21-1*, the involvement of PACAP and VIP in various centrally mediated physiological responses and behaviors is suggested not only by anatomical, physiological, and

**Table 21-1**
**PACAP, VIP, PAC1, and VPAC receptor knockouts and their phenotypes**

| Gene | Knockout phenotype |
|---|---|
| PACAP(−/−) | **Cardiovascular** |
| | *Heart Failure* (Cummings et al., 2003), ↓Catecholamine secretion (Hamelink et al., 2002) |
| | **Nociception** |
| | ↓Inflammatory pain response (Mabuchi et al., 2004) |
| | **Behavior and Cognition** |
| | ↑*Psychomotor behavior*, ↓ *Anxiety and fear* (Hashimoto et al., 2001) |
| | ↓ *LTP* (Matsuyama et al., 2003) |
| | **Reproduction** ♀ |
| | ↓ *Fertility*, ↓ *Mating*, ↓ *Crouching* (Shintani et al., 2002) |
| | **Circadian Function** |
| | ↓ Sustaining of normal circadian rhythms (Colwell et al., 2003) |
| | **Feeding and Thermogenesis** |
| | ↓ *Carbohydrate intake and metabolism*, ↓ *Fat metabolism* (Nakata et al., 2004, Gray et al., 2001) |
| | ↑*Insulin induced hyperglycemia* (Hamelink et al., 2002) |
| | ↑*Body temperature loss* (Gray et al., 2002) |
| PAC1(−/−) | **Cardiovascular** |
| | *Heart failure*, ↑*Hypertension* ↑*Hypertrophy* (Otto et al., 2004) |
| | **Nociception** |
| | ↓ Visceral and Chronic pain response (Jongsma et al., 2001) |
| | **Behavior and Cognition** |
| | ↓ *Affiliative*,↓ *Social*, ↓*Psychomotor*, ↓ *Anxiety and fear* (Nicot et al., 2004, Otto et al., 2001) |
| | ↓ *LTP*, ↓ *Associative learning* (Matsuyama et al., 2003, Otto et al., 2001) |
| | **Reproduction** ♀ |
| | ↓ *Litter size* (Jamen et al., 2000) |
| | **Circadian Function** |
| | ↓ Sustaining of normal circadian rhythms, ↓ Clock gene expression (Hannibal et al., 2001) |
| | **Feeding** |
| | ↓ Glucose stimulated *insulin secretion* (Jamen et al., 2002, Jamen et al., 2000) |
| | **Immunity** |
| | ↑*LPS shock and mortality*, ↑ *inflammation* (Martinez et al., 2005, Martinez et al., 2002) |
| VIP(−/−) | **Circadian Function** |
| | ↓ *Sustaining of normal circadian rhythms*, ↓ *Clock gene expression* |
| VPAC1(−/−) | ? |
| VPAC2(−/−) | **Growth** ↓ (Asnicar et al., 2002) |
| | **Reproduction** ♂ |
| | ↓*Fertility and hypospermia* (Asnicar et al., 2002) |
| | **Circadian Function** |
| | ↓ *Sustaining of normal circadian rhythms*, ↓ *Clock gene expression* (Cutler et al., 2003, Harmar et al., 2002) |
| | **Immunity** |
| | ↓ $T_H2/T_H1$ *cytokine ratio maintenance* (Voice et al., 2004) |
| | ↓ *T cell differentiation and function*, ↑ *Delayed type hypersensitivity* (Goetzl et al., 2001) |
| | **Feeding** |
| | ↑ *Insulin sensitivity*, ↑*Metabolic rate*, ↓ *Fat mass* (Asnicar et al., 2002) |

pharmacological experiments, but by phenotypes of PACAP- ,VIP-, PAC1-, and VPAC2-deficient mice (Goetzl et al., 2001; Martinez et al., 2002; Voice et al., 2004). Interestingly, both PACAP and PAC1 knockouts have corresponding deficits in hippocampal long-term potentiation (Otto et al., 2001a; Matsuyama et al., 2003). This is consistent with a role for PACAP neurotransmission through the PAC1 receptor in hippocampus, but not for amygdala-dependent associative learning (Otto et al., 2001a). Increased locomotor activity in a novel environment in PACAP-deficient mice (Hashimoto et al., 2001) and increased psychomotor activity with decreased anxiety and fear in PAC1-deficient mice (Otto et al., 2001b) suggest that deficiency in PACAP is phenocopied by the loss of the receptor that is specific for this ligand. Localizing these phenotypes to deficiencies in PACAP neurotransmission in substantia nigra, hippocampus, cortex, and hypothalamus will contribute greatly to delineating the anatomical fine structure of peptidergic chemical coding in spatial, associative and emotional memory, and learning. Despite the role of PACAP in modulating biogenic amine biosynthesis at the adrenomedullary synapse, Hashimoto et al. reported no abnormalities in brain biogenic amines or metabolites in PACAP-deficient mice, suggesting at least initially that PACAP's roles in psychomotor behavior and learning is not mediated through the biogenic amines (Hashimoto et al., 2001). Thus at this time, effects of PACAP deficiency cannot be traced to a biogenic amine loss, although PACAP seems to support catecholamine biosynthesis under stress in the peripheral nervous system.

## 4.7 Pain

The presence of PACAP and VIP in small-diameter sensory fibers (Moller et al., 1993), and their upregulation in sensory neurons after nerve injury, or in neuropathic and inflammatory pain (Hokfelt et al., 1994; Zhang et al., 1998) suggests that these two neuropeptides participate as neurotransmitters or local effectors in sensory pain responses (Dickinson and Fleetwood-Walker, 1999). PACAP-deficient mice do not respond to inflammatory pain, although nociceptive pathways are apparently intact (Mabuchi et al., 2004). Visceral and chronic, but not acute, somatic pain perception is also decreased in PAC1-deficient mice (Jongsma et al., 2001; Martin et al., 2003), as well as mice deficient in PACAP (Mabuchi et al., 2004). These observations support an important role for PACAP/VIP signaling in sensory pathways as potential targets for the management of chronic pain (Dickinson and Fleetwood-Walker, 1999).

## 4.8 Metabolic, Respiratory, and Cardiovascular Regulation

VIP is an important regulator of glucose metabolism in the brain through stimulation of glycogen hydrolysis in astrocytes (Magistretti et al., 1983). PACAP exerts effects on metabolism in a variety of ways, including stimulation of glucose-induced insulin secretion in pancreas (Filipsson et al., 1999) via PAC1 receptor stimulation (Jamen et al., 2000a), epinephrine release from adrenal medulla (Hamelink et al., 2003), and stimulation of the thyroid axis (Arimura, 1998). It is not clear to what extent metabolic effects of PACAP and VIP are mediated through the hypothalamo–pituitary axis. PACAP can release several hormones from the superfused pituitary gland, including LH, ACTH, GH, and PRL (Miyata et al., 1989) but not TSH, and is a candidate hypophysiotropic hormone in some species (Rawlings and Hezareh, 1996). However, major alterations in pituitary function have not been reported in PACAP-deficient or PAC1- or VPAC2-deficient mice in vivo (Brabet et al., 2003; Sherwood et al., 2003), despite the initial discovery of PACAP as a modulator of pituitary secretion of multiple pituitary hormones (Miyata et al., 1989), with the exception of possible dysregulation of gonadotropin secretion in female PAC1-deficient mice (see Sect. 4.9). Both VIP and PACAP exert lipolytic effects on adipocytes through VPAC2 receptor-dependent cAMP elevation, thus antagonizing insulin effects on these cells (Akesson et al., 2005), and VPAC2-deficient mice show retarded growth and increased basal metabolic rate (Asnicar et al., 2002). Behaviorally, PACAP-deficient mice show decreased preference for a high-carbohydrate diet, and this may be due to lack of activation of neuropeptide Y (NPY)-containing arcuate neurons mediating feeding behavior (Nakata et al., 2004). Thus, PACAP and VIP act at multiple behavioral, metabolic, and catabolic steps, in brain, pituitary,

autonomic nervous system, and fuel-handling organs such as liver and fat cells, to affect fuel consumption in mammals.

Diving turtles have unusually high levels of PACAP in their brains (Reglodi et al., 2001), which is consistent with a role for PACAP in maintenance of respiratory homeostasis and perhaps neuronal protection under conditions of low oxygen availability (Rabl et al., 2002). PACAP is both thermoregulatory and involved in lipid metabolism as evidenced from defects in both in PACAP-deficient mice (Gray et al., 2001, 2002). Sherwood and colleagues have drawn attention to a respiratory syndrome in PACAP-deficient mice that may be associated with respiratory responsiveness to hypoxia (Cummings et al., 2004a, b). Related to a possible global neuroprotective function in hypoxic brain, PACAP reduces infarct size and is functionally neuroprotective following middle cerebral artery occlusion in rodents (Reglodi et al., 2000; Chen et al., 2002, 2005; Reglodi et al., 2002).

We have already alluded to PACAP's indirect cardiovascular effects via control of adrenomedullary catecholamine secretion (Hamelink et al., 2002b). It is also been reported that PAC1-deficient C57Bl/6 mice do not generally survive the second week of life due to pulmonary hypertension and right heart failure, presumably secondary to autonomic regulation of pulmonary vascular tone (Otto et al., 2004). It is noteworthy that such cardiovascular failure does not occur in PACAP-deficient C57Bl/6 mice (C Hamelink and R Damadzic, personal communication), suggesting the possibility that a ligand other than PACAP for the PAC1 receptor exists in the lung.

### 4.9 Fertility and Reproductive Behavior

It has been noted that PACAP-deficient mice are at risk for premature death during weaning (Arimura, 2002) and also that fertility is significantly decreased in both PAC1- and PACAP-deficient female mice (Jamen et al., 2000b; Shintani et al., 2002). PACAP may affect female fertility through regulation of estrus cycling, possibly through disrupted circadian signaling, since PAC1-deficient mice display disrupted estrus cycling although ovulatory function is locally normal (Brabet et al., 2003). PACAP-deficient female mice may also be less fertile as a result of impaired affiliative behavior during male courtship (Shintani et al., 2002). This decreased affiliative behavior could be related to defects in pheromone signal processing and pheromone-driven regulation of social interaction between female and male mice (Nicot et al., 2004). Male fertility may also be regulated by PACAP and/or VIP, as demonstrated by seminiferous tubule degeneration and decreased fertility, albeit only in older male mice, due to VPAC2 receptor knockout (Asnicar et al., 2002).

## 5 The VIP/PACAP Receptor Family

### 5.1 PACAP and PACAP-Preferring Receptors and Their Agonists/Antagonists

There are a total of three distinct receptors in mammals with physiological affinity for PACAP and VIP but not for SEC, PHM/PHI, GLP1/2,GLUC, GRF, and GIP. Harmar et al. summarized the properties of the three receptors, for which VIP and PACAP have overlapping specificity, and proposed a nomenclature for them in 1998 (Harmar et al., 1998). PACAP binds all three receptors, PAC1, VPAC1, and VPAC2 with high affinity, while VIP binds and activates only the VPAC1 and VPAC2 receptors at high affinity and can activate only one PAC1 receptor variant at nanomolar concentrations. VPAC1 (Ishihara et al., 1992) and VPAC2 (Lutz et al., 1993) were initially cloned and characterized as VIP receptors, distributed in brain and peripheral tissues, that also recognize PACAP and have no known splice variants. PAC1 was molecularly characterized independently by several groups in 1993 (Hashimoto et al., 1993; Hosoya et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993; Svoboda et al., 1993). PAC1 exists as several splice variants with differential second messenger coupling. VPAC1, VPAC2, and PAC1 receptors belong to the secretin family of Group II GPCRs that includes receptors for SEC, GLUC, GLP-1, GLP-2, GRF, and GIP. It is hypothesized that following a duplication of an ancestral gene into the PAC1/VPAC1 and VPAC2 "precursors," the PAC1/

VPAC1 gene duplicated again and diverged to yield the modern VPAC1 and PAC1 receptors (Chow et al., 2003). Therefore in addition to PACAP, VPAC receptors are also capable of recognizing to some degree other naturally occurring peptides that belong to the secretin family of peptides, which are structurally related to VIP, including SEC, GRF, and PHI (see *Table 21-2*).

**Table 21-2**
**PACAP and VIP receptor agonists and antagonists and their affinities**

| Receptor subtype | Ligand affinity | Agonists | Antagonist |
|---|---|---|---|
| PAC1 | PACAP-38≥PACAP-27>>>VIP | Maxadilan | PACAP-6-27 |
| | | | PACAP-6-38 |
| | | | M65 |
| VPAC1 | General: VIP>PACAP-38=PHI>PACAP-27>>GRF>Secretin | [$Ala^{11,22,28}$]-VIP | PG-97-269 |
| | Rat: VIP=PACAP-38=PACAP-27>PHI>GRF>>Secretin | [$K^{15}R^{16}L^{27}$]VIP(1-7)GRF(8-27) | |
| | Human: VIP=PACAP-27> | $VIP_{4-28}$ | |
| | PACAP38>Helodermin>GRF=PHI>Secretin | [$A^{22}$]-secretin | |
| | | Ro 25-1392 | |
| VPAC2 | Rat: VIP>PACAP38=PHI>PACAP27>>>GRF | Ro 25-1553 | |
| | | [$Ala^{11,22,28}$]-VIP | VIP4-28 |
| | Human: VIP=PACAP-38=PACAP-27>PHI>>GRF | Hexanoyl[$A^{19}$, $K^{27,28}$]VIP | PG-99-465 |

As demonstrated in *Table 21-2*, VPAC1 and VPAC2 receptors (rat) bind these natural ligands with the same relative rank order of potency, except for SEC that displays higher affinity for the VPAC1 receptor. The order of ligand potency for the human VPAC receptors is very similar to that of the rat receptors with the major exception that rat VPAC1 binds both PHI and SEC with lower affinity than human VPAC1. The PACAP selective receptor, PAC1, recognizes both PACAP-38 and PACAP-27 with high affinity (~0.5–1 nM). However it binds VIP at ~1000-fold lower affinity and does not recognize other VIP-related peptides at concentrations less than 1 μM. In some studies, PACAP-38 has been shown to bind with least tenfold higher affinity to the PAC1 receptor compared with PACAP-27 (Vaudry et al., 2000). These minor discrepancies in receptor specificities may be related to the different pharmacological assays employed (radioligand-binding assays vs generation of cAMP) or due to the different cell lines employed to express the recombinant receptors. These can introduce variations in G protein expression, glycosylation, trafficking, accessory protein expression, receptor desensitization, and plasma membrane microdomain composition.

By using only natural ligands, it would be difficult to pharmacologically discriminate between VPAC1 and VPAC2 receptors, without the development of more selective analogs, both agonists and antagonists. Ro 25-1392 and Ro 25-1553, cyclic analogues of VIP containing a lactam ring between positions 21 and 25, were developed and shown to be selective VPAC2 receptor agonists (O'Donnell et al., 1994a, b; Gourlet et al., 1997b; Xia et al., 1997). Large scale screening of mutated analogs led to the discovery of a VIP analog containing three mutations, V19A, L27K, and N28K that conferred VPAC2 selectivity (Yung et al., 2003). Langer and colleagues then combined hexanoylation of the N terminus of a VIP analog substituted at positions 19, 27, 28, to develop Hexanoyl[A19, K27,28]VIP, an even more selective VPAC2 receptor agonist (Langer et al., 2004). Total alanine scanning of the VIP peptide revealed that a combination of three substitutions at positions 11, 22, and 38 results in a highly selective VPAC1 receptor agonist, [$Ala^{11,22,28}$]-VIP (Nicole et al., 2000a, b). Introduction of an arginine residue on position 16 in chicken secretin also lead to the development of a highly selective VPAC1 receptor agonist (Gourlet et al., 1997a). In these same studies Gourlet and colleagues designed a chimeric VIP/GRF analogue, [$K^{15}$,$R^{16}$,$L^{27}$]VIP(1-7)/GRF(8-27)

that to date appears to be the most selective agonist at VPAC1 receptors. Manipulation of the chimeric VIP/GRF analog later led to the discovery of the first highly selective VPAC1 receptor antagonist, PG 97-269 (Gourlet et al., 1997a). Interestingly, lymphocytes are capable of breaking down VIP and generating truncated VIP fragments. Of these fragments, $VIP_{4–28}$ was also shown to act as a potent VPAC1 agonist while acting as a potent antagonist at the VPAC2 receptor (Summers et al., 2003). An important issue in future, in the context of drug development, will be the behavior and cross-reactivity of these agonists and antagonists tested in cell systems containing endogenously expressed human VPAC1 and VPAC2 receptors and PAC1 receptors and their splice variants.

In addition to PACAP, maxadilan, a potent vasodilatory peptide isolated from the salivary glands of the sand fly, *Lutzomyia longipalpis* (Lerner and Shoemaker, 1992), is also a potent agonist at the PAC1 receptor with affinity similar to PACAP-38 and PACAP-27 (Moro and Lerner, 1997). Interestingly maxadilan shares no sequence homology to PACAP and does not appear to be endogenously expressed in mammalian tissue. Deletion of 17 amino acids (from 25 to 41) of maxadilan has been shown to result in the generation of a potent PAC1 receptor antagonist known as M65 (Uchida et al., 1998). N-terminal truncations of PACAP also led to the discovery of the analogs, PACAP(6-38) and PACAP(6-27), which are specific PAC1 receptor antagonists (Robberecht et al., 1992).

## 5.2 PAC1 Receptor Isoforms

In comparison to both VPAC1 and VPAC2 receptors and other receptors belonging to the secretin/glucagon superfamily of seven transmembrane GPCRs, the PAC1 receptor is one of most the extensively spliced GPCRs identified to date. Alternative splicing of both the N-, and C- terminal domains gives rise to at least eight receptor variants exhibiting alternative distributions, ligand-binding properties, and the ability to couple to different signal transduction pathways.

The first cloned PAC1 receptor (PAC1null), from a pancreatic acinar carcinoma cell line, encoded a 495-amino-acid protein, with seven putative membrane spanning domains, sharing a high degree of sequence identity with other secretin/glucagon superfamily GPCRs (Pisegna and Wank, 1993). Five different subtypes of the PAC1 receptor were then identified and were shown to originate from alternative splicing of two cassettes, hip or hop1(28)/hop2(27), within the third intracellular region, giving rise to $PAC1_{hip}$, $PAC1_{hop1}$, $PAC1_{hop2}$, $PAC1_{hiphop1}$ and $PAC1_{hiphop2}$ (Spengler et al., 1993; Journot et al., 1995). Of these receptors, the $PAC1_{hop1}$ appears to be the most abundant and commonly identified next to the $PAC1_{null}$ in a number of species and tissues. It shares similar binding affinity for both PACAP-38 and PACAP-27 and has the ability to potently activate AC and phospholipase C (PLC). The rat $PAC1_{hip}$ variant does not stimulate PLC, and when expressed together with the hop cassettes ($PAC1_{hiphop1}$ and $PAC1_{hiphop2}$) reduces the signal transduction efficacy of the receptor. More recently it has also been shown that the $PAC1_{hop1}$ variant specifically activates phospholipase D (PLD), unlike the $PAC1_{null}$ (McCulloch et al., 2001; Ronaldson et al., 2002). This signal transduction pathway is thought to be mediated by direct interaction of the GTP-binding, ADP-ribosylation factor 6 with the hop cassette (Ronaldson et al., 2002). This is the first line of evidence to date highlighting a functional significance of the cassette in the IC3 domain that enables the receptor to couple to novel signal transduction pathways, in addition to maintaining its classical effector coupling.

N-terminal domain splice variants of the PAC1 receptor have been identified that contain either 21- (PAC1s) or 57- (PAC1vs)-amino-acid deletions (Pantaloni et al., 1996; Dautzenberg et al., 1999). These variants did not contain any hip or hop cassettes. As expected, deletion of 57 amino acids, containing three of the seven conserved cysteine residues, reduced binding of both PACAP-38 and PACAP-27 (>100-fold) and their ability to activate AC. Unexpectedly, removal of 21 amino acids resulted in a receptor that was able to recognize and bind VIP with similar affinity to PACAP. VIP was also capable of potently stimulating AC through the PAC1 receptor (Dautzenberg et al., 1999). These findings suggest that the 21-amino-acid motif induces structural changes to the extracellular domain of the PAC1 receptor that results in a PACAP specific receptor, but prevents VIP binding. The fact that the PAC1s variant is expressed endogenously in the brain raises a significant physiological issue concerning the actions of VIP through the PAC1 receptor, i.e. that VIP would be physiologically active on cells expressing this PAC1 variant in the absence of either PACAP

or VPAC1/2 receptors. Recently, Daniel and colleagues identified a PAC1 variant containing a 24-amino-acid insert in the N-terminal extracellular domain that was preferentially expressed in the seminiferous tubules during spermatogenesis (Daniel et al., 2001). This variant (PAC13a) displays a sixfold higher affinity for PACAP-38 than PACAP-27, while exhibiting a reduced potency of PACAP-38 for AC stimulation and IP turnover.

Another PAC1 receptor variant (PAC1TM4) was reported as a rat cerebellum-derived cDNA clone that failed to couple to AC or PLC yet conferred PACAP-dependent calcium influx through putative voltage-sensitive calcium channels (VSCCs) in Chinese hamster ovary (CHO) cells. This variant is said to have three separate substitutions that distinguish it from the $PAC1_{null}$ receptor reported previously (Hashimoto et al., 1993; Hosoya et al., 1993; Pisegna and Wank, 1993; Spengler et al., 1993). These are positions 136 at the N terminus (D to N), 190 in the second transmembrane domain (N to D), and 279–282 in the fourth transmembrane domain (CVIV to SA). It seems remarkable that minor modifications in these transmembrane domain regions, which are not critical in ligand binding or involved in direct interaction with G proteins, can significantly alter receptor coupling to AC, PLC, and dihydropyridine-sensitive L-type calcium channels. Although the TM4 variant was originally reported to be present in the cerebral cortex, cerebellum, and brainstem, Ajpura and colleagues could only detect an amplicon corresponding to PAC1TM4 in rat pancreatic islet cells (Ajpru et al., 2002), and not anywhere in the brain. Another study failed to confirm the expression of the TM4 transcript even in rat pancreatic islets cells (Jamen et al., 2002). The TM4 variant, as described by Chatterjee et al., is furthermore difficult to be envisaged as a naturally occuring mRNA transcript: all three of the presumptive sequence variations are centrally located within nonduplicated exons in the rat gene, and thus cannot arise from alternative splicing. This cDNA would thus have to represent a transcript from a *Rattus norvegicus* multivariant allele, or else a transcript from the normal rat allele, with extensively edited RNA. Caution should be exercised in considering TM4 to be a naturally occuring allelic or RNA-edited PAC1 receptor variant until further evidence of its existence is adduced.

The VPAC1 was first identified and isolated from rat lung in 1992 (Ishihara et al., 1992), and the human homolog was cloned soon after, from HT-29 cells (Sreedharan et al., 1993) as well as from human small intestinal epithelium (Couvineau et al., 1994). The rat VPAC2 receptor was then cloned from the pituitary gland (Lutz et al., 1993), human SUP-TI lymphocytes (Svoboda et al., 1994), and placenta (Adamou et al., 1995). As mentioned previously, both VPAC1 and VPAC2 bind both VIP and PACAP with similar affinity, belong to the class II family of G-protein receptors, and share the common feature of strongly coupling to adenylate cyclase. Until recently there was no evidence to support the existence of naturally occurring VPAC1 or VPAC2 receptor variants, despite the prediction of possible truncated VPAC2 receptor variants based on intron/exon boundary gene analysis. A variant of the VPAC2 receptor was recently characterized in the mouse spleen and thymus which lacks 14 amino acids in the carboxyl terminal end of the seventh transmembrane region of the receptor and binds VIP without subsequent activation of adenylate cyclase (Grinninger et al., 2004).

Other as yet uncharacterized receptor variants with unique physiological and signaling profiles may exist. For example PAC1 receptor variants have been identified with truncated N-terminal domains containing hip or hop cassettes within the third intracellular domain that are yet to be characterized (Dautzenberg et al., 1999).

## 5.3 PACAP Receptor Signaling: G-Protein Coupling and Downstream Signaling

The PAC1 receptor is capable of activating dual signal transduction pathways by coupling to different G proteins and probably also by physical association with proteins or complexes involved in distinct signaling cascades. The PAC1 receptor activates AC through its association with the heterotrimeric G protein, Gαs. All known PAC1 receptor variants are capable of potently stimulating AC. The presence of the hip cassette ($PAC1_{hip}$, $PAC1_{hip1hop}$) does however perturb the ability of the rat receptor to stimulate AC compared with $PAC1_{null}$ and $PAC1_{hop1}$ receptor subtypes (Spengler et al., 1993). In contrast, all these human receptor variants stimulated AC with similar potencies (Pisegna and Wank, 1996) (❯ *Table 21-3*). Generation of

**Table 21-3**
**PAC1 receptor splice variants and their signaling properties**

| Receptor subtype | Ligand binding - $IC_{50}$ [nM] | | | cAMP production - $EC_{50}$ [nM] | | | IP production - $EC_{50}$ [nM] | | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | PACAP38 | PACAP27 | VIP | PACAP38 | PACAP27 | VIP | PACAP38 | PACAP27 | VIP | |
| $PAC1_{null}$ | | | | | | | | | | |
| Rat | 0.1 (Ki) | 0.08 (Ki) | 80 (Ki) | 0.4 | 0.1 | – | 15 | >1μM | – | (Spengler et al., 1993) (Ciccareli et al., 1995) (Pisegna & Wank, 1996) |
| Human | 20 (Ki) | 30 (Ki) | – | 0.6 | 0.8 | – | 35 | 50 | – | |
| $PAC1_{hip}$ | | | | | | | | | | |
| Rat | – | – | – | 6 | 1.7 | – | NS | NS | – | (Spengler et al., 1993) |
| Human | 40 (Ki) | 60 (Ki) | – | 0.8 | 1 | | 35 | 50 | – | (Pisegna & Wank, 1996) |
| $PAC1_{hop1}$ | | | | | | | | | | |
| Rat | 0.1 (Ki) | 0.1 (Ki) | 80 | 0.4 | 0.1 | – | 15 | >1 μM | – | (Spengler et al., 1993) |
| Human | 20 (Ki) | 30 (Ki) | – | 1.0 | 1.0 | – | 35 | 50 | – | (Pisegna & Wank, 1996) |
| $PAC1_{hop2}$ | | | | | | | | | | |
| Rat | – | – | – | 0.4 | 0.1 | – | 15 | > 1μM | – | (Spengler et al., 1993) |
| Human | – | – | – | – | – | – | – | – | – | |
| $PAC1_{hiphop1}$ | | | | | | | | | | |
| Rat | – | – | – | 2.5 | 0.7 | – | 50 | >> 1 μM | – | (Spengler et al., 1993) |
| Human | – | – | – | – | – | | – | – | – | (Journet et al., 1995) (Pantaloni et al., 1996) |
| $PAC1_{s}$ | 1.7–2.8 | 3.1–4.4 | 4.4 | 0.2–0.7 | 0.3–1 | 2.1 | 10 | 26 | – | (Dautzenberg et al., 1999) (Pantaloni et al., 1996) |
| $PAC1_{vs}$ | 121 | 129 | >1000 | 29 | 37 | 373 | – | – | – | (Dautzenberg et al., 1999) |
| $PAC1_{3a}$ | 0.8 | 8.21 | – | 27.4 | 8.29 | – | 53.4 | 31.2 | – | (Daniel et al., 2001) |

*$EC_{50}$*: Values are the effective concentration (nM) required to produce 50% of the maximal effect of peptides to stimulate cAMP or IP production. *$IC_{50}$*: The concentration of unlabelled ligand that inhibits the binding of 50%. *Ki*: Equilibirum dissociation constant of a ligand determined in inhibition studies. NS: Does not stimulate at any dose tested. (–): Yet to be determined.

cAMP and activation of PKA appears to be one of the key downstream receptor signal transduction pathways following PAC1 receptor activation and can be mimicked by the use of cAMP-elevating agents such as forskolin. Both VPAC1 and VPAC2 receptors also induce robust increases in AC activity and cAMP production mediated by coupling to Gαs (Couvineau et al., 1990; Kermode et al., 1992). As with PAC1 receptors, stimulation of AC by VPAC receptors also leads to subsequent activation of PKA, which serves as a key downstream signaling molecule associated with VPAC1/2 receptor signaling.

The second classical signal transduction pathway associated with the PAC1 receptor is activation of PLC through Gαq resulting in IP3-mediated intracellular calcium mobilization and DAG-mediated activation of protein kinase C. The major PAC1 receptor isoforms, $PAC1_{null}$ and $PAC1_{hop}$, have been shown to potently stimulate PLC and IP turnover with similar efficacy, except in human tissues where $PAC1_{hop}$ stimulated IP turnover more than the $PAC1_{null}$ variant (Pisegna and Wank, 1996). Additionally, rodent pancreatic islets coexpressing $PAC1_{null}$, $PAC1_{hop}$, and $PAC1_{vs}$, however show only stimulation of AC, and not PLC, with PACAP (Jamen et al., 2002). In astrocytes the $PAC1_{null}$ receptor is also not coupled to PLC activation (Grimaldi and Cavallaro, 1999). These discrepancies highlight tissue- and cell-dependent differences. The presence of the hip cassette in the IC3 region of the rat receptor completely abolishes PLC signaling (Spengler et al., 1993), while the human form of the $PAC1_{hip}$ receptor is capable of stimulating IP with similar efficacy as other variants (*Table 21-3*). Additionally when expressed in combination with the hop cassettes, e.g. the rat $PAC1_{hiphop}$, the hip cassette reduces the ability of the receptor to activate PLC (Spengler et al., 1993; Journot et al., 1995). Such discrepancies may be attributed to the use of different cell lines (NIH-3T3 vs LLC PK1) and possibly sequence differences between species that could ultimately change the coupling patterns of the different variants to different signal transduction molecules.

The ability of VPAC1/2 receptors to activate PLC also appears to be variable in different tissues. The rat VPAC1 receptor was shown to induce IP production through a pertussis toxin sensitive pathway suggesting the involvement of G proteins, Gαq and Gαi/o (Diehl et al., 1996; Van Rampelbergh et al., 1997), while in rat alveolar macrophages, coupling occurs through both Gαs and Gαi (Shreeve et al., 2000). Interestingly human VPAC1-mediated PLC activation was shown to couple to Gαs and not Gαi or Gαq G proteins (Shreeve et al., 2000), suggesting species dependent differences in G-protein coupling for activating PLC. VPAC2-mediated IP3 production was only partially inhibited by pertussis toxin, suggesting that the VPAC2 receptor may also couple to PLC through Gαi/o or Gαq in addition to activation of Gαs (MacKenzie et al., 2001).

Dual coupling of the PAC1 receptor to AC and PLC ultimately leads to the activation of a number of downstream signaling cascades. One of these is the MAPK/ERK pathway that seems to be linked with the neurotrophic role (neuroprotection and differentiation) of PACAP. PACAP has been shown to stimulate ERK1/2 activation through MEK activity in a PKA-dependent (Frödin et al., 1994; Tanaka et al., 1997b; Villalaba et al., 1997; Le Pechon-Vallee et al., 2000) and PKA-independent manner that may involve PKC and calcium (Barrie et al., 1997; Tanaka et al., 1997a; Lazarovici et al., 1998) or cAMP (Vaudry et al., 2002a, b, 2003). Although ERK can be activated in a PKA-dependent manner, studies have shown PACAP-mediated activation of some genes in bovine chromaffin cells to occur in a cAMP-dependent, ERK-dependent but PKA-independent manner (Hamelink et al., 2002a). Activation of Rap1 through cAMP-regulated guanine nucleotide exchange factor (Epac), may account for this observation, although Bos and coworkers report that specific activation of Epac does not lead to ERK activation in PC12 cells (Enserink et al., 2002; Bos, 2003). PACAP also activates p38 MAPK in PC12 cells, through activation of PLC, mobilization of intracellular calcium stores, and calcium influx through L-type VSCCs that seems to be cAMP-and PKA-independent (Sakai et al., 2002).

The $PAC1_{null}$ and $PAC1_{hop}$ receptor variants were recently shown to activate PLD, in addition to AC and PLC. The ability of the $PAC1_{hop}$ receptor to stimulate PLD activity was threefold greater than the $PAC1_{null}$ variant. In these studies the $PAC1_{hop}$ receptor variant was sensitive to brefeldin A, an inhibitor of the GTP exchange factor ARF, and was not affected by a PLC inhibitor, unlike the $PAC_{null}$ isoform (McCulloch et al., 2001). This finding suggests that the $PAC1_{null}$ variant was most likely stimulating PLD through a PLC–PKC mediated pathway, while $PAC1_{hop}$ operates through an ARF-dependent pathway. This was clearly demonstrated by the ability of ARF6 to specifically interact with the hop cassette of $PAC1_{hop}$ variant but not the $PAC1_{null}$ variant that lacks the cassette (Ronaldson et al., 2002). VPAC1 and VPAC2 receptors expressed

endogenously or exogenously in cell lines also activated PLD, which was sensitive to the ARF inhibitor, brefeldin A (BFA) that is also consistent with the physical association between VPAC receptors and ARF (McCulloch et al., 2000, 2001; Ronaldson et al., 2002).

It is well established that ARF6 can directly bind to and activate PLD that leads to the production of phosphatidic acid (PA) and generation of phosphatidylinositol 4,5-bisphosphate (PIP2) (Donaldson, 2003). These findings open up new possibilities for PAC1 receptor signaling through ARF6 or potentially other novel interacting proteins that may provide feedback to the existing pathways already associated with PAC1 signaling and also open up new pathways that can be directly attributed to PAC1–ARF6 signaling. For example ARF6 may play a role in PAC1-mediated secretion at the adrenomedullary synapse. It may also play a role in receptor trafficking and desensitization. PACAP is also thought to activate PI3K/Akt through transactivation of TrkA receptors, but the possibility now exists that the $PAC1_{hop}$ receptor can alone activate this pathway in an ARF6–PLD-dependent manner. This research provides the first line of clear evidence documenting a functional significance of the hop cassette in the PAC1 receptor, which could ultimately serve to couple the receptor to novel signal transduction pathways through interacting proteins.

As mentioned above, PACAP mediates intracellular calcium mobilization, triggered by activation of PLC, which in turn triggers IP3 production, thereby leading to release of $Ca^{2+}$ from IP3 receptor operated endoplasmic reticulum (ER) intracellular calcium stores. It has also been suggested that PACAP may be able to regulate calcium release through caffeine/ryanodine operated ER calcium stores (Tanaka et al., 1998). However the ability of PACAP to increase cytosolic calcium concentrations, in particular in secretory cells such as chromaffin cells and pancreatic islet cells, largely depends on extracellular calcium influx through VSCCs (Tanaka et al. 1996a, b; O'Farrell and Marley, 1997) that could also involve cAMP/PKA (Perrin et al., 1995; Przywara et al., 1996), PKC (Chik et al., 1996), or PLC (Taupenot et al., 1999). Calcium influx via PLC/IP3-dependent nonselective conductance changes at Trp channels (Beaudet et al., 2000), receptor-operated calcium stores (Morita et al., 2002), or a combination of several of these mechanisms (Isobe et al., 1993; Chik et al., 1996; Osipenko et al., 2000; Fukushima et al., 2001) may also occur. In chromaffin cells it is clear that cAMP stimulation alone is insufficient to cause acute/short-term secretion (1–30 min) (Eiden et al., 1998). PACAP-stimulated histamine release from ECL cells of the gut requires calcium influx mainly through L-type, and also receptor-operated calcium channels (Lindstrom et al., 2001). Therefore, even though the signaling networks involved in PACAP regulation of calcium influx leading to secretion are complex, they ultimately seem to involve primarily VSCCs, at least in the well-studied chromaffin and pancreatic cell systems.

The mechanism by which PACAP can regulate VSCCs and the receptor isoform with which it interacts to do so, remain to be elucidated. An important clue as to the PAC1 receptor isoform that couples to voltage-sensitive calcium channel opening can be found in bovine chromaffin cells, which are a primary site of PACAP-mediated calcium influx via VSCCs. Bovine chromaffin cells express the $PAC1_{hop}$ receptor variant (Tanaka et al., 1998), and significantly, this is the only PAC1 splice variant expressed in these primary neuroendocrine cells (Mustafa T, personal observation). Coupling of PAC1 to calcium influx may also be indirect if PACAP causes opening or closure of membrane cation channels other than VSCCs, and the subsequent depolarization leads to opening of the VSCCs. PACAP has been reported to cause depolarization of chromaffin cells (Tanaka et al., 1996b), suprachiasmatic neurons (Dziema and Obrietan, 2002), pinealocytes (Darvish and Russell, 1998), and superior cervical ganglion neurons (May et al., 1998; Beaudet et al., 2000) through mechanisms of depolarization that include inhibition of outwardly directed $K^+$ currents, induction of sodium-dependent membrane depolarization, and opening of nonselective plasma membrane cation channels.

In concluding this section, it should be remarked that receptor splice variants coexpressed in the same cell may couple to different signaling pathways or to the same pathway with different efficacy. Thus, future research may well uncover segregation of PAC1 receptor variants, for example, in lipid rafts, caveolae, and signaling vesicles where they are concentrated with different signaling molecules, defining the signaling route taken by the receptor variant. In addition, RAMP (receptor activity-modifying proteins) association, glycosylation, trafficking, and endocytosis kinetics may all affect receptor-signaling behavior. Recently, RAMP2 interaction with VPAC1 leading to enhanced IP3 signaling has been reported (Christopoulos et al., 2003), and similar interactions could occur with other members of the VIP/PACAP receptor family.

## 6 Concluding Remarks

### 6.1 Neurotransmitter, Hormone, Neuromodulatory, and Paracrine Functions

VIP and PACAP are unique members of the secretin superfamily in that they appear to act primarily as neurotransmitters and paracrine factors, and not as hormones. The question of whether neuropeptides are neuromodulators, affecting the action of a primary transmitter, or are primary neurotransmitters on their own, appears to be definitively answered in favor of the latter for PACAP at the adrenomedullary synapse, although it should be pointed out that even here PACAP may also have neuromodulatory properties, at least in some species (Inoue et al., 2000).

### 6.2 Importance of Mapping Ligand to Receptor In Vivo

Given the existence of overlapping receptors for VIP and PACAP and the affinity for VIP of some newly discovered N-terminal splice variants of the PAC1 receptor, the concerted study of PACAP-, VIP-, VPAC1-, VPAC2-, and PAC1-knockout mice, the precise chemical neuroanatomy of PACAPergic and VIPergic synapses in vivo, and the deployment of increasingly specific ligands for VIP/PACAP receptors will be required to unravel the role(s) of each neuropeptide in physiological regulation in the brain and periphery.

**Figure 21-3**
**Schematic illustration of PAC1 receptor splice variants and their sequences**

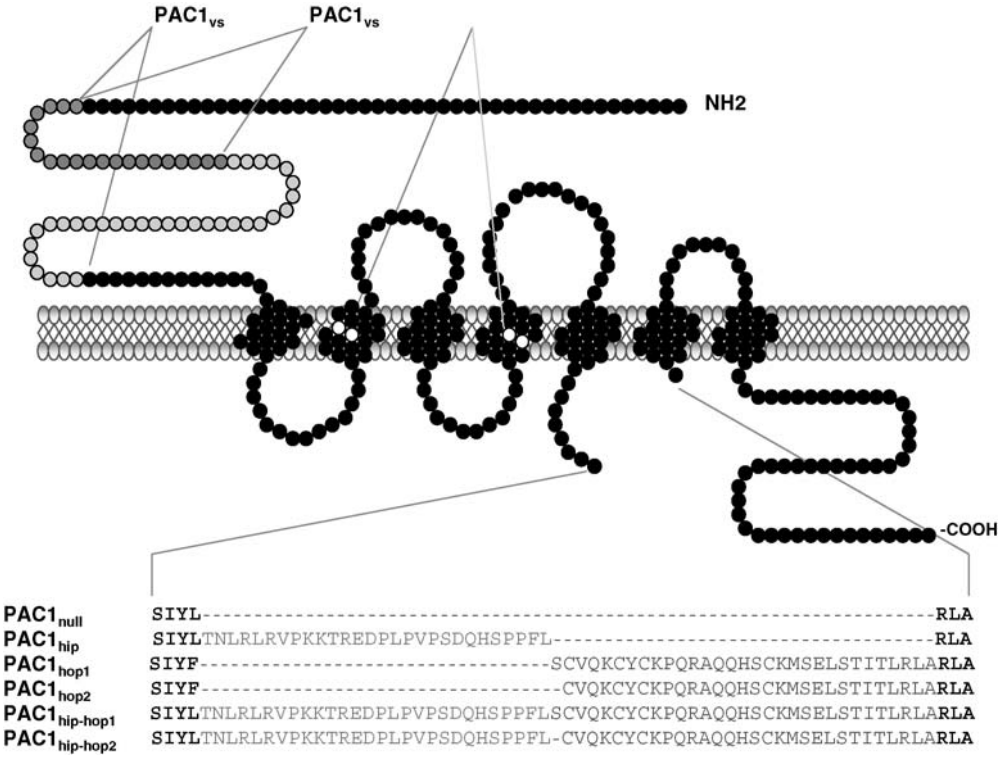
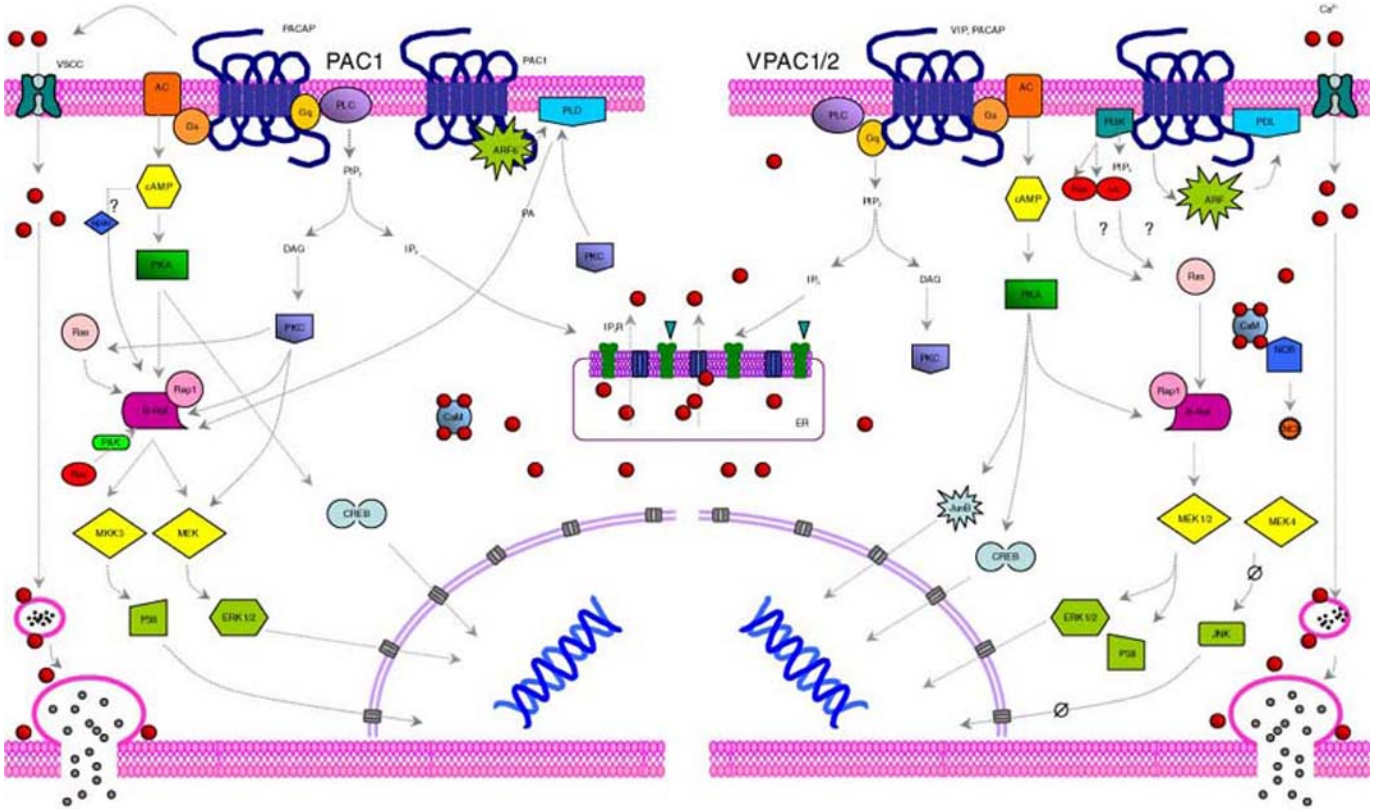


**Figure 21-4**

**Intracellular signaling by pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) a. PACAP signaling through the PAC1 receptor b. PACAP/VIP signaling through the VPAC1 and VPAC2 receptors**



### 6.3 Importance of Reconciling Knockout and Pharmacological Phenotypes

An unresolved conundrum is the seeming importance of VIP and PACAP in development, based on cell culture and in vivo pharmacological studies, and the absence of evidence of developmental impairment in VIP-, PACAP-, PAC1-, or VPAC2-knockout mice. Several possibilities exist. One is that VIP and PACAP act not in sculpting normal development, but as neuroprotective and/or antitumorigenic factors during development. In support of such a role, Vaudry et al. have observed enhanced neuronal damage upon oxidative (ethanol-induced) stress in neuron cultures from normal mice compared with PACAP-deficient mice (Vaudry et al., 2005). A second possibility is that, like the tyro receptor-related factors, VIP and PACAP act in concert, perhaps even with additional factors, to regulate cell differentiation (Lemke and Lu, 2003). The reports of Muller and Waschek and others of the concerted action of VIP and PACAP on cross-modulation of receptor expression, and proliferation and differentiation of neuroblastoma cell lines, support both possibilities (Waschek et al., 1995, 1997; Lelièvre et al., 1996; Waschek, 1996). Double and triple knockouts will be required to address this question via phenotyping of mice with appropriately engineered neuropeptide deficiencies.

### 6.4 Neuropeptides in the Evolutionary Vanguard?

One of the most daunting challenges of modern neuropeptide neurochemistry (indeed, of modern neurochemistry) is integrating the cellular and molecular biology of neuropeptide (first messenger) signaling with anatomically precise, chemically coded neurotransmission, and its linkage to specific aspects of highly integrated brain functions such as cognition and circadian regulation, homeostatic responses such as programmed cell death and neuroprotection, and events that involve hundreds of other effectors such as development, injury response, inflammation, and innate and acquired immunity. A general feature of the VIP/PACAP system seems to be that PACAP signals in a combinatorial fashion, through calcium, cAMP, IP3, and phosphatidic acid, to activate a very broad range of third messenger systems such as Akt, ERK, JNK and p38, PKC, PKA, and PI3K, with VIP signaling restricted mainly to activation of AC and PLC.

That PACAP signaling, mainly through PAC1, is so much more diffuse than that of VIP is consistent with its role in paraphysiological adaptation, i.e. its "emergency response" function in transducing, gating, and disseminating sensory stimuli including light and pain, and metabolic, environmental, and psychogenic stressors. Consistent with its anatomical position downstream of PACAP at two major synaptic systems, the retinohypothalamic and splanchnicoadrenomedullary systems, VIP plays an "effector" role as a secretagogue and vasodilator peripherally, and in synchronizing circadian activity centrally. The physiological role of VIP in the cerebral cortex remains somewhat mysterious and that of PACAP in complex centrally mediated processes including memory and conditioned behavior even more so. Focusing on the roles of neuropeptides and their receptors in stabilizing emergent adaptive homeostatic responses to altered environmental conditions, to create new mechanisms for chronic adaptation to previously intolerable stressors, may provide a way to discover species-dependent as well as conserved functions for these peptides in humans, other mammals, and other animals.

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## References

Adamou JE, Aiyar N, Van Horn S, Elshourbagy NA. 1995. Cloning and functional characterization of the human vasoactive intestinal peptide (VIP)-2 receptor. Biochem Biophys Res Commun 209: 385-392.

Agoston DV, Conlon JM, Whittaker VP. 1988. Selective depletion of the acetylcholine and vasoactive intestinal polypeptide of the guinea-pig myenteric plexus by differential mobilization of distinct transmitter pools. Exp Brain Res 72: 535-542.

Ajpru S, McArthur AJ, Piggins HD, Sugden D. 2002. Identification of PAC1 receptor isoform mRNAs by real-time PCR in rat suprachiasmatic nucleus. Brain Res Mol Brain Res 105: 29-37.

Akesson L, Ahren B, Edgren G, Degerman E. 2005. VPAC2-R mediates the lipolytic effects of pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide in primary rat adipocytes. Endocrinology 146: 744-750.

Anlauf M, Schäfer MK-H, Eiden LE, Weihe E. 2003. Chemical coding of the human gastrointestinal nervous system: cholinergic, VIPergic and catecholaminergic phenotypes. J Comp Neurol 459: 90-111.

Arimura A. 1992. Pituitary adenylate cyclase-activating polypeptide (PACAP): Discovery and current status of research. Regul Pept 37: 287-303.

Arimura A. 1998. Perspectives on pituitary adenylate cyclase activating polypeptide (PACAP) in the neuroendocrine, endocrine, and nervous systems. Jpn J Physiol 48: 301-331.

Arimura A. 2002. Editorial: impaired adaptive thermogenesis in pituitary adenylate cyclase-activating polypeptide-deficient mice. Endocrinology 143: 3715-3716.

Arimura A, Somogyvari-Vigh A, Miyata A, Mizuno K, Coy DH, et al. 1991. Tissue distribution of PACAP as determined by RIA: Highly abundant in the rat brain and testes. Endocrinology 129: 2787-2789.

Armstrong BD, Hu Z, Abad C, Yamamoto M, Rodriguez WI, et al. 2003. Lymphocyte regulation of neuropeptide gene expression after neuronal injury. J Neurosci Res 74: 240-247.

Asmus SE, Parsons S, Landis SC. 2000. Developmental changes in the transmitter properties of sympathetic neurons that innervate the periosteum. J Neurosci 20: 1495-1504.

Asnicar MA, Koster A, Heiman ML, Tinsley F, Smith DP, et al. 2002. Vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor 2 deficiency in mice results in growth retardation and increased basal metabolic rate. Endocrinology 143: 3994-4006.

Aton SJ, Colwell CS, Harmar AJ, Waschek J, Herzog ED. 2005. Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. Nat Neurosci 8(4): 476-483.

Babinski K, Bodart V, Roy M, De Léan A, Ong H. 1996. Pituitary adenylate-cyclase activating polypeptide (PACAP) evokes long-lasting secretion and de novo biosynthesis of bovine adrenal medullary neuropeptides. Neuropeptides 30: 572-582.

Barrie AP, Clohessy AM, Buensuceso CS, Rogers MV, Allen JM. 1997. Pituitary adenylyl cyclase-activating peptide stimulates extracellular signal-regulated kinase 1 or 2 (ERK1/2) activity in a ras-independent, mitogen-activated protein kinase/ERK kinase 1 or 2-dependent manner in PC12 cells. J Biol Chem 272: 19666-19671.

Basille M, Gonzalez BJ, Leroux P, Jeandel L, Fournier A, et al. 1993. Localization and characterization of PACAP receptors in the rat cerebellum during development: evidence for a stimulatory effect of PACAP on immature cerebellar granule cells. Neuroscience 57: 329-338.

Beaudet MM, Braas KM, May V. 1998. Pituitary adenylate cyclase activating polypeptide (PACAP) expression in sympathetic preganglionic projection neurons to the superior cervical ganglion. J Neurobiol 36: 325-336.

Beaudet MM, Parsons RL, Braas KM, May V. 2000. Mechanisms mediating pituitary adenylate cyclase-activating polypeptide depolarization of rat sympathetic neurons. J Neurosci 20: 7353-7361.

Bennett MR. 1997. Non-adrenergic non-cholinergic (NANC) transmission to smooth muscle: 35 years on. Prog Neurobiol 52: 159-195.

Berisha HI, Bratut M, Bangale Y, Colasurdo G, Paul S, Said SI. 2002. New evidence for transmitter role of VIP in the airways: Impaired relaxation by a catalytic antibody. Pulm Pharmacol Therap 15: 121-127.

Bloom SR, Christofides ND, Delamarter J, Buell G, Kawashima E, Polak JM. 1983. Diarrhoea in vipoma patients associated with cosecretion of a second active peptide (peptide histidine isoleucine) explained by single coding gene. Lancet 2: 1163-1165.

Bloom SR, Yiangou Y, Polak JM. 1988. Vasoactive intestinal peptide secreting tumors. Pathophysiological and clinical correlations. Ann N Y Acad Sci 527: 518-527.

Bodnar M, Sarrieau A, Deschepper CF, Walker CD. 1997. Adrenal vasoactive intestinal peptide participates in neonatal corticosteroid production in the rat. Am J Physiol 273: R1163-1172.

Bornstein SR, Haidan A, Ehrhart-Bornstein M. 1996. Cellular communication in the neuroadrenocortical axis: role of vasoactive intestinal polypeptide (VIP). Endocrine Res 22: 819-829.

Bos JL. 2003. Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol 4: 733-738.

Brabet P, Jamen F, Rodriguez-Henche N, Bertrand G, Bockaert J. 2003. PACAP receptor knockout and transgenics: what have we learnt? Pituitary adenylate cyclase-activating polypeptide. Vaudry H, Arimura A, editors. Norwell, Massachusetts: Kluwer Academic Publishers; pp. 323-346.

Brenneman DE, Eiden LE. 1986. Vasoactive intestinal peptide and electrical activity influence neuronal survival. Proc Natl Acad Sci USA 83: 1159-1162.

Brenneman DE, Neale EA, Foster GA, d'Autremont SW, Westbrook GL. 1987. Nonneuronal cells mediate neurotrophic action of vasoactive intestinal peptide. J Cell Biol 104: 1603-1610.

Bryant MG, Polak MM, Modlin I, Bloom SR, Albuquerque RH, et al. 1976. Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter substance. Lancet 1: 991-993.

Burnstock G, Hökfelt T, Gershon MD, Iversen LL, Kosterlitz HW, et al. 1979. Non-adrenergic, non-cholinergic autonomic neurotransmission mechanisms. Neurosci Res Prog Bull 17: 377-519.

Chen D, Buchanan GF, Ding JM, Hannibal J, Gillette MU. 1999. Pituitary adenylyl cyclase-activating peptide: a pivotal modulator of glutamatergic regulation of the suprachiasmatic circadian clock. Proc Natl Acad Sci USA 96: 13468-13473.

Chen Y, Hamelink C, Chen Y, Hallenbeck JM, Eiden LE. 2002. Mechanism for neuroprotective effects of PACAP in cerebral ischemic insult in PACAP-deficient mice, Washington, DC: Society for Neuroscience, 2002. Online Abstract Viewer/Itinerary Planner 2002.

Chen Y, Hamelink C, Vaudry D, Xiang C, Brownstein M, et al. 2004. Expression profiling of cerebrocortical transcripts during middel cerebral artery occlusion and treatment with pituitary adenylate cyclase-activating polypeptide (PACAP) in the mouse. Pharmacology of cerebral ischemia. Krieglstein J, Klumpp S, editors. Stuttgart, Germany: Medpharm Scientific Publishers; pp. 267-277.

Chen Y, Hamelink C, Vaudry D, Xiang C, Brownstein MJ, et al. 2005. Expression profiling of cerebrocortical transcripts during middle cerebral artery occlusion and treatmenttwith pituitary adenylate cyclase activating polypeptide (PACAP) in the mouse. Pharmacology of Cerebral Ischemia. Klumpp S, editor. Stuttgart, Germany: MedPharm Scientific Publishers.

Cherruau M, Morvan FO, Schirar A, Saffar JL. 2003. Chemical sympathectomy-induced changes in TH-, VIP-, and CGRP-immunoreactive fibers in the rat mandible periosteum: influence on bone resorption. J Cell Physiol 194: 341-348.

Chik CL, Li B, Ogiwara T, Ho AK, Karpinski E. 1996. PACAP modulates L-type Ca2+ channel currents in vascular smooth muscle cells: involvement of PKC and PKA. FASEB J 10: 1310-1317.

Chow BK-C, Pang RTK, Ng SS-M. 2003. Molecular evolution of PACAP precursor and PACAP receptors. Pituitary adenylate cyclase-activating polypeptide. Vaudry H, Arimura A, editors. Norwell, Massachusetts: Kluwer Academic Publishers; pp. 25-47.

Christopoulos A, Christopoulos G, Morfis M, Udawela M, Laburthe M, et al. 2003. Novel receptor partners and function of receptor activity-modifying proteins. J Biol Chem 278: 3293-3297.

Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, et al. 2003. Disrupted circadian rhythms in VIP and PHI deficient mice. Am J Physiol Regul Integr Comp Physiol 285: R939-R949.

Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, et al. 2004. Selective deficits in the circadian light response in mice lacking PACAP. Am J Physiol Regul Integr Comp Physiol 287: R1194-R1201.

Colwell CS, Waschek JA. 2001. Role of PACAP in circadian function of the SCN. Regul Pept 102: 49-68.

Couvineau A, Rouyer-Fessard C, Darmoul D, Maoret JJ, Carrero I, et al. 1994. Human intestinal VIP receptor: cloning and functional expression of two cDNA encoding proteins with different N-terminal domains. Biochem Biophys Res Commun 200: 769-776.

Couvineau A, Rouyer-Fessard C, Voisin T, Laburthe M. 1990. Functional and immunological evidence for stable association of solubilized vasoactive-intestinal-peptide receptor and stimulatory guanine-nucleotide-binding protein from rat liver. Eur J Biochem 187: 605-609.

Cucavelli E, Svoboda M, DeNeef P, DiPaola E, Bollen A, Dubeaux C, Vilardaga JP, Wadbroeck M, Robberecht P. 1995. Pharmacological properties of two recombinant splice variants of PACAP type receptor, translected and stably expressed in CHO cells. Eur J Pharm 288: 259-267.

Cummings KJ, Pendlebury JD, Jirik FR, Sherwood NM, Wilson RJ. 2004a. A SIDS-like phenotype is associated with reduced respiratory chemoresponses in PACAP deficient neonatal mice. Adv Exp Med Biol 551: 77-83.

Cummings KJ, Pendlebury JD, Sherwood NM, Wilson RJ. 2004b. Sudden neonatal death in PACAP-deficient mice is associated with reduced respiratory chemoresponse and susceptibility to apnoea. J Physiol 555: 15-26.

Cutler DJ, Haraura M, Reed HE, Shen S, Sheward WJ, et al. 2003. The mouse VPAC2 receptor confers suprachiasmatic nuclei cellular rhythmicity and responsiveness to vasoactive intestinal polypeptide in vitro. Eur J Neurosci 17: 197-204.

Daniel PB, Kieffer TJ, Leech CA, Habener JF. 2001. Novel alternatively spliced exon in the extracellular ligand-binding domain of the pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 receptor (PAC1R) selectively increases ligand affinity and alters signal transduction coupling during spermatogenesis. J Biol Chem 276: 12938-12944.

Darvish N, Russell JT. 1998. Neurotransmitter-induced novel modulation of a nonselective cation channel by a cAMP-dependent mechanisms in rat pineal cells. J Neurophysiol 79: 2546-2556.

Dautzenberg FM, Mevenkamp G, Wille S, Hauger RL. 1999. N-terminal splice variants of the type I PACAP receptor:

isolation, characterization and ligand binding/selectivity determinants. J Neuroendocrinol 11: 941-949.

Delgado M, Abad C, Martinez C, Juarranz MG, Leceta J, et al. 2003. PACAP in immunity and inflammation. Ann N Y Acad Sci 992: 141-157.

Delgado M, Abad C, Marinez C, Leceta J, Gomariz RP. 2001. Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. Nat Med 7: 563-568.

Delgado M, Martinez C, Pozo D, Calvo JR, Leceta J, et al. 1999a. Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP) protect mice from lethal endotoxemia through the inhibition of TNFalpha and IL6. J Immunol 162: 1200-1205.

Delgado M, Pozo D, Ganea D. 2004. The significance of vasoactive intestinal peptide in immunomodulation. Pharmacol Rev 56: 249-290.

Delgado M, Pozo D, Martinez C, Leceta J, Calvo JR, et al. 1999b. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibit endotoxin-induced TNF-alpha production by macrophages: in vitro and in vivo studies. J Immunol 162: 2358-2367.

Deschodt-Lanckman M, Robberecht P, Christophe J. 1977. Characterization of VIP-sensitive adenylate cyclase in guinea pig brain. FEBS Lett 83: 76-80.

DiCicco-Bloom E, Lu NR, Pintar JE, Wang JW. 1998. The PACAP ligand/receptor system regulates cerebral cortical neurogenesis. Ann N Y Acad Sci 865: 74-289.

DiCicco-Bloom E, Deutsch PJ, Maltzman J. Zhang J, Pintar JE, et al. 2000. Autocrine expression and ontogenetic functions of the PACAP ligand/receptor system during sympathetic development. Dev Biol 219: 197-213.

Dickinson T, Fleetwood-Walker SM. 1999. VIP and PACAP: very important in pain? TIPS 20: 324-329.

Diehl NL, Kermode JC, Shreeve SM. 1996. Direct evidence for functional coupling of the vasoactive intestinal peptide receptor to Gi3 in native lung membranes. Mol Pharmacol 50: 624-630.

Donaldson JG. 2003. Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. J Biol Chem 278: 41573-41576.

Duckles SP, Said SI. 1982. Vasoactive intestinal peptide as a neurotransmitter in the cerebral circulation. Eur J Pharmacol 78: 371-374.

Dun NJ, Tang H, Dun SL, Huang R, Dun EC, et al. 1996. Pituitary adenylate cyclase activating polypeptide-immunoreactive sensory neurons innervate rat adrenal medulla. Brain Res 716: 11-21.

Dziema H, Obrietan K. 2002. PACAP potentiates L-type calcium channel conductance in suprachiasmatic nucleus neurons by activating the MAPK pathway. J Neurophysiol 88: 1374-1386.

Eckenstein F, Baughman RW. 1984. Two types of cholinergic innervation in cortex, one co-localized with vasoactive intestinal polypeptide. Nature 309: 153-155.

Edwards AV, Jones CT. 1993. Adrenal cortical and medullary responses to acetylcholine and vasoactive intestinal peptide in conscious calves. J Physiol 468: 515-527.

Edwards AV, Jones CT. 1994. Adrenal responses to the peptide PACAP in conscious functionally hypophysectomized calves. Am J Physiol 266: E870-E876.

Eiden LE. 1987. The enkephalin-containing cell: strategies for polypeptide synthesis and secretion throughout the neuroendocrine system. Cell Mol Neurobiol 7: 339-352.

Eiden LE. 2003. Signaling during exocytosis. Handbook of cell signaling. Bradshaw R, Dennis E, editors. New York: Academic Press; Vol. 3, pp. 375-392.

Eiden LE, Anouar Y, Hsu C-M, Mac Arthur L, Hahm SH. 1998. Transcription regulation coupled to calcium and protein kinase signaling systems through TRE- and CRE-like sequences in neuropeptide genes. Adv Pharmacol 42: 264-269.

Eiden LE, Eskay RL, Scott J, Pollard H, Hotchkiss AJ. 1983. Primary cultures of bovine chromaffin cells synthesize and secrete vasoactive intestinal polypeptide (VIP). Life Sci 33: 687-693.

Eiden LE, Giraud P, Dave J, Hotchkiss JA, Affolter H-U. 1984. Nicotinic receptor stimulation activates both enkephalin release and biosynthesis in adrenal chromaffin cells. Nature 312: 661-663.

Eiden LE, Hokfelt T, Brownstein MJ, Palkovits M. 1985. Vasoactive intestinal polypeptide afferents to the bed nucleus of the stria terminalis in the rat: an immunohistochemical and biochemical study. Neuroscience 15: 999-1013.

Eipper BA, Mains RE, Glembotski C. 1983. Identification in pituitary tissue of a peptide alpha-amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper and ascorbic acid. Proc Natl Acad Sci USA 80: 5144-5148.

Elde R, Haber S, Ho R, Holets V, de Lanerolle N, et al. 1980. Interspecies conservation and variation in peptidergic neurons. Peptides 1: 21-26.

Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. 2000. The sympathetic nerve — an integrative interface between two supersystems: the brain and the immune system. Pharmacol Rev 52: 595-638.

Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, et al. 2002. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol 4: 901-906.

Fahrenkrug J. 1979. Vasoactive intestinal polypeptide: measurement, distribution and putative neurotransmitter function. Digestion 19: 149-169.

Feany MB, Quinn WG. 1995. A neuropeptide gene defined by the Drosophila memory mutant amnesiac. Science 268: 869-873.

Feldman SA, Eiden LE. 2003. The chromogranins: Their roles in secretion from neuroendocrine cells and as markers for neuroendocrine neoplasia. Endocr Pathol 14: 3-23.

Filipsson K, Sundler F, Ahren B. 1999. PACAP is an islet neuropeptide which contributes to glucose-stimulated insulin secretion. Biochem Biophys Res Commun 256: 664-667.

Frechilla D, Garcia-Osta A, Palacios S, Cenarruzabeitia E, Del Rio J. 2001. BDNF mediates the neuroprotective effect of PACAP-38 on rat cortical neurons. Neuroreport 12: 919-923.

Frödin M, Peraldi P, Van Obberghen E. 1994. Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells. J Biol Chem 269: 6207-6214.

Fukushima Y, Nagayama T, kawashima H, Hikichi H, Yoshida M, et al. 2001. Role of calcium channels and adenylate cyclase in the PACAP-induced adrenal catecholamine secretion. Am J Physiol Regul Integr Comp Physiol 281: R495-R501.

Geng G, Gaspo R, Trabelsi F, Yamaguchi N. 1997. Role of L-type $Ca^{2+}$ channel in PACAP-induced adrenal catecholamine release in vivo. Regul Integr Comp Physiol 42: R1339-R1345.

Ghatei M.A, Takahashi K, Suzuki Y, Gardiner J, Jones PM, Bloom SR. 1993. Distribution, molecular characterization of pituitary adenylate cyclase-activating polypeptide and its precursor encoding messenger RNA in human and rat tissues. J Endocrinol 136: 159-166.

Giachetti A, Said SI, Reynolds RC, Koniges FC. 1977. Vasoactive intestinal polypeptide in brain: localization in and release from isolated nerve terminals. Proc Natl Acad Sci USA 74: 3424-3428.

Girard BM, May V, Bora SH, Fina F, Braas KM. 2002. Regulation of neurotrophic peptide expression in sympathetic neurons: quantitative analysis using radioimmunoassay and real-time quantitative polymerase chain reaction. Regul Pept 109: 89-101.

Goetzl EJ, Voice JK, Shen S, Dorsam G, Kong Y, et al. 2001. Enhanced delayed-type hypersensitivity and diminished immediate-type hypersensitivity in mice lacking the inducible VPAC(2) receptor for vasoactive intestinal peptide. Proc Natl Acad Sci USA 98: 13854-13859.

Gonzalez BJ, Basille M, Mei YA, Vaudry D, Fournier A, et al. 1996. Ontogeny of PACAP and PACAP receptors in the rat brain: role of PACAP in the cerebellum during development. Ann N Y Acad Sci 805: 302-313.

Gourlet P, Vandermeers A, Vertongen P, Rathe J, De Neef P, Cnudde J, Waelbroeck M, Robberecht P. 1997a. Development of high affinity selective VIP1 receptor agonists. Peptides 18: 1539-1545.

Gourlet P, Vertongen P, Vandermeers A, Vandermeers-Piret MC, Rathe J, et al. 1997b. The long-acting vaso active intestinal polypeptide agonist RO 25-1553 is highly selective of the VIP2 receptor subclass. Pept 18: 403-408.

Goyal RK, Rattan S, Said SI. 1980. VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones. Nature 288: 378-380.

Gray SL, Cummings KJ, Jirik FR, Sherwood NM. 2001. Targeted disruption of the pituitary adenylate cyclase-activating polypeptide gene results in early postnatal death associated with dysfunction of lipid and carbohydrate metabolism. Mol Endocri 15: 1739-1747.

Gray SL, Yamaguchi N, Vencova P, Sherwood NM. 2002. Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. Endocrinology 143: 3946-3954.

Greengard P. 2001. The neurobiology of slow synaptic transmission. Science 294: 1024-1030.

Gressens P, Hill JM, Gozes I, Fridkin M, Brenneman DE. 1993. Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos. Nature 362: 155-158.

Grimaldi M, Cavallaro S. 1999. Functional and molecular diversity of PACAP/VIP receptors in cortical neurons and type I astrocytes. Eur J Neurosci 11: 2767-2772.

Grinninger C, Wang W, Oskoui KB, Voice JK, Goetzl EJ. 2004. A natural variant type II G protein-coupled receptor for vasoactive intestinal peptide with altered function. J Biol Chem 279: 40259-40262.

Gudermann T, Schöneberg T, Schultz G. 1997. Functional and structural complexity of signal transduction via G-protein-coupled receptors. Annu Rev Neurosci 20: 399-427.

Habecker BA, Asmus SE, Francis N, Landis SC. 1997. Target regulation of VIP expression in sympathetic neurons. Ann N Y Acad Sci 814: 198-208.

Hahm SH, Hsu C-M, Eiden LE. 1998. PACAP activates calcium influx-dependent and -independent pathways to couple met-enkephalin secretion and biosynthesis in chromaffin cells. J Mol Neurosci 11: 1-15.

Hamelink C, Lee H-W, Grimaldi M, Eiden LE. 2002a. Coincident elevation of cyclic AMP and calcium influx by PACAP-27 synergistically regulates VIP gene transcription through a novel PKA-independent signaling pathway. J Neurosci 22: 5310-5320.

Hamelink C, Tjurmina O, Damadzic R, Young WS, Weihe E, Lee HW, Eiden LE. 2002b. Pituitary adenylate cyclase activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. Proc Natl Acad Sci USA 99: 461-466.

Hamelink C, Weihe E, Eiden LE. 2003. PACAP: an 'emergency response' co-transmitter in the adrenal medulla. Pituitary adenylate cyclase-activiting polypeptide. Vaudry H, Arimura A, editors. New York: Kluwer Academic Press; pp. 227-250.

Hannibal J, Hindersson P, Knudsen SM, Georg B, Fahrenkrug JSM, et al. 2002. The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. J Neurosci 22: RC191.

Hannibal J, Jamen F, Nielsen HS, Journot L, Brabet P, et al. 2001. Dissociation between light-induced phase shift of the circadian rhythm and clock gene expression in mice lacking the pituitary adenylate cyclase activating polypeptide type 1 receptor. J Neurosci 21: 4883-4890.

Hannibal J, Mikkelsen JD, Clausen H, Holst JJ, Wulff BS, et al. 1995. Gene expression of pituitary adenylate cyclase activating polypeptide (PACAP) in the rat hypothalamus. Regul Pept 55: 133-148.

Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, et al. 1998. International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol Rev 50: 265-270.

Harmar AJ, Marston HM, Shen S, Spratt C, West KM, et al. 2002. The VPAC2 receptor is essential for circadian function in the mouse suprachiasmatic nuclei. Cell 109: 497-508.

Hashimoto H, Ishihara T, Shigemoto R, Mori K, Nagata S. 1993. Molecular cloning and tissue distribution of a receptor for pituitary adenylate cyclase-activating polypeptide. Neuron 11: 333-342.

Hashimoto H, Shintani N, Tanaka K, Mori W, Hirose M, et al. 2001. Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). Proc Natl Acad Sci USA 98: 13355-13360.

Haycock JW. 1996. Short- and long-term regulation of tyrosine hydroxylase in chromaffin cells by VIP and PACAP. Ann N Y Acad Sci 805: 219-230.

Henriksen JH, de Muckadell OB. 2000. Secretin, its discovery, and the introduction of the hormone concept. Scand J Clin Lab Invest 60: 463-471.

Hökfelt T, Lundberg JM, Schultzberg M, Fahrenkrug J. 1981. Immunohistochemical evidence for a local VIP-ergic neuron system in the adrenal gland of the rat. Acta Physiol Scand 113: 575-576.

Hokfelt T, Zhang X, Wiesenfeld-Hallin Z. 1994. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. Trends Neurosci 17: 22-30.

Holgert H, Holmberg K, Hannibal J, Fahrenkrug J, Brimijoin S, et al. 1996. PACAP in the adrenal gland—relationship with choline acetyltransferase, enkephalin and chromaffin cells and effects of immunological sympathectomy. Neuroreport 20: 297-301.

Holzwarth MA. 1984. The distribution of vasoactive intestinal peptide in the rat adrenal cortex and medulla. J Auton Nerv Sys 11: 269-283.

Hosoya M, Onda H, Ogi K, Masuda Y, Miyamoto Y, et al. 1993. Molecular cloning and functional expression of rat cDNAs encoding the receptor for pituitary adenylate cyclase activating polypeptide (PACAP). Biochem Biophys Res Commun 194: 133-143.

Inoue M, Fijishiro N, Ogawa K, Muroi M, Sakamoto Y, et al. 2000. Pituitary adenylate cyclase-activating polypeptide may function as a neuromodulator in guinea-pig adrenal medulla. J Physiol 528(3): 473-487.

Ishihara T, Shigemoto R, Mori K, Takahashi K, Nagata S. 1992. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. Neuron 8: 811-819.

Isobe K, Hakai T, Takuwa Y. 1993. $Ca^{2+}$-dependent stimulatory effect of pituitary adenylate cyclase-activating polypeptide on catecholamine secretion from cultured porcine adrenal chromaffin cells. Endocrinology 132: 1757-1765.

Itri J, Michel S, Waschek JA, Colwell CS. 2004. Circadian rhythm in inhibitory synaptic transmission in the mouse suprachiasmatic nucleus. J Neurophysiol 92: 311-319.

Jamen F, Persson K, Bertrand G, Rodriguez-Henche N, Puech R, et al. 2000a. PAC1 receptor-deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. J Clin Invest 105: 1307-1315.

Jamen F, Puech R, Bockaert J, Brabet P, Bertrand G. 2002. Pituitary adenylate cyclase-activating polypeptide receptors mediating insulin secretion in rodent pancreatic islets are coupled to adenylate cyclase but not to PLC. Endocrinology 143: 1253-1259.

Jamen F, Rodriguez-Henche N, Pralong F, Jegou B, Gaillard R, et al. 2000b. PAC1 null females display decreased fertility. Ann N Y Acad Sci 921: 400-404.

Jessop DS. 2002. Neuropeptides in the immune system: functional roles in health and disease. Front Horm Res 29: 50-68.

Jongsma H, Pettersson LM, Zhang Y, Reimer MK, Kanje M, et al. 2001. Markedly reduced chronic nociceptive response in mice lacking the PAC1 receptor. Neuroreport 12: 2215-2219.

Journot L, Waeber C, Pantaloni C, Holsboer F, Seeburg PH, et al. 1995. Differential signal transduction by six splice variants of the pituitary adenylate cyclase-activating peptide (PACAP) receptor. Biochem Soc Trans 23: 133-137.

Kermode JC, De Luca AW, Zilberman A, Valliere J, Shreeve SM. 1992. Evidence for the formation of a functional complex between vasoactive intestinal peptide, its receptor, and Gs in lung membranes. J Biol Chem 267: 3382--3388.

Kim W-K, Kan Y, Ganea D, Hart RP, Gozes I, et al. 2000. Vasoactive intestinal peptide and pituitary adenylyl cyclase-activating polypeptide inhibit tumor necrosis factor-alpha production in injured spinal cord and in activated microglia via a cAMP-dependent pathway. J Neurosci 20: 3622-3630.

Klimaschewski L. 1997. VIP - a 'very important peptide' in the sympathetic nervous system? Anat Embryol (Berl) 196: 269-277.

Koves K, Kantor O, Vereczki V, Kausz M, Nemeskeri A, et al. 2000. PACAP and VIP in the photoneuroendocrine system. From the retina to the pituitary gland. Ann N Y Acad Sci 921: 321-326.

Lamouche S, Martineau D, Yamaguchi N. 1999. Modulation of adrenal catecholamine release by PACAP in vivo. Regul Integr Comp Physiol 45: R162-R170.

Langer I, Gregoire F, Nachtergael I, De Neef P, Vertongen P, et al. 2004. Hexanoylation of a VPAC2 receptor-preferring ligand markedly increased its selectivity and potency. Peptides 25: 275-278.

Lazarovici P, Jiang H, Fink Jr. D 1998. The 38-amino-acid form of pituitary adenylate cyclase-activating polypeptide induces neurite outgrowth in PC12 cells that is dependent on protein kinase C and extracellular signal-regulated kinase but not on protein kinase A, nerve growth factor receptor tyrosine kinase, p21ras G protein, and pp60c-src cytoplasmic tyrosine kinase. Mol Pharmacol 54: 547-558.

Le Pechon-Vallee C, Magalon K, Rasolonjanahary R, Enjalbert A, Gerard C. 2000. Vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptides stimulate mitogen-activated protein kinase in the pituitary cell line GH4C1 by a 3′,5′-cyclic adenosine monophosphate pathway. Neuroendocrinology 72: 46-56.

Lelièvre V, Becq-Giraudon L, Meunier A-C, Muller J-M. 1996. Switches in the expression and function of PACAP and VIP receptors during phenotypic interconversion in human neuroblastoma cells. Neuropeptides 30: 313-322.

Lelievre V, Hu Z, Byun JY, Ioffe Y. Waschek JA. 2002. Fibroblast growth factor-2 converts PACAP growth action on embryonic hindbrain precursors from stimulation to inhibition. J Neurosci Res 67: 566-573.

Lemke G, Lu Q. 2003. Macrophage regulation by Tyro 3 family receptors. Curr Opin Immunol 15: 31-36.

Lerner EA, Shoemaker CB. 1992. Maxadilan. Cloning and functional expression of the gene encoding this potent vasodilator peptide. J Biol Chem 267: 1062-1066.

Lindberg PT, Hamelink C, Damadzic R, Eiden LE, Gillette MU. 2004. Pituitary adenylate cyclase-activating peptide plays a time-dependent role in light-induced phase shifts of circadian rhythms. Abstract Viewer/Itinerary Planner. Washington, DC: Society for Neuroscience, 2004. Online. Program No. 195.14. (2004)

Lindstrom E, Eliasson L, Bjorkqvist M, Hakanson R. 2001. Gastrin and the neuropeptide PACAP evoke secretion from rat stomach histamine-containing (ECL) cells by stimulating influx of $Ca^{2+}$ through different $Ca^{2+}$ channels. J Physiol 535: 663-677.

Loren I, Emson PC, Fahrenkrug J, Bjorklund A, Alumets J, et al. 1979. Distribution of vasoactive intestinal polypeptide in the rat and mouse brain. Neuroscience 4: 1953-1976.

Lundberg JM, Anggard A, Fahrenkrug J, Hökfelt T, Mutt V. 1980. Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: functional significance of coexisting transmitters for vasodilation and secretion. Proc Natl Acad Sci USA 77: 1651-1655.

Lutz EM, Sheward WJ, West KM, Morrow JA, Fink G, et al. 1993. The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. FEBS Lett 334: 3-8.

Lyu RM, Germano PM, Choi JK, Le SV, Pisegna JR. 2000. Identification of an essential amino acid motif within the C terminus of the pituitary adenylate cyclase-activating polypeptide type I receptor that is critical for signal transduction but not for receptor internalization. J Biol Chem 275: 36134-36142.

Mabuchi T, Shintani N, Matsumura S, Okuda-Ashitaka E, Hashimoto H, et al. 2004. Pituitary adenylate cyclase-activating polypeptide is required for the development of spinal sensitization and induction of neuropathic pain. J Neurosci 24: 7283-7291.

Mac Kenzie CJ, Lutz EM, Johnson MS, Robertson DN, Holland PJ, et al. 2001. Mechanisms of phospholipase C activation by the vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating polypeptide type 2 receptor. Endocrinology 142: 1209-1217.

Magistretti PJ, Manthorpe M, Bloom FE, Varon S. 1983. Functional receptors for vasoactive intestinal polypeptide in cultured astroglia from neonatal rat brain. Regul Pept 6: 71-80.

Mahapatra NR, Mahata M, O'Connor DT, Mahata SK. 2003. Secretin activation of chromogranin A gene transcription. Identification of the signaling pathways in cis and in trans. J Biol Chem 278: 19986-19994.

Martin M, Otto C, Santamarta MT, Torrecilla M, Pineda J, et al. 2003. Morphine withdrawal is modified in pituitary adenylate cyclase-activating polypeptide type I-receptor-deficient mice. Brain Res Mol Brain Res 110: 109-118.

Martinez. 2005. Analysis of the role of the PAC1 receptor in neutrophil recruitment, acute-phase response, and nitric oxide production in septic shock. J Leukoc Biol (in press): 1–10.

Martinez C, Abad C, Delgado M, Arranz A, Juarranz MG, et al. 2002. Anti-inflammatory role in septic shock of

pituitary adenylate cyclase-activating polypeptide receptor. Proc Natl Acad Sci USA 99: 1053-1058.

Masuo Y, Tokito F, Matsumoto Y, Shimamoto N, Fujino M. 1994. Ontogeny of pituitary adenylate cyclase-activating polypeptide (PACAP) and its binding sites in the rat brain. Neurosci Lett 170: 43-46.

Matsuyama S, Matsumoto A, Hashimoto H, Shintani N, Baba A. 2003. Impaired long-term potentiation in vivo in the dentate gyrus of pituitary adenylate cyclase-activating polypeptide (PACAP) or PACAP type 1 receptor-mutant mice. Neuroreport 14: 2095-2098.

May V, Beaudet MM, Parsons RL, Hardwick JC, Gauthier EA, et al. 1998. Mechanisms of pituitary adenylate cyclase activating polypeptide (PACAP)-induced depolarization of sympathetic superior cervical ganglion (SCG) neurons. Ann N Y Acad Sci 865: 164-175.

McCulloch DA, Lutz EM, Johnson MS, Mac Kenzie CJ, Mitchell R. 2000. Differential activation of phospholipase D by VPAC and PAC1 receptors. Ann N Y Acad Sci 921: 175-185.

McCulloch DA, Lutz EM, Johnson MS, Robertson DN, Mac Kenzie CJ, et al. 2001. ADP-ribosylation factor-dependent phospholipase D activation by VPAC receptors and a PAC(1) receptor splice variant. Mol Pharmacol 59: 1523-1532.

Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, et al. 1989. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. Biochem Biophys Res Commun 164: 567-574.

Mizushima H, Banks WA, Dohi K, Shioda S, Matsumoto H, Matsumoto K. 1999. The effect of cardiac arrest on the permeability of the mouse blood-brain and blood-spinal cord barrier to pituitary adenylate cyclase activating polypeptide (PACAP). Peptides 20: 1337-1340.

Mohney RP, Zigmond RE. 1998. Vasoactive intestinal peptide enhances its own expression in sympathetic neurons after injury. J Neurosci 18: 5285-5293.

Moller K, Reimer M, Ekblad E, Hannibal J, Fahrenkrug J, et al. 1997. The effects of axotomy and preganglionic denervation on the expression of pituitary adenylate cyclase activating peptide (PACAP), galanin and PACAP type 1 receptors in the rat superior cervical ganglion. Brain Res 775: 166-182.

Moller K, Zhang Y-Z, Hakanson R, Luts A, Sjölund B, et al. 1993. Pituitary adenylate cyclase activating peptide is a sensory neuropeptide: immunocytochemical and immunochemical evidence. Neuroscience 57: 725-732.

Moller M, Baeres FM. 2003. PACAP-containing intrapineal nerve fibers originate predominantly in the trigeminal ganglion: a combined retrograde tracing- and immunohistochemical study of the rat. Brain Res 984: 160-169.

Morales MA, Holmberg K, Xu ZQ, Cozzari C, Hartman BK, et al. 1995. Localization of choline acetyltransferase in rat peripheral sympathetic neurons and its coexistence with nitric oxide synthase and neuropeptides. Proc Natl Acad Sci USA 92: 11819-11823.

Morita K, Sakakibara A, Kitayama S, Kumagai K, Tanne K, Dohi T. 2002. Pituitary adenylate cyclase-activating polypeptide induces a sustained increase in intracellular free Ca(2+) concentration and catechol amine release by activating Ca(2+) influx via receptor-stimulated Ca(2+) entry, independent of store-operated Ca(2+) channels, and voltage-dependent Ca(2+) channels in bovine adrenal medullary chromaffin cells. J Pharmacol Exp Ther 302: 972-982.

Moro O, Lerner EA. 1997. Maxadilan, the vasodilator from sand flies, is a specific pituitary adenylate cyclase activating peptide type I receptor agonist. J Biol Chem 272: 966-970.

Mulder H, Jongsma H, Zhang Y, Gebre-Medhin S. Sundler F, et al. 1999. Pituitary adenylate cyclase-activating polypeptide and islet amyloid polypeptide in primary sensory neurons. Functional implications from plasticity in expression on nerve injury and inflammation. Mol. Neurobiol 19: 229-253.

Mulder H, Uddman R, Moller K, Zhang Y-Q, Ekblad E, et al. 1994. Pituitary adenylate cyclase activating polypeptide expression in sensory neurons. Neuroscience 63: 307-312.

Nahin RL, Ren K, De Leon M, Ruda M. 1994. Primary sensory neurons exhibit altered gene expression in a rat model of neuropathic pain. Pain 58: 95-108.

Nakata M, Kohno D, Shintani N, Nemoto Y, Hashimoto H, et al. 2004. PACAP deficient mice display reduced carbohydrate intake and PACAP activates NPY-containing neurons in the rat hypothalamic arcuate nucleus. Neurosci Lett 370: 252-256.

Neves SR, Ram PT, Iyengar R. 2002. G protein pathways. Science 296: 1636-1639.

Nicole P, Lins L, Rouyer-Fessard C, Drouot C, Fulcrand P, et al. 2000a. Identification of key residues for interaction of vasoactive intestinal peptide with human VPAC1 and VPAC2 receptors and development of a highly selective VPAC1 agonist. Alanine scanning and molecular modeling of the peptide. J Biol Chem 275: 24003-24012.

Nicole P, Rouyer-Fessard C, Couvineau A, Drouot C, Fulcrand P, et al. 2000b. Alanine scanning of VIP. Structure-function relationship for binding to human recombinant VPAC1 receptor. Ann N Y Acad Sci 921: 352-356.

Nicot A, Otto T, Brabet P, Dicicco-Bloom EM. 2004. Altered social behavior in pituitary adenylate cyclase-activating polypeptide type I receptor-deficient mice. J Neurosci 24: 8786-8795.

Nielsch U, Keen P. 1989. Reciprocal regulation of tachykinin- and vasoactive intestinal peptide-gene expression in rat sensory neurones following cut and crush injury. Brain Res 481: 25-30.

Nielsen HS, Hannibal H, Fahrenkrug J. 1997. Developmental expression of the pituitary adenylate cyclase activating polypeptide (PACAP) in rat autonomic ganglia and spinal cord. Regul Pept 71: 125.

Nielsen HS, Hannibal J, Fahrenkrug J. 1998. Embryonic expression of pituitary adenylate cyclase-activating polypeptide in sensory and autonomic ganglia and in spinal cord of the rat. J Comp Neurol 394: 403-415.

O'Donnell M, Garippa RJ, Rinaldi N, Selig WM, Simko B, et al. 1994a. Ro 25-1553: a novel, long-acting vasoactive intestinal peptide agonist. Part I: In vitro and in vivo bronchodilator studies. J Pharmacol Exp Ther 270: 1282-1288.

O'Donnell M, Garippa RJ, Rinaldi N, Selig WM, Tocker JE, et al. 1994b. Ro 25-1553: a novel, long-acting vasoactive intestinal peptide agonist. Part II: Effect on in vitro and in vivo models of pulmonary anaphylaxis. J Pharmacol Exp Ther 270: 1289-1294.

O'Farrell M, Marley PD. 1997. Multiple calcium channels are required for pituitary adenylate cyclase-activating polypeptide-induced catecholamine secretion from bovine cultured adrenal chromaffin cells. Naunyn Schmiedebergs Arch Pharmacol 356: 536-542.

Osipenko ON, Barrie AP, Allen JM, Gurney AM. 2000. Pituitary adenylyl cyclase-activating peptide activates multiple intracellular signaling pathways to regulate ion channels in PC12 cells. J Biol Chem 275: 16626-16631.

Otto C, Hein L, Brede M, Jahns R, Engelhardt S, et al. 2004. Pulmonary hypertension and right heart failure in pituitary adenylate cyclase-activating polypeptide type I receptor-deficient mice. Circulation 110: 3245-3251.

Otto C, Kovalchuk Y, Wolfer DP, Gass P, Martin M, et al. 2001a. Impairment of mossy fiber long-term potentiation and associative learning in pituitary adenylate cyclase activating polypeptide type I receptor-deficient mice. J Neurosci 21: 5520-5527.

Otto C, Martin M, Wolfer DP, Lipp HP, Maldonado R, et al. 2001b. Altered emotional behavior in PACAP-type-I-receptor-deficient mice. Brain Res Mol Brain Res 92: 78-84.

Pantaloni C, Brabet P, Bilanges B, Dumuis A, Houssami S, et al. 1996. Alternative splicing in the N-terminal extracellular domain of the pituitary adenylate cyclase-activating polypeptide (PACAP) receptor modulates receptor selectivity and relative potencies of PACAP-27 and PACAP-38 in phospholipase C activation. J Biol Chem 271: 22146-22151.

Perrin D, Germeshausen A, Soling HD, Wuttke W, Jarry H. 1995. Enhanced cAMP production mediates the stimulatory action of pituitary adenylate cyclase activating polypeptide (PACAP) on in vitro catecholamine secretion from bovine adrenal chromaffin cells. Exp Clin Endocrinol Diabetes 103: 81-87.

Pisegna JR, Wank SA. 1993. Molecular cloning and functional expression of the pituitary adenylate cyclase-activating polypeptide type I receptor. Proc Natl Acad Sci USA 90: 6345-6349.

Pisegna JR, Wank SA. 1996. Cloning and characterization of the signal transduction of four splice variants of the human pituitary adenylate cyclase activating polypeptide receptor. Evidence for dual coupling to adenylate cyclase and phospholipase C. J Biol Chem 271: 17267-17274.

Przywara DA, Xi G, Angelilli L, Wakade TD, Wakade AR. 1996. A noncholinergic transmitter, pituitary adenylate cyclase activating polypeptide, utilizes a novel mechanism to evoke catecholamine secretion in rat adrenal chromaffin cells. J Biol Chem 271: 10545-10550.

Rabl K, Reglodi D, Banvolgyi T, Somogyvari-Vigh A, Lengvari I, et al. 2002. PACAP inhibits anoxia-induced changes in physiological responses in horizontal cells in the turtle retina. Regul Pept 109: 71-74.

Rawlings SR, Hezareh M. 1996. Pituitary adenylate cyclase activating polypeptide (PACAP) and PACAP/vasoactive intestinal polypeptide receptors: action on the anterior pituitary gland. Endocr Rev 17: 4-29.

Reglodi D, Somogyvari-Vigh A, Vigh J, Li M, Lengvari I, et al. 2001. Pituitary adenylate cyclase activating polypeptide is highly abundant in the nervous system of anoxia-tolerant turtle, Pseudemys scripta elegans. Peptides 22: 873-878.

Reglodi D, Somogyvari-Vigh A, Vigh S, Kozicz T, Arimura A. 2000. Delayed systemic administration of PACAP38 is neuroprotective in transient middle cerebral artery occlusion in the rat. Stroke 31: 1411-1417.

Reglodi D, Tamas A, Somogyvari-Vigh A, Szanto Z, Kertes E, et al. 2002. Effects of pretreatment with PACAP on the infarct size and functional outcome in rat permanent focal cerebral ischemia. Peptides 23: 2227-2234.

Robberecht P, Gourlet P, De Neef P, Woussen-Colle MC, Vandermeers-Piret MC, et al. 1992. Structural requirements for the occupancy of pituitary adenylate-cyclase-activating-peptide (PACAP) receptors and adenylate cyclase activation in human neuroblastoma NB-OK-1 cell membranes. Discovery of PACAP(6–38) as a potent antagonist. Eur J Biochem 207: 239-246.

Ronaldson E, Robertson DN, Johnson MS, Holland PJ, Mitchell R, et al. 2002. Specific interaction between the hop1 intracellular loop 3 domain of the human PAC(1) receptor and ARF. Regul Pept 109: 193-198.

Rostene WH. 1984. Neurobiological and neuroendocrine functions of the vasoactive intestinal peptide (VIP). Prog Neurobiol 22: 103-129.

Said SI, Mutt V. 1970. Polypeptide with broad biological activity: isolation from small intestine. Science 169: 1217-1218.

Said SI, Rosenberg RN. 1976. Vasoactive intestinal polypeptide: abundant immunoreactivity in neural cell lines and normal nervous tissue. Science 192: 907-908.

Sakai Y, Hashimoto H, Shintani N, Ichibori A, Tomimoto S, et al. 2002. Involvement of intracellular $Ca^{2+}$ elevation but not cyclic AMP in PACAP-induced p38 MAP kinase activation in PC12 cells. Regul Pept 109: 149-153.

Samson WK, Said SI, Graham JW, McCann SM. 1978. Vasoactive-intestinal-polypeptide concentrations in median eminence of hypothalamus. Lancet 2: 901-902.

Samson WK, Said SI, McCann SM. 1979. Radioimmunologic localization of vasoactive intestinal polypeptide in hypothalamic and extrahypothalamic sites in the rat brain. Neurosci Lett 12: 265-269.

Schemann M, Schaaf C, Mäder M. 1995. Neurochemical coding of enteric neurons in the guinea pig stomach. J Comp Neurol 353: 161-178.

Sherwood NM, Gray SL, Cummings KJ. 2003. Consequences of PACAP gene knockout. Pituitary adenylate cyclase-activating polypeptide. Vaudry H, Arimura A, editors. Norwell, Massachusetts: Kluwer Acadmic Publishers; pp. 347-360.

Sherwood NM, Krueckl SL, McRory JE. 2000. The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. Endocr Rev 21: 619-670.

Sheward WJ, Lutz EM, Copp AJ, Harmar AJ. 1998. Expression of PACAP, and PACAP type 1 (PAC1) receptor mRNA during development of the mouse embryo. Dev Brain Res 109: 245-253.

Shintani N, Mori W, Hashimoto H, Imai M, Tanaka K, et al. 2002. Defects in reproductive functions in PACAP-deficient female mice. Regul Pept 109: 45-48.

Shioda S, Ozawa H, Dohi K, Mizushima H, Matsumoto K, et al. 1998. PACAP protects hippocampal neurons against apoptosis: involvement of JNK/SAPK signaling pathway. Ann N Y Acad Sci 865: 111-117.

Shivers BD, Görcs TJ, Gottschall PE, Arimura A. 1991. Two high affinity binding sites for pituitary adenylate cyclase-activating polypeptide have different tissue distributions. Endocrinology 128: 3055-3065.

Shreeve SM, Sreedharan SP, Hacker MP, Gannon DE, Morgan MJ. 2000. VIP activates G(s) and G(i3) in rat alveolar macrophages and G(s) in HEK293 cells transfected with the human VPAC(1) receptor. Biochem Biophys Res Commun 272: 922-928.

Sims KB, Hoffman DL, Said SI, Zimmerman EA. 1980. Vasoactive intestinal polypeptide (VIP) in mouse and rat brain: an immunocytochemical study. Brain Res 186: 165-183.

Somogyvari-Vigh A, Reglodi D, Li M, Lengvari I, Vigh S, et al. 2000. Tissue distribution of PACAP27 and -38 in oligochaeta: PACAP27 is the predominant form in the nervous system of Lumbricus polyphemus. Peptides 21: 1185-1191.

Spengler D, Bweber C, Pantaloni C, Hlsboer F, Bockaert J, et al. 1993. Differential signal transduction by five splice variants of the PACAP receptor. Nature 365: 170-175.

Sreedharan SP, Patel DR, Huang JX, Goetzl EJ. 1993. Cloning and functional expression of a human neuroendocrine vasoactive intestinal peptide receptor. Biochem Biophys Res Commun 193: 546-553.

Summers MA, O'Dorisio MS, Cox MO, Lara-Marquez M, Goetzl EJ. 2003. A lymphocyte-generated fragment of vasoactive intestinal peptide with VPAC1 agonist activity and VPAC2 antagonist effects. J Pharmacol Exp Ther 306: 638-645.

Sun Y, Rao MS, Zigmond RE, Landis SC. 1994. Regulation of vasoactive intestinal peptide expression in sympathetic neurons in culture and after axotomy: the role of cholinergic differentiation factor/leukemia inhibitory factor. J Neurobiol 25: 415-430.

Sundler F, Ekblad E, Hannibal J, Moller K, Zhang Y-Z, et al. 1996. Pituitary adenylate cyclase-activating peptide in sensory and autonomic ganglia: localization and regulation. Ann N Y Acad Sci 805: 410-428.

Svoboda M, Tastenoy M, Ciccarelli E, Stievenart M, Christophe J. 1993. Cloning of a splice variant of the pituitary adenylate cyclase-activating polypeptide (PACAP) type I receptor. Biochem Biophys Res Commun 195: 881-888.

Svoboda M, Tastenoy M, Van Rampelbergh J, Goossens JF, De Neef P, et al. 1994. Molecular cloning and functional characterization of a human VIP receptor from SUP-T1 lymphoblasts. Biochem Biophys Res Commun 205: 1617-1624.

Tanaka J, Koshimura K, Murakami Y, Kato Y. 1996a. Stimulatory effect of PACAP on neuronal cell survival. Ann N Y Acad Sci: 473–475.

Tanaka J, Koshimura K, Murakami Y, Sohmiya M, Yanaihara N, et al. 1997a. Neuronal protection from apoptosis by pituitary adenylate cyclase-activating polypeptide. Regul Pept 72: 1-8.

Tanaka K, Shibuya I, Harayama N, Nomura M, Kabashima N, et al. 1997b. Pituitary adenylate cyclase-activating polypeptide potentiation of $Ca^{2+}$ entry via protein kinase C and A pathways in melanotrophs of the pituitary pars intermedia of rats. Endocrinology 138: 4086-4095.

Tanaka K, Shibuya I, Nagamoto T, Yamasha H, Kanno T. 1996b. Pituitary adenylate cyclase-activating polypeptide causes rapid $Ca^{2+}$ release from intracellular stores and long lasting $Ca^{2+}$ influx mediated by $Na^{+}$ influx-dependent membrane depolarization in bovine adrenal chromaffin cells. Endocrinology 137: 956-966.

Tanaka K, Shibuya I, Uezono Y, Ueta Y, Toyohira Y, et al. 1998. Pituitary adenylate cyclase-activating polypeptide causes $Ca^{2+}$ release from ryanodine/caffeine stores through a novel pathway independent of both inositol trisphosphates

and cyclic AMP in bovine adrenal medullary cells. J Neurochem 70: 1652-1661.

Tatsuno I, Somogyvari-Vigh A, Mizuno K, Gottschall PE, Hidaka H, et al. 1991. Neuropeptide regulation of interleukin-6 production from the pituitary: stimulation by pituitary adenylate cyclase activating polypeptide and calcitonin gene-related peptide. Endocrinology 129: 1797-1804.

Tatsuno I, Uchida D, Tanaka T, Saeki N, Hirai A, et al. 2001. Maxadilan specifically interacts with PAC1 receptor, which is a dominant form of PACAP/VIP family receptors in cultured rat cortical neurons. Brain Res 889: 138-148.

Taupenot L, Mahata M, Mahata SK, O'Connor DT. 1999. Time-dependent effects of the neuropeptide PACAP on catecholamine secretion. Stimulation and desensitization. Hypertension 34: 1152-1162.

Tse DL, Pang RT, Wong AO, Chan SM, Vaudry H, et al. 2002. Identification of a potential receptor for both peptide histidine isoleucine and peptide histidine valine. Endocrinology 143: 1327-1336.

Tyrrell S, Landis SC. 1994. The appearance of NPY and VIP in sympathetic neuroblasts and subsequent alterations in their expression. J Neurosci 14: 4529-4547.

Uchida D, Arimura A, Somogyvari-Vigh A, Shioda S, Banks WA. 1996. Prevention of ischemia-induced death of hippocampal neurons by pituitary adenylate cyclase activating polypeptide. Brain Res 736: 280-286.

Uchida D, Tatsuno I, Tanaka T, Hirai A, Saito Y, et al. 1998. Maxadilan is a specific agonist and its deleted peptide (M65) is a specific antagonist for PACAP type 1 receptor. Ann N Y Acad Sci 865: 253-258.

Uddman R, Fahrenkrug J, Malm L, Alumets J, Hakanson R, et al. 1980. Neuronal VIP in salivary glands: distribution and release. Acta Physiol Scand 110: 31-38.

Uhl GR, Snyder SH. 1979. Neurotensin: a neuronal pathway projecting from amygdala through stria terminalis. Brain Res 161: 522-526.

Van Rampelbergh J, Poloczek P, Francoys I, Delporte C, Winand J, et al. 1997. The pituitary adenylate cyclase activating polypeptide (PACAP I) and VIP (PACAP II VIP1) receptors stimulate inositol phosphate synthesis in transfected CHO cells through interaction with different G proteins. Biochim Biophys Acta 1357: 249-255.

Vaudry D, Falluel-Morel A, Basille M, Pamantung TF, Fontaine M, et al. 2003. Pituitary adenylate cyclase-activating polypeptide prevents C2-ceramide-induced apoptosis of cerebellar granule cells. J Neurosci Res 72: 303-316.

Vaudry D, Gonzalez BJ, Basille M, Fournier A, Vaudry H. 1999. Neurotrophic activity of pituitary adenylate cyclase-activating polypeptide on rat cerebellar cortex during development. Proc Natl Acad Sci USA 96: 9415-9420.

Vaudry D, Gonzalez BJ, Basille M, Yon L, Fournier A, et al. 2000. Pituitary adenylate cyclase-activating polypeptide and its receptors: From structure to functions. Pharmacol Rev 52: 269-324.

Vaudry D, Hamelink C, Damadzic R, Eskay EL, Gonzalez B, et al. 2005. Endogenous PACAP acts as a stress response peptide to protect cerebeller neurons from ethanol or oxidative insult. Peptides (In press).

Vaudry D, Rousselle C, Basille M, Falluel-Morel A, Pamantung TF, et al. 2002a. Pituitary adenylate cyclase-activating polypeptide protects rat cerebellar granule neurons against ethanol-induced apoptotic cell death. Proc Natl Acad Sci USA 99: 6398-6403.

Vaudry D, Stork PJS, Lazarovici P, Eiden LE. 2002b. Signaling Pathways for PC12 cell Differentaition: making the right connections. Science 296: 1648-1649.

Villalaba M, Bockaert J, Journot L. 1997. Pituitary adenylate cyclase-activating polypeptide (PACAP-38) protects cerebellar granule neurons from apoptosis by activating the mitogen-activated protein kinase (MAP kinase) pathway. J Neurosci 17: 83-90.

Voice J, Donnelly S, Dorsam G, Dolganov G, Paul S, et al. 2004. c-Maf and JunB mediation of Th2 differentiation induced by the type 2 G protein-coupled receptor (VPAC2) for vasoactive intestinal peptide. J Immunol 172: 7289-7296.

Voice JK, Dorsam G, Chan RC, Grinninger C, Kong Y, et al. 2002. Immunoeffector and immunoregulatory activities of vasoactive intestinal peptide. Regul Pept 109: 199-208.

Wakade AR. 1988. Non-cholinergic transmitter(s) maintains secretion of catecholamines from rat adrenal medulla for several hours of continuous stimulation of splanchnic neurons. J Neurochem 50: 1302-1308.

Waschek JA. 1995. Vasoactive intestinal peptide: an important trophic factor and developmental regulator? Dev Neurosci 17: 1-7.

Waschek JA. 1996. VIP and PACAP receptor-mediated actions on cell proliferation and survival. Ann N Y Acad Sci 805: 290-300.

Waschek JA, Cassillas RA, Nguyen TB, DiCicco-Bloom EM, Carpenter EM, et al. 1998. Neural tube expression of pituitary adenylate cyclase-activating peptide (PACAP) and receptor: potential role in patterning and neurogenesis. Proc Natl Acad Sci USA 95: 9602-9607.

Waschek JA, Lelievre V, Bravo DT, Nguyen T, Muller J-M. 1997. Retinoic acid regulation of the VIP and PACAP autocrine ligand and receptor system in human neuroblastoma cell lines. Peptides 18: 835-841.

Waschek JA, Pruss RM, Siegel RE, Eiden LE, Bader M-F, et al. 1987. Regulation of enkephalin, VIP and chromogranin A biosynthesis in actively secreting chromaffin cells: multiple strategies for multiple peptides. Ann N Y Acad Sci 493: 308-323.

Waschek JA, Richards ML, Bravo DT. 1995. Differential expression of VIP/PACAP receptor genes in breast, intestinal, and pancreatic cell lines. Cancer Lett 92(2): 143-149.

Watanabe T, Masuo Y, Matsumoto H, Suzuki N, Ohtaki T, et al. 1992. Pituitary adenylate cyclase activating polypeptide provokes cultured rat chromaffin cells to secrete adrenaline. Biochem Biophys Res Commun 182: 403-411.

Weihe E, Nohr D, Michel S, Muller S, Zentel HJ, et al. 1991. Molecular anatomy of the neuro-immune connection. Int J Neurosci 59: 1-23.

Willis EA, Ottesen B, Wagner G, Sundler F, Fahrenkrug J. 1983. Vasoactive intestinal polypeptide (VIP) as a putative neurotransmitter in penile erection. Life Sci 33: 383-391.

Xia M, Sreedharan SP, Bolin DR, Gaufo GO, Goetzl EJ. 1997. Novel cyclic peptide agonist of high potency and selectivity for the type II vasoactive intestinal peptide receptor. J Pharmacol Exp Ther 281: 629-633.

Yiangou Y, Di Marzo V, Spokes RA, Panico M, Morris HR, et al. 1987a. Isolation, characterization, and pharmacological actions of peptide histidine valine 42, a novel prepro-vasoactive intestinal peptide-derived peptide. J Biol Chem 262: 14010-14013.

Yiangou Y, Williams SJ, Bishop AE, Polak JM, Bloom SR. 1987b. Peptide histidine-methionine immunoreactivity in plasma and tissue from patients with vasoactive intestinal peptide-secreting tumors and watery diarrhea syndrome. J Clin Endocrinol Metab 64: 131-139.

Yung SL, Dela Cruz F, Hamren S, Zhu J, Tsutsumi M, et al. 2003. Generation of highly selective VPAC2 receptor agonists by high throughput mutagenesis of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide. J Biol Chem 278: 10273-10281.

Zhang Y, Danielsen N, Sundler F, Mulder H. 1998. Pituitary adenylate cyclase-activating peptide is upregulated in sensory neurons by inflammation. Neuroreport 9: 2833-2836.

Zhong Y. 1995. Mediation of PACAP-like neuropeptide transmission by coactivation of Ras/Raf and cAMP signal transduction pathways in Drosophila. Nature 375: 588-592.

Zhou CJ, Shioda S, Shibanuma M, Nakajo S, Funahashi H, et al. 1999a. Pituitary adenylate cyclase-activating polypeptide receptors during development: expression in the rat embryo at primitive streak stage. Neuroscience 93: 375-391.

Zhou X, Rodriguez WI, Casillas RA, Ma V, Tam J, et al. 1999b. Axotomy-induced changes in pituitary adenylate cyclase activating polypeptide (PACAP) and PACAP receptor gene expression in the adult rat facial motor nucleus. J Neurosci Res 57: 953-961.

Zigmond RE. 2000. Neuropeptide action in sympathetic ganglia. Evidence for distinct functions in intact and axotomized ganglia. Ann N Y Acad Sci 921: 103-108.

Zigmond RE, Sun Y. 1997. Regulation of neuropeptide expression in sympathetic neurons. Paracrine and retrograde influences. Ann N Y Acad Sci 814: 181-197.

# 22 Hypocretins/Orexins in Brain Function

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**Abstract:** The hypocretins (also called the orexins) are two C-terminally amidated neuropeptides of related sequence. They are produced from a common precursor whose expression is restricted to a few thousand neurons of the rat dorsolateral hypothalamus. Two G-protein-coupled receptors (GPCRs) for the hypocretins have been identified, and these have different distributions within the CNS and differential affinities for the two hypocretins. The hypocretins have been detected in secretory vesicles at synapses of fibers that project to areas within the posterior hypothalamus that are implicated in feeding behaviors and hormone secretion. Hypocretin fibers also project to diverse targets in other brain regions and the spinal cord, including several areas implicated in cardiovascular function and sleep–wake regulation. The peptides are excitatory when applied directly in vivo and to cultured neurons and slices, although there is also evidence for some inhibitory signaling. Administration of the hypocretins stimulates food intake, affects blood pressure, hormone secretion, and locomotor activity, and increases wakefulness while suppressing rapid eye movement (REM) sleep. Inactivating mutations in the hypocretin receptor 2 gene (hcrtr 2) in dogs result in narcolepsy. Mice whose hypocretin gene has been inactivated exhibit a narcolepsy-like phenotype. Most human patients with narcolepsy have greatly reduced levels of hypocretin peptides in their cerebrospinal fluid (CSF) and no or barely detectable hypocretin neurons in their hypothalami, suggestive of autoimmune attack. One aspect of hypocretin activity is the direct excitation of cholinergic forebrain neurons, brainstem monoaminergic REM-off neurons in the locus coeruleus and dorsal raphe nucleus, and histaminergic tuberomammillary nucleus (TMN), which together suppress slow-wave sleep. The hypocretins also modulate the activity of cholinergic REM-on neurons in the brainstem, which gate REM entry. The effects on wakefulness appear to be the dominant activities of the hypocretin system and are twofold: maintenance of the waking state and suppression of REM entry.

**List of Abbreviations:** AgRP, agouti-related peptide; CRF, corticotropin-releasing factor; CSF, cerebrospinal fluid; DMH, dorsomedial hypothalamus; EEG, electroencephalogram; GPCR, G-protein-coupled receptor; Hcrt, hypocretin; Hcrtr1, 2, hypocretin receptors 1, 2; ICV, intracerebroventricular administration; LC, locus coeruleus; LDT, laterodorsal tegmentum; LH, lateral hypothalamus; LHSS, lateral hypothalamus self-stimulation; MCH, melanin-concentrating hormone; PKC, protein kinase C; PPT, pedunculopontine nucleus; PVN, paraventricular nucleus; REM, rapid eye movement sleep; SCN, suprachiasmatic nucleus; TMN, tuberomammillary nucleus; VTA, ventral tegmental area

## 1 Historical Aspects of the Lateral Hypothalamus

Early notions about the role of the lateral hypothalamus (LH) were based on observations of humans and experimental animals with localized hypothalamic lesions. von Economo (1930), studying patients with encephalitis lethargica, proposed that the posterior hypothalamus (including the LH) was required for maintaining the awake state. Ablations of the monkey LH led to coma and hypophagia, whereas ablations of the medial hypothalamus led to hyperphagia (Ranson, 1939; Hetherington and Ranson, 1940; Anand and Brobeck, 1951a, b). Thus, the LH was known to play a role in both arousal and energy balance, both important aspects of motivated behavior. It had additionally been shown that animals lever-press for electrical stimulation when electrodes are placed within the LH (Olds, 1962), further implicating it as a reward-mediating structure. The signaling molecules and circuitry responsible for coordinating these behaviors remained unknown until the advent of molecular biological techniques for studying the nervous system and the discoveries of the hypocretin (Hcrt) and melanin-concentrating hormone (MCH) systems.

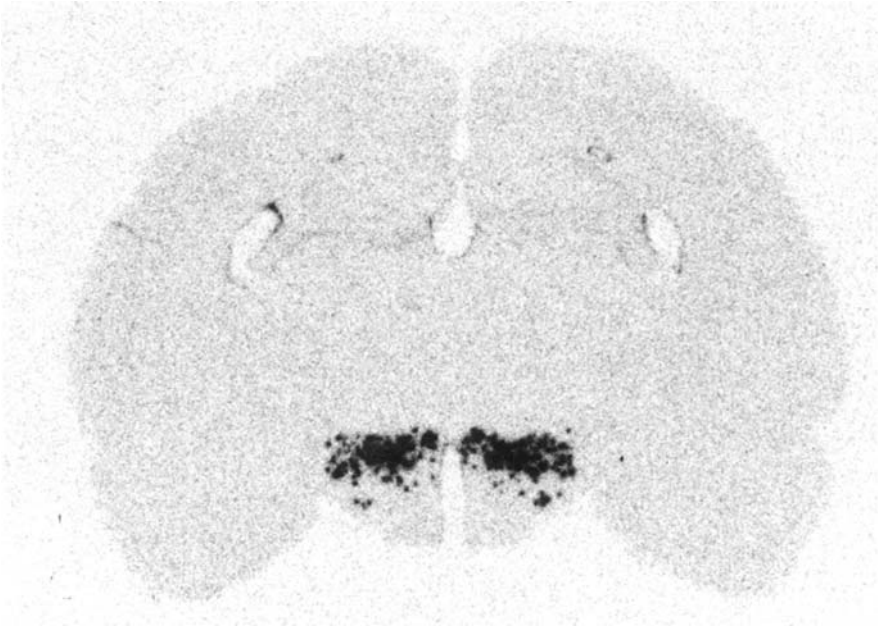
## 2 Discovery and Biochemical Aspects of the Hypocretins

### 2.1 Isolation of cDNA Clones

The first glimpse of the hypocretins came in an open-system search for undiscovered hypothalamic regulatory peptides. Gautvik et al. (1996) conducted a systematic subtractive hybridization survey aimed

at identifying messenger RNA (mRNA) species whose expression was restricted to discrete nuclei within the rat hypothalamus. Among the novel hypothalamic mRNAs that were identified by that study was a species whose expression, as detected by in situ hybridization analyses, was restricted to a few thousand neurons that were bilaterally distributed within the dorsolateral hypothalamus (*Figure 22-1*; Gautvik et al., 1996;

**Figure 22-1**
**The first glimpse of the hypocretin system. In situ hybridization of rat coronal section with cDNA isolated in subtractive hybridization study detecting a few thousand neurons in the dorsal–lateral hypothalamus (From Gautvik et al., 1996).**



de Lecea et al., 1998). In Northern blots, the mRNA migrated at ~700 nucleotides, detectable during brain development at low concentrations as early as embryonic day 18, and increasing dramatically in concentration after the third postnatal week.

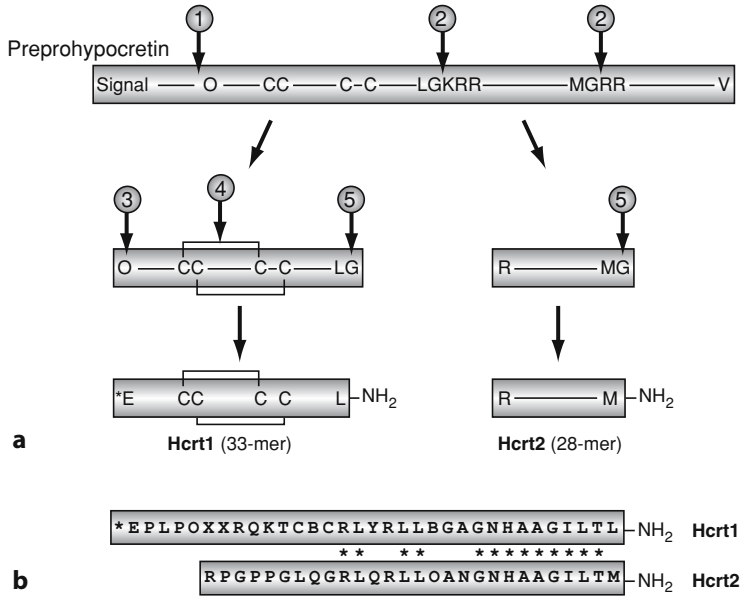
The peptides were discovered independently in a large collaborative study to identify endogenous ligands for orphan G-protein-coupled receptors (GPCRs) (Sakurai et al., 1998). This group referred to the peptides as orexins because they stimulated food intake when administered to rats during the daytime. In this chapter, we will refer to the peptides by their first-used name, the hypocretins, but the terms are interchangeable and are both used extensively in the large literature that has grown up around the peptides.

## 2.2 Structure of mRNA/Protein

The sequences of the rat (569 nonpoly(A) nucleotides) and homologous mouse (582 nucleotides) mRNAs each encoded a 130-residue putative secretory protein with an apparent signal sequence and two additional phylogenically conserved sites for potential proteolytic maturation followed by modification of the carboxy-terminal glycines by peptidylglycine α-amidating monooxygenase (de Lecea et al., 1998). These features suggested that the product of this hypothalamic mRNA served as a preprohormone for two C-terminally amidated, secreted peptides (*Figure 22-2a*). One of these, hypocretin 2 (Hcrt2 or OxB),

**Figure 22-2**

**a. Maturation of preprohypocretin. Only amino acid residues key to the processing of the prepropeptide are shown. After removal of the secretion signal (*1*), the prohypocretin is cleaved at two pairs of tandem basic amino acids (KR, RR) (*2*). The genetically encoded glutamine (Q) is derivatized to form pyroglutamate (*E) (*3*), two intrachain disulfide bonds (C–C) are formed (*4*), and the C-terminal glycines (G) are modified by peptidyl-glycine alpha-amidating monooxygenase (*5*), leaving C-terminal amides on the resulting 33-mer and 28-mer peptides, Hcrt1 and Hcrt2, respectively. b. The primary amino acid sequences of rat Hcrt1 and Hcrt2. The *E at the N terminus represents the pyroglutamate residue; the *asterisks* between the sequences indicate the positions of identity between the two peptides. The disulfide bonds are as in Figure 22-2a. Notice the C-terminal amide groups (-$NH_2$). (From Sutcliffe and de Lecea, 2002).**




on the basis of the putative preprohormone amino acid sequence, was predicted to contain precisely 28 residues. The other, hypocretin 1 (Hcrt1 or OxA), had a defined predicted amidated C terminus but, because of uncertainties as to how the amino terminus might be proteolytically processed, an undefined N-terminal extent (de Lecea et al., 1998). The C-terminal 19 residues of these two putative peptides shared 13-amino-acid identities (*Figure 22-2b*), suggesting that the peptides had related structures and functions. This region of hcrt2 contained a 7-amino-acid match with secretin.

Antisera generated against synthetic peptides corresponding to regions of the deduced prohypocretin sequence and to bacterially expressed preprohypocretin have been generated (de Lecea et al., 1998; Date et al., 1999; van den Pol, 1999). The antisera are specific for the hypocretin-related peptides and have been used extensively to characterize the protein in both anatomical and ELISA-type studies, described below.

## 2.3 Detection of Endogenous Peptides

The detection of the two hypocretin peptides within the brain allowed the exact structures of these endogenous peptides to be determined by mass spectroscopy (*Figure 22-2*; Sakurai et al., 1998). The sequence of endogenous Hcrt2 was the same as that predicted from the cDNA sequence. The N terminus of Hcrt1 was found to correspond to a genetically encoded glutamine that was derivatized as pyroglutamate.

Hcrt1 (33 residues) contains two intrachain disulfide bonds. Human Hcrt1 is identical to the rodent peptide, whereas human Hcrt2 differs from rodent Hcrt2 at two residues (Sakurai et al., 1998).

### 2.4 Phylogeny/Genomics

The mouse hypocretin gene, *HCRT*, is located on chromosome 11, and the human *HCRT* gene maps to chromosome arm 17q21–q24. Phylogenic studies have detected genes that encode conserved preprohypocretins in pufferfish and frog species, suggesting that the gene arose early in the chordate lineage (Alvarez and Sutcliffe, 2002). Sequence similarities with various members of the incretin family, especially secretin, suggest that the preprohypocretin gene was formed from the secretin gene by three genetic rearrangements: first, a duplication of the secretin gene; second, deletions of the N-terminal portion of the 5′-duplicate and the C-terminal portion of the 3′-duplicate to yield a secretin with its N- and C-termini leapfrogged (circularly permuted); and third, a further duplication of the permuted gene, followed by modifications, to form a secretin derivative that encoded two related hypocretin peptides (Alvarez and Sutcliffe, 2002).

### 2.5 3D Structure

Consistent with the hypothesis that the hypocretins and secretin are phylogenically related, portions of their three-dimensional solution structures, as determined by nuclear magnetic resonance, are similar despite their leapfrogged primary sequence, consisting of two adjacent α-helices (6 to 7- and 9 to 14-amino-acids long) separated by a short 2–3-amino-acid turn (Gronenborn et al., 1987; Lee et al., 1999). The longer helix corresponds to the region of identity between the two peptides.

## 3 Hypocretin Receptors

### 3.1 Identification

Sakurai et al. (1998) prepared transfected cell lines stably expressing each of 50 orphan GPCRs and then measured calcium fluxes in these cell lines in response to fractions from tissue extracts. One of these transfected cell lines responded to a substance in a brain extract. Mass spectroscopy showed that this substance was a peptide whose sequence was later identified as that of endogenous Hcrt1.

### 3.2 Binding Properties

The initial orphan GPCR, Hcrtr1 (also referred to as OX1R), bound Hcrt1 with high affinity but Hcrt2 with 100 to 1000-fold lower affinity. A related GPCR, Hcrtr2 (OX2R), sharing 64% identity with Hcrtr1 was identified by searching database entries with the Hcrtr1 sequence, had a high affinity for both Hcrt2 and Hcrt1 (Sakurai et al., 1998). These two receptors are highly conserved (95%) across species. Radioligand-binding studies and calcium flux measurements have shown Hcrt1 to have equal affinity for Hcrtr1 and Hcrtr2, whereas Hcrt2 has (tenfold greater affinity for Hcrtr2 than Hcrtr1 (Upton, 2005).

### 3.3 Agonists and Antagonists

Substitution of alanine for leucine at position 11 of human Hcrt2 ($[A^{11}]$Hcrt2) produces a modified agonist 100-fold more selective than native Hcrt2 for Hcrtr2 over Hcrtr1 (Asahi et al., 2000). Several Hcrt2 analogues with >1000-fold selectivity for Hcrtr2 and a truncated form of Hcrt1 (residues 2–23) with modest Hcrtr1 preference have also been produced (Lang et al., 2004).

SB-334867 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]napthyridin-4-yl urea) is an antagonist that has an affinity of 40 nM for Hcrtr1 and is more than 50-fold selective over Hcrtr2 and other GPCRs and ion channels (Duxon et al., 2001; Porter et al., 2001; Smart et al., 2001). SB-408124 (1-(6,8-difluoro-2-methylquinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea) is slightly more potent and has greater Hcrtr1 selectivity than SB-334867 (Upton, 2005). Both compounds are systemically bioavailable and brain penetrant. *N*-Acyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline is the first Hcrtr2-selective antagonist (Hirose et al., 2003).

## 4 Anatomical Considerations

### 4.1 Where Peptides Are Made

In situ hybridization and immunohistochemistry using antisera to the hypocretins have revealed a few thousand neurons highly positive for Hcrt seen between the rat fornix and the mammillothalamic tracts and a prominent network of axons that project from these cells to other neurons in the perifornical and posterior hypothalamus (Gautvik et al., 1996; Broberger et al., 1998; Elias et al., 1998; de Lecea et al., 1998; Peyron et al., 1998). There are 50,000–80,000 hypocretin neurons in the human lateral hypothalamus (Moore et al., 2001). With the exception of one report on Hcrt in the enteric nervous system (Kirchgessner and Liu, 1999), there is no other place in the brain or periphery where Hcrt-producing neuronal perikarya have been found. Hcrt neurons are 20–30 μm in diameter and are multipolar or fusiform in shape, with 2–4 primary dendrites bearing few spines. In addition to rats, mice, and humans, Hcrt neurons with a similar restricted hypothalamic distribution have been detected in monkeys, hamsters, cats, sheeps, pigs, chicken, various amphibians, and zebrafishes.

The perifornical hypothalamus contains a collection of neurons that express MCH, a peptide that has been implicated in feeding-related behavior (Qu et al., 1996). Double-label colocalization studies (Broberger et al., 1998; Elias et al., 1998; Peyron et al., 1998; Hakansson et al., 1999) have shown that the MCH and hypocretin neurons are distinct, but spatially intermingled, each set with a different topological distribution. There is a nearly one-to-one correspondence between neurons in the lateral hypothalamus that express the opioid receptor agonist dynorphin and the hypocretin neurons (Chou et al., 2001). Nearly all Hcrt neurons express secretogranin II (Bayer et al., 2002b). Glutamate, the excitatory amino acid transporter EAAT3, and the vesicular glutamate transporters VGLUT1 and VGLUT2 are expressed by Hcrt neurons (Abrahamson et al., 2001; Li et al., 2002; Collin et al., 2003; Rosin et al., 2003; Torrealba et al., 2003), and thus Hcrt neurons are likely to be glutamatergic. Other proteins detected in Hcrt neurons include the $GABA_A$ receptor epsilon subunit, $5\text{-}HT_{1A}$ receptor, μ-opioid receptor, pancreatic polypeptide Y4 receptor, adenosine A1 receptor, leptin receptor, precursor-protein convertase, transcription factor Stat-3, and the neuronal pentraxin Narp implicated in clustering of ionotropic glutamate receptors (Hakansson et al., 1999; Horvath et al., 1999b; Reti et al., 2002; Thakkar et al., 2002; Campbell et al., 2003; Georgescu et al., 2003; Moragues et al., 2003; Nilaweera et al., 2003; Muraki et al., 2004).

### 4.2 Hcrt Projections

Hcrt-immunoreactive axons can be observed emerging from the Hcrt cell bodies, and projecting throughout the brain, with the highest density of terminal fields seen in the hypothalamus (Broberger et al., 1998; Elias et al., 1998; de Lecea et al., 1998; Peyron et al., 1998). Hypothalamic regions receiving projections include the LH and posterior hypothalamic areas (regions of Hcrt and MCH neuronal populations), the dorsomedial hypothalamus (DMH), the paraventricular hypothalamic nucleus, and arcuate nucleus. Hcrt is reciprocally connected with neuropeptide Y receptor (NPY)- and leptin receptor-positive neurons in the arcuate nucleus (Horvath et al., 1999a), an area important in feeding behaviors and endocrine regulation. Hcrt neurons also make reciprocal synaptic contact with neighboring MCH neurons (Guan et al., 2002).

Prominent Hcrt fibers project from the LH to apparent terminal fields in many areas of the brain. Peyron et al. (1998) referred to four Hcrt efferent pathways: dorsal and ventral ascending pathways and

dorsal and ventral descending pathways. The dorsal ascending pathway projects through the zona incerta to the paraventricular nucleus of the thalamus, central medial nucleus of the thalamus, lateral habenula, substantia innominata, bed nucleus of the stria terminalis, septal nuclei, dorsal anterior nucleus of the olfactory bulb, and cerebral cortex. The ventral ascending pathway projects to the ventral pallidum, vertical and horizontal limb of the diagonal band of Broca, medial part of the accumbens nucleus, and olfactory bulb. The dorsal descending pathway projects through the mesencephalic central gray to the superior and inferior colliculi and the pontine central gray, locus coeruleus (LC), dorsal raphe nucleus, and laterodorsal tegmental nucleus (LDT). A second bundle of fibers projects through the dorsal tegmental area to the pedunculopontine nucleus (PPT), parabrachial nucleus, subcoeruleus area, nucleus of the solitary tract, parvocellular reticular area, dorsal medullary region, and the caudal spinal trigeminal nucleus. This tract continues to all levels of the spinal cord (van den Pol, 1999). The ventral descending pathway runs through the interpeduncular nucleus, ventral tegmental area (VTA), substantia nigra pars compacta, raphe nuclei and the reticular formation, gigantocellular reticular nuclei, ventral medullary area, raphe magnus, lateral paragigantocellular nucleus, and ventral subcoeruleus. The cumulative set of projections is consistent with the combined patterns of expression of the two hypocretin GPCRs (discussed below). The projection fields in humans are comparable to those in rodents (Moore et al., 2001). The diffuse nature of Hcrt projections provided the first evidence of the potential for multiple physiological roles for the peptides.

## 4.3 Detection at Synapses

Electron microscopic examination revealed that hypocretin immunoreactivity is associated with the rough endoplasmic reticulum, the Golgi network, and cytoplasmic dense core vesicles (de Lecea et al., 1998; Peyron et al., 1998; Horvath et al., 1999a). The latter have been observed along myelinated axons, at presynaptic boutons apposed to dendritic shafts in both the periaqueductal gray and in the LC, where the synapses are with tyrosine-hydroxylase-positive noradrenergic dendrites. The accumulation of the hypocretins within dense core vesicles at axon terminals suggested that they might have intercellular signaling activity.

## 4.4 Hcrt Afferents

In addition to reciprocal connections with the MCH neurons of the LH and with NPY/agouti-related peptide neurons of the arcuate, corticotropin-releasing factor (CRF)-positive inputs arising from the paraventricular hypothalamic nucleus have been detected in close apposition to Hcrt perikarya (Winsky-Sommerer et al., 2004). Hypocretin neurons receive direct projections positive for arginine-vasopressin and vasoactive intestinal peptide from neurons in the suprachiasmatic nucleus (SCN) (Abrahamson et al., 2001), which is responsible for generating the circadian rhythm, and also innervation from the DMH, which itself responds to SCN input (Chou et al., 2003).

Hcrt neurons are predominantly controlled by local glutamatergic excitatory interneurons (Li et al., 2002). Perikaryal GABA input onto the Hcrt neurons is very minimal, approximately 10% of the glutamatergic input. Other afferents include brain stem noradrenergic and dorsal raphe nucleus serotonergic inputs, as well as cholinergic afferents (Li et al., 2002; Yamanaka et al., 2003).

## 4.5 Receptor Distributions

The mRNAs that encode the two hypocretin receptors and the receptor proteins themselves, detected by immunohistochemistry, are both enriched in the brain and moderately abundant in the hypothalamus but have different distributions within the brain (Trivedi et al., 1998; Marcus et al., 2001). Hcrtr1 mRNA is prominent in the prefrontal and intralimbic cortex, hippocampus, paraventricular thalamic nucleus,

ventromedial hypothalamic nucleus, dorsal raphe nucleus, LC, the laterodorsal and pedunculopontine tegmental nuclei (at higher density than Hcrtr2), pontine raphe, raphe magnus, raphe obscurus, dorsal motor vagal complex, and spinal cord. Additional forebrain regions expressing moderate to high levels of Hcrtr1 mRNA are the tenia tecta, bed nucleus of the stria terminalis, horizontal limb of the diagonal band of Broca, and medial amygdala. Immunoreactive terminals have been detected in the LH.

Hcrtr2 mRNA is detected in the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and various nuclei of the hypothalamus, including the tuberomammillary nucleus (TMN), dorsomedial nucleus, paraventricular nucleus (PVN), ventral premammillary nucleus, ventral periaqueductal gray, midbrain reticular formation, dorsal interpeduncular nucleus, Barrington's nucleus, sensory trigeminal nucleus, ventrolateral medulla, and dorsal vagal nucleus. Hcrtr2 mRNA was prominent in the medial septum, vertical and horizontal limbs of the diagonal band of Broca, substantia innominata, and cortical amygdala.

Both receptors are detected in dorsal raphe, the ventral tegmental area, paraventricular thalamic nucleus, and the intergeniculate leaflet, with lesser density in the rhomboid, reuniens, and other midline nuclei. In the cerebral cortex, Hcrtr1 mRNA is expressed primarily in layers II, III, and V, whereas Hcrtr2 mRNA is found at higher density in layers II and VI and more diffusely in other layers. In the hippocampus, Hcrtr1 is expressed mainly in the CA2 region and medial dentate gyrus, while Hcrtr2 was most abundant in CA3 (Hervieu et al., 2001).

The distribution of Hcrt receptors is largely consistent with Hcrt axon innervation patterns. The composite distribution of the two Hcrt receptors strongly resembles the distribution of the MCH receptor (Kilduff and de Lecea, 2001). In the LC, amygdala, and other brainstem noradrenergic groups, MCH receptor mRNA distribution is similar to that of the Hcrtr1. In regions such as the septum, hypothalamus, and much of the brainstem, the distribution of MCH receptor mRNA resembles that of the Hcrtr2 (Kilduff and de Lecea, 2001).

The Hcrt receptors are not restricted to the central nervous system: in the periphery, they are widely expressed, especially in endocrine tissues. Hcrtr1 or Hcrtr2 have been detected in the pituitary, adrenal gland, testis, gastrointestinal tract, pancreas, and pineal gland.

## 5 Neurotransmitter Properties

### 5.1 Electrophysiological Properties

The detection of Hcrt neurons in a specialized region of the hypothalamus, processing of the precursor into two related peptides, and the detection of the peptides in vesicles at synapses suggested that the peptides might possess neurotransmitter activities. Bath application of synthetic Hcrt2 to mature hypothalamic neurons evoked increases in the frequency of postsynaptic currents (de Lecea et al., 1998). Hypocretin-mediated excitation has been found in a large number of brain regions; many of them involved in arousal. Hypocretin increases activity in the hypothalamus (van den Pol et al., 1998, 2001; Shirasaka et al., 2001), LC (Hagan et al., 1999; Horvath et al., 1999b; Bourgin et al., 2000), dorsal raphe (Brown et al., 2002; Liu et al., 2002), dorsal horn of the spinal cord (Grudt et al., 2002), spinal motoneurons (Yamuy et al., 2004), nucleus of the solitary tract (Smith et al., 2002), dorsal motor nucleus (Hwang et al., 2001), dorsolateral tegmentum (Burlet et al., 2002), TMN (Eriksson et al., 2001), basal forebrain (Eggermann et al., 2001; Wu et al., 2004), midline thalamus (Bayer et al., 2000), and trigeminal nucleus (Peever et al., 2003).

Hcrt2 has a potent effect at both presynaptic and postsynaptic receptors (van den Pol et al., 1998): in the presence of tetrodotoxin, the hypocretins increases the frequency, but not the amplitude, of miniature postsynaptic currents (presynaptic effect) and evoke an increase in cytoplasmic calcium by opening plasma membrane $Ca^{2+}$ channels in arcuate postsynaptic neurons (postsynaptic effect). Most synaptic activity in hypothalamic circuits is attributable to axonal release of GABA or glutamate. Hypocretin, acting directly at axon terminals, can increase the release of each of these amino acid transmitters, as demonstrated by whole-cell patch-clamp recording (van den Pol et al., 1998).

### 5.2 Coupling

Both Hcrt1 and Hcrt2 evoke rises in $Ca^{2+}$, as measured by fura-2 imaging, in about one-third of hypothalamic neurons, probably by opening a calcium channel (van den Pol et al., 1998; Kukkonen and Akerman, 2001). Responses to hypocretin are completely blocked by the protein kinase C-specific inhibitor bisindolylmaleide and by phospholipase C inhibitors, suggesting that the hypocretins work through a family of GTP-binding proteins ($G_q$) that activate protein kinase C (PKC) and mobilization of intracellular calcium. $G_q$-activated signaling cascades result in phosphorylation of $Ca^{2+}$ channels, which can increase $Ca^{2+}$ conductance and neuronal excitability (Smart et al., 1999; Uramura et al., 2001). The nonamidated forms of the peptides are not electrophysiologically active (Smart et al., 1999). In the substantia nigra pars reticulate, hypocretin stimulation was sensitive to an inhibitor of protein kinase A, which mediates effects of cAMP, but insensitive to blockers of PKC (Korotkova et al., 2002), implicating a role of the $G_s$ system.

The adrenal glands also express Hcrtr2 (Karteris et al., 2001; Randeva et al., 2001). Treatment of human adrenal membranes from fetal or adult tissue with Hcrt1 increased the labeling of $G_s$ and $G_i$ in both preparations and additionally $G_q$ in the adult preparation. Thus, although the majority of hypocretin signaling is excitatory, it may be inhibitory in some cases (Martin et al., 2002). Acting as excitatory peptides, the hypocretins can enhance the activities of both excitatory and inhibitory neurons.

## 6 Administration of Peptides

Administration of the hypocretins to experimental animals stimulates food intake, affects autonomic and endocrine parameters, and increases arousal. These are discussed below in that order, but because the disease of the hypocretin system is the sleep disorder narcolepsy, the arousal aspects are the most important and will be discussed in a later section of the chapter.

### 6.1 Feeding and Metabolism

Sakurai et al. (1998) found that intracerebroventricular (ICV) administration of either Hcrt1 or Hcrt2 increased short-term food consumption in rats. Furthermore, rats that had been deprived of food for 48 h showed increased concentrations of hypocretin mRNA and peptides in the hypothalamus (Sakurai et al., 1998; Mondal et al., 1999). Feeding responses can be elicited by local administration of Hcrt1 to the PVN, the dorsomedial nucleus, the lateral hypothalamus, or the perifornical area (Dube et al., 1999). ICV administration of Hcrt2 also increases food intake in sheep (Sartin et al., 2001).

Many observations leave little doubt that the hypocretin system influences and is influenced by primary nutritional homeostasis circuits, but other findings suggest that the hypocretins are not critical players in feeding activities but rather play roles in increasing arousal and motivation levels so that feeding can take place. Hypocretin-immunoreactive fibers form synapses with neurons in the arcuate nucleus that contain NPY, an important orexigenic (appetite-stimulating) peptide, and with POMC-expressing neurons, which produce α-melanocyte-stimulating hormone, a satiety factor (Broberger et al., 1998; Elias et al., 1998; Horvath et al., 1999). Hypocretin neurons express leptin receptors (Hakansson et al., 1999; Horvath et al., 1999), and preprohypocretin mRNA expression is reduced in *obese* (*ob*/*ob*) mice (Yamamoto et al., 1999), which lack leptin. Hypocretin neurons receive inputs from NPY- and agouti-related peptide (AgRP)-positive neurons in the arcuate nucleus, which themselves express leptin receptors, and NPY stimulates *c-fos* expression by hypocretin neurons, whereas NPY receptor antagonists block the feeding effect of hypocretins (Jain et al., 2000; Yamanaka et al., 2000). Administration of an antiserum directed against Hcrt1 attenuates the feeding response to ICV NPY, and pharmacological blockade of the NPY receptor reduces the feeding stimulatory effect elicited by Hcrt1 (Jain et al., 2000; Lopez et al., 2000; Yamanaka et al., 2000). Hcrt-expressing cells respond to circulating leptin by reducing Hcrt1 concentrations and *c-fos* expression (Beck and Richy, 1999; Lopez et al., 2000; Niimi et al., 2001). Also supporting the idea that the hypocretin neurons are involved in feeding is the observation that they express STAT3, a transcription

factor that is induced by leptin (Hakansson et al., 1999). However, whereas NPY-induced feeding is completely inhibited by leptin, the Hcrt feeding response is only partially suppressed by leptin (Zhu et al., 2002).

Hcrt cells are sensitive to glucose and food deprivation (Griffond et al., 1999; Moriguchi et al., 1999; Bayer et al., 2000; Cai et al., 2001; Muroya et al., 2001): the activity of hypocretin neurons and their expression of hypocretin mRNA and *c-fos* increases during hypoglycemia; hypocretin mRNA decreases during glucopenia; and *c-fos* expression increases during fasting. Acute administration of the Hcrtr1-selective antagonist SB-334867 suppresses food intake in rats. Interestingly, SB-334867 also reduced weight gain for 3 to 5 days posttreatment (Rodgers et al., 2001; Ishii et al., 2004), despite being no longer detectable after 12 h, and repeated administration of the antagonist reduced the food intake and weight gain over 14 days in *ob*/*ob* mice (Haynes et al., 2002). These results are consistent with a complex circuitry of appetite-controlling signaling molecules in the arcuate and lateral hypothalamus in which hypocretin might play a role.

Not all data support this notion, though, particularly when the other activities of the hypocretins are considered (see below). Hcrt1-induced increases in food intake were small relative to those induced by NPY infusion (Edwards et al., 1999; Ida et al., 1999). No alteration in Hcrt1 peptide concentration or Hcrt mRNA in the hypothalamus in response to either fasting or a high-fat diet, and no effect on Hcrt mRNA levels in experimentally induced diabetes, have been observed in rats (Taheri et al., 1999; Tritos et al., 2001; Swart et al., 2001). However, Hcrt mRNA increases after leptin administration to fasted mice, and increases in response to a high-fat diet.

It is difficult to attribute physiological effects to ICV administration of high doses of hypocretin, which might activate circuits other than those that would be activated by local axonal release of the transmitter (van den Pol et al., 1998). And, the Hcrts might be orexigenic only under some physiological states (perhaps related to circadian rhythms or stress). In this regard, it might be significant that the hypocretins activate dopamine-mediated stereotypic behaviors. Hcrt1 peptide concentrations in the hypothalamus are under circadian control and are highest during the awake, dark period in nocturnal rodents (Yoshida et al., 2001). During fasting, Hcrt1 accumulation in the CSF does not exceed concentrations normal for the waking period, suggesting that some of the food-uptake effect may result from arousal rather than direct feeding pressure (Fujiki et al., 2001). Continuous administration of Hcrt1 for 7 days in rats does not significantly alter daily food intake, body weight, blood glucose, total cholesterol, or free fatty acid levels (Yamanaka et al., 1999), suggesting that many of hypocretin's effects may be limited to short-term, immediate stimulation of feeding behavior due to increased wakefulness. That is, animals eat more and are motivated to eat when they are awake.

## 6.2 Autonomic and Endocrine Effects

Hypocretin neurons receive inputs from brainstem areas that are associated with cardiovascular function, and project to the ventrolateral medulla, the locus coeruleus, the lateral paragigantocellular nucleus, the nucleus of the solitary tract and other areas that have been implicated in the regulation of blood pressure and heart rate (Dampney, 1994). Projections to the arcuate nucleus also suggested a role in the regulation of hormone release. In the ovine hypothalamus, there are hypocretin terminals on the neurons that produce gonadotropin-releasing hormone, suggesting that hypocretin might particularly modulate reproductive endocrinology (Iqbal et al., 2001). In addition, projections to the raphe magnus and subcoeruleus suggested a role for hypocretins in the regulation of body temperature. The dense hypocretinergic projections to the ventrolateral preoptic area, TMN, pontine reticular formation, PPT, LDT area, and LC, suggested their involvement in states of arousal (Sherin et al., 1996; Peyron et al., 1998; Hagan et al., 1999). Very strong hypocretin-immunoreactive projections have been described in regions of the spinal cord that are related to modulation of pain (van den Pol, 1999), and hypocretin-like immunoreactivity has also been detected in the intestinal epithelium (Kirchgessner and Liu, 1999).

In accordance with the wide distribution of hypocretin terminals, ICV administration of the hypocretins affects not only feeding, but also several other functions. Both Hcrt1 and Hcrt2 elevate mean arterial

blood pressure, heart rate (both suppressed by SB334867), and oxygen consumption (Samson et al., 1999; Shirasaka et al., 1999; Chen et al., 2000; Wang et al., 2001). Hcrt1 increases body temperature independent of peripheral thermogenesis (Yoshimichi et al., 2001), increases water consumption, and stimulates gastric acid secretion in the gut (Kunii et al., 1999; Takahashi et al., 1999). Hcrt1 also increases locomotor activity and wakefulness, while decreasing slow wave and depressing REM sleep (Hagan et al, 1999; Bourgin et al., 2000; Piper et al., 2000; Espana et al., 2001; Thakkar et al., 2001).

The peptides also stimulate the secretion of luteinizing hormone in ovarectomized and proestrus female rats (suppressed by central administration of SB334867) and in hypothalamic explants from male pituitaries (Pu et al., 1998; Russell et al., 2001b). Hcrt1 decreases the concentrations of circulating growth hormone and prolactin, while increasing corticosterone, ACTH, and insulin levels (Hagan et al., 1999; Malendowicz et al., 1999; Ida et al., 2000; Nowak et al., 2000; Russell et al., 2001a). Hcrt2, but not Hcrt1, increases circulating thyroid-stimulating hormone (Jones et al., 2001) and has direct effects on the pituitary, adrenal, and pineal glands (Mikkelsen et al., 2001; Randeva et al., 2001; Samson and Taylor, 2001). Both peptides depolarize CRF neurons in the paraventricular hypothalamic nucleus and increase CRF and arginine-vasopressin mRNA concentrations in the PVN, and thus have clear effects on the HPA axis and stress-related physiology (Kuru et al., 2000; Al Barazanji et al., 2001; Shirasaka et al., 2001; Follwell and Ferguson, 2002; Samson et al., 2002). Hcrt2 is directly excitatory on superficial dorsal horn neurons of the spinal cord (Grudt et al., 2002) and exhibits an analgesic effect in models of pain (Bingham et al., 2001). There are several examples in which either Hcrt1 or Hcrt2, but not both peptides, effects a response, suggesting that the two peptides are not redundant. In some cases, the effects can be explained by differential involvement of Hcrtr1 or Hcrtr2; in others, differential resistance of the peptides to degradation may provide the explanation.

## 6.3 Motivation/Addiction

Hcrt neurons are highly responsive to morphine and are activated by naltrexone-precipitated withdrawal. However, the response of these neurons is heterogeneous, suggesting that there might be different populations of Hcrt cells (Fadel and Deutch, 2002). The expression of the *Hcrt* gene increases only after precipitated withdrawal. The neurons express the μ-opioid receptor; hence their response may be directly to morphine and naltrexone (Georgescu et al., 2003). These observations might explain why animals self-administer heroin to the LH (Corrigall, 1987). Hcrt knockout mice exhibit dramatically attenuated morphine withdrawal symptoms (Georgescu et al., 2003). Hcrt neurons have extensive projections to the mesolimbic dopamine and noradrenergic (LC) pathways, regions well studied for their roles in drug addiction. These neurons also project to and inhibit nucleus accumbens neurons (Martin et al., 2002).

Rats can be trained to turn a wheel to deliver electrical current to the LH: LH self-stimulation (LHSS). LHSS thresholds measure brain reward systems; lower thresholds represent increased reward. Most drugs of abuse lower LHSS thresholds. LHSS is thought to be rewarding in part because it activates cholinergic neurons in the LDT and the PPT nuclei that consequently activate dopaminergic neurons in the VTA (Forster and Blaha, 2000). Hypocretins excite LDT cholinergic neurons both directly and indirectly, acting synergistically with glutamatergic afferents (Burlet et al., 2002) to drive dopamine release in the nucleus accumbens by exciting dopaminergic neurons in the VTA. Thus, the Hcrt system acts as a modulator of brain reward function.

## 6.4 Effects of Anesthetics

Hcrt1 decreases barbiturate anesthesia time in rats by 15–40%, an action reversed by SB-334867 (Kushikata et al., 2003). In vitro, barbiturates inhibit Hcrt-induced norepinephrine release although they do not interact directly with Hcrt receptors. In isoflorane-anesthetized animals, Hcrt1 elicits arousal without cardiovascular activation, in contrast to its effect on awake animals (Yasuda et al., 2003).

## 7 Narcolepsy Is a Disease of the Hypocretin System

### 7.1 Sleep, Arousal, and Narcolepsy

Sleep is characterized by complex patterns of neuronal activity in thalamocortical systems (Steriade et al., 1993; Jones, 1994; McCormick and Bal, 1997). The fast, low-amplitude electroencephalogram (EEG) activity of the aroused state is replaced by synchronized high-amplitude waves that characterize slow-wave sleep. This pattern develops further into high-frequency waves that define paradoxical, or REM, sleep. Switching among these states is controlled in part by the activities of neurons in the hypothalamic ventrolateral preoptic nucleus and a series of areas referred to as the ascending reticular activating system, which is distributed among the pedunculopontine and laterodorsal tegmental nuclei (PPT–LDT), LC, dorsal raphe nucleus and TMN, and regulates cortical activity and arousal (Saper et al., 2001).

The first case of human narcolepsy was reported in 1877 by Westphal, and the sleep disorder acquired its name from Gélineau in 1880. Narcolepsy affects around 1 in 2000 adults, appears between the ages of 15 to 30 years, and shows four characteristic symptoms: (1) excessive daytime sleepiness with irresistible sleep attacks during the day; (2) cataplexy (brief episodes of muscle weakness or paralysis precipitated by strong emotions such as laughter or surprise); (3) sleep paralysis, a symptom considered to be an abnormal episode of REM sleep atonia, in which the patient suddenly finds himself unable to move for a few minutes, most often upon falling asleep or waking up; and (4) hypnagogic hallucinations, or dream-like images that occur at sleep onset. These latter symptoms have been proposed as pathological equivalents of REM sleep. The disorder is considered to represent a disturbed distribution of sleep states rather than an excessive amount of sleep.

Studies with monozygotic twins have shown that narcolepsy is weakly penetrant i.e; in only 25% of cases does the monozygotic twin of an affected individual also develop the disorder. Sporadic narcolepsy (which accounts for 95% of human cases) is highly correlated with particular class II HLA-DR and -DQ histocompatibility haplotypes in about 90% of patients, but most people with these haplotypes are not narcoleptic (Mignot et al., 2001). Because many autoimmune disorders are HLA-linked and because of the late and variable age of disease onset, narcolepsy has long been considered a likely autoimmune disorder, but the targets of the immune attack are not known.

### 7.2 Canine Narcolepsy

Both sporadic and heritable narcolepsies are observed in dogs, and the symptoms resemble those exhibited by humans suffering from narcolepsy. The first link between the hypocretins and narcolepsy came from genetic linkage studies in a colony of Doberman pinschers in which narcolepsy was inherited as an autosomal recessive, fully penetrant phenotype. Fine mapping and cloning of the defective canine narcolepsy gene showed it to be the gene that encodes the hypocretin receptor, Hcrtr2 (Lin et al., 1999). The mutation in the Doberman lineage is an insertion of a short interspersed repeat element (SINE) into the third intron of *HCRTR2* that causes aberrant splicing of the Hcrtr2 mRNA (exon 4 is skipped) and results in a truncated receptor protein. In cells that have been transfected with the mutant gene, the truncated Hcrtr2 protein does not properly localize to the membrane and, therefore does not bind its ligands (Hungs et al., 2001). Analysis of a colony of narcoleptic Labradors revealed that their *HCRTR2* gene contained a distinct mutation that resulted in the skipping of exon 6, also leading to a truncated receptor protein. A third family of narcoleptic Dachshunds carries a point mutation in *HCRTR2* that results in a receptor protein that reaches the membrane but cannot bind the hypocretins.

### 7.3 Mouse Knockout Mutants

Continuous recording of the behavior of knockout mice in which the hypocretin gene was inactivated by homologous recombination in embryonic stem cells revealed periods of ataxia, which were especially

frequent during the dark period (Chemelli et al., 1999). EEG recordings showed that these episodes were not related to epilepsy and that the mice suffered from cataplectic attacks, a hallmark of narcolepsy. In addition, the mutant mice spent almost twice as much time in REM sleep during the dark period as did their wild-type littermates, and their EEGs showed episodes of direct transition from wakefulness to REM sleep, another event that is unique to narcolepsy. Similar observations were made in rats in which the hypocretin neurons of the lateral hypothalamus were inactivated by saporin targeting (Gerashchenko et al., 2001), although in this model cataplexy was not observed. Mice with an inactivated *HCRTR2* gene have a milder narcoleptic phenotype than the *HCRT* knockouts; *HCRTR1* knockouts exhibit only a sleep fragmentation phenotype, whereas double *HCRTR1* and *HCRTR2* mutants recapitulate the full *HCRT* knockout phenotype (Willie et al., 2003), suggesting that signaling through both receptors contributes to normal arousal, although the role of *HCRTR2* is greater than that of *HCRTR1*.

### 7.4 Human Narcolepsy

Nishino et al. (2000) studied hypocretin concentrations in the CSF of normal controls and patients with narcolepsy by radioimmunoassay. In control CSF, hypocretin concentrations were highly clustered, suggesting that tight regulation of the substance is important. However, of nine patients with narcolepsy, only one had a hypocretin concentration within the normal range. One patient had a greatly elevated concentration, while seven patients had no detectable circulating hypocretin. In an expanded study, hypocretin was undetectable in 37 of 42 patients with narcolepsy and in a few patients with Guillain-Barré syndrome (Ripley et al., 2001). CSF hypocretin was in the normal range for most neurological diseases, but was low, although detectable in some patients with CNS infections, brain trauma, and brain tumors.

Peyron (2000), Thannickal (2000) and their teams of collaborators found that, in the brains of narcolepsy patients, they could detect few or no hypocretin-producing neurons. Whether the hypocretin neurons are selectively depleted, as is most likely, or only no longer expressing hypocretin is not yet known, although one report showed some indications of gliosis (Thannickal et al., 2000). The codistributed MCH neurons were unaffected. Furthermore, a single patient with a nonHLA-linked narcolepsy carried a mutation within the hypocretin gene itself. The mutation results in a dominant negative amino acid substitution in the secretion signal sequence that sequesters both the mutant and heterozygous wild-type hypocretin nonproductively to the smooth endoplasmic reticulum (Peyron et al., 2000).

These findings leave no doubt as to the central role of the hypocretin system in this sleep disorder. Because most cases are sporadic, mutations in the hypocretin gene or those for its receptors can account for no more than a small subset of the human narcolepsies. The HLA association, loss of neurons with signs of gliosis, and age of disease onset are consistent with autoimmune destruction of the hypocretin neurons accounting for the majority of narcolepsy (Lin et al., 2001), although a nonimmune-mediated degenerative process has not been ruled out. Whether hypocretin itself or some other protein that is selectively expressed by the hypocretin neurons is the target antigen is yet to be determined. The precipitating factor for the development of the autoimmunity is also unknown, but there must be one because only a small percentage of individuals with the predisposing HLA haplotypes develop the disorder. The narcolepsies as a group are probably a collection of disorders that are caused by defects in the production or secretion of the hypocretins or in their signaling, and these could have numerous genetic, traumatic, viral, and/or autoimmune causes.

## 8. Hypocretin and Arousal Circuitry

Because narcolepsy is the consequence of a defective hypocretin system, it follows that the dominant role of the system is in maintenance of the waking state and suppression of REM entry, and data about the hypocretins give insights as to how this is accomplished. The hypocretin neurons project to various brainstem structures of the ascending reticular activating system that express one or both of the hypocretin

receptors and have been implicated in regulating arousal. The noradrenergic neurons of the LC, the serotonergic neurons of the dorsal raphe, and the histaminergic neurons of the TMN are all so-called REM-off cells: each group fires rapidly during wakefulness, slowly during slow-wave sleep, and hardly at all during REM (Saper et al., 2001). Each of these structures sends projections to a diverse array of targets in the forebrain, and their firing stimulates cortical arousal. The activity state of these groups of monoaminergic neurons is one of the features that distinguishes wakefulness from REM. Additionally, and importantly, the hypocretin neurons project to other brain areas that have been implicated in arousal. For instance, the hypocretins, acting through Hcrtr2, excite cholinergic neurons of the basal forebrain, which produce the cortical acetylcholine characteristic of the desynchronized EEG associated with wakefulness and REM (Xi et al., 2001). Direct infusion of the hypocretins into the basal forebrain produces dramatic increases in wakefulness (Espana et al., 2001; Thakkar et al., 2001).

## 8.1 LH Neurons

Among the neurons of the perifornical lateral hypothalamus, 53% increase their firing rates during both wakefulness and REM but decrease their activities during slow-wave sleep (Alam et al., 2002). An additional 38% of the neurons in this area are activated only during the awake phase. Hypocretin neurons express *c-fos* during the waking period (night time in rats), and *c-fos* expression is increased by sleep deprivation and methamphetamine (Estabrooke et al., 2001). The stimulant modafinil, which is commonly used to treat the drowsiness associated with narcolepsy, greatly elevates *c-fos* expression in hypocretin neurons (Chemelli et al., 1999). Hypocretin levels fluctuate circadianly, being highest during waking, and peptide concentrations increase as a consequence of forced sleep deprivation (Yoshida et al., 2001), suggesting that the hypocretins and the activity of the hypocretin neurons serve as pressures that oppose sleep. Other perturbations that increase *c-fos* expression in Hcrt cells include treatment with NPY, leptin, ghrelin, hypoglycemia, and food and sleep deprivation.

## 8.2 Noradrenergic Systems

The noradrenergic LC neurons fire constantly during wakefulness. In addition to their projections to the forebrain, these neurons send inhibitory projections to cholinergic REM-on (fire during wakefulness and more rapidly during REM, but do not fire during slow-wave sleep) generator neurons in the PPT–LDT that project to the pontine reticular formation (Hobson et al., 1975; Aston-Jones and Bloom, 1981). Hypocretin axons form synapses on these LC neurons, which express Hcrtr1 postsynaptically (Horvath et al., 1999b; Bourgin et al., 2000). Local administration of Hcrt1, but not Hcrt2, to the LC suppresses REM in a dose-dependent manner and increases wakefulness (Bourgin et al., 2000). These effects are neutralized by an antibody that prevents binding of Hcrt1 to Hcrtr1. Administration of Hcrt1 increases the firing rate of noradrenergic LC neurons and induces expression of *c-fos* in these cells (Bourgin et al., 2000; Ivanov and Aston-Jones, 2000).

Hcrt terminals are also found on GABA interneurons in the LC (Zhu et al., 2003). GABA inhibits LC neurons (Ennis and Aston-Jones, 1989), suggesting that Hcrt has both direct excitatory effects on LC noradrenergic neurons and inhibitory effects on these same neurons via a feedforward circuit involving local GABA interneurons. Hcrt neurons are responsive to noradrenalin, thus providing a feedback loop between LC and Hcrt neurons. Whether the noradrenalin effect is excitatory, inhibitory, or state-dependent is presently a matter of debate.

## 8.3 Serotonergic Systems

Serotonin neurons of the dorsal raphe in the brainstem are part of modulatory ascending and descending pathways that gate sleep–wake states (Hobson and Pace-Schott, 2002) These neurons express both Hcrtr1

and Hcrtr2 (Brown et al., 2002) and receive input from Hcrt fibers. Hypocretins increase the activity of serotonin cells in the dorsal raphe primarily by activation of nonselective cation currents (Brown et al., 2002; Liu et al., 2002).

## 8.4 Histaminergic Systems

Histamine neurons are wake-active neurons, located exclusively in the TMN of the hypothalamus. They project to the hypothalamus, basal forebrain, thalamus, cortex, and brainstem. TMN neurons express Hcrtr2 receptors and receive inputs from Hcrt-containing axons. Both Hcrt1 and Hcrt2 depolarize histaminergic TMN neurons by activation of an electrogenic sodium–calcium exchanger and a $Ca^{2+}$ current, associated with a small decrease in input resistance and increases in spontaneous firing (Eriksson et al., 2001). Knockout mice deficient in histamine receptor 1 are impervious to hypocretin administration, suggesting that at least some of the effects of the Hcrts are caused by release of histamine and activation of postsynaptic H1 receptors (Bayer et al., 2001; Huang et al., 2001; Yamanaka et al., 2002).

One of the features of narcolepsy is cataplexy, which is a sudden loss of skeletal muscle tone, often triggered by emotions or laughter. During cataplectic episodes, although narcoleptic individuals enter a REM-like state of muscle atonia, they are awake, aware of their environment, and otherwise conscious. Thus, vigilance and the control of muscle tone are dissociated in this pathological state. Studies in Hcrtr2-deficient narcoleptic dogs (John et al., 2004) showed that histamine neurons, in contrast to noradrenergic and serotonergic REM-off cell groups, are active during cataplexy. Activity of histamine neurons is thus linked to the maintenance of waking, whereas that of noradrenergic and serotonergic neurons is tightly coupled to the maintenance of muscle tone in waking and its loss in REM sleep and cataplexy.

## 8.5 Dopaminergic Systems

The dopaminergic neurons of the VTA and ventral periaqueductal gray do not change their activity greatly throughout the sleep–wake cycle. Dopaminergic and GABAergic neurons in the VTA receive inputs from Hcrt neurons of the LH (Fadel and Deutch, 2002). The hypocretins excite these neurons via $Ca^{2+}$-, PLC-, and PKC-dependent pathways (Uramura et al., 2001), promoting arousal.

## 8.6 Cholinergic Systems

The hypocretin neurons project to cholinergic brainstem REM-on neurons, including those in the LDT and the PRF whose projections contribute to cholinergic tone in the forebrain. This tone is elevated during both wakefulness and REM leading to desynchronization of the EEG. Cholinergic tone is low in slow-wave sleep and during which acetylcholine activity is further inhibited by the sleep-promoting peptide of forebrain interneurons, cortistatin (de Lecea et al., 1996), contributing to the slow-wave synchrony of the EEG. Local injection of Hcrt1 into the LDT of freely moving cats increases wakefulness and decreases the number of REM episodes, but does not influence episode length (Xi et al., 2001), suggesting that the hypocretin system influences the gate (or switch) to REM by reducing the firing rates of the brainstem REM-on cells, but does not itself operate during REM. This and the fact that deficiencies in the hypocretin system lead to increases in REM make it more likely that action at REM-on structures by hypocretin occurs only during waking periods (Sutcliffe and de Lecea, 2002). The role of hypocretin in regulating the REM gate is a complex one in that, paradoxically, the REM-on structures receive both indirect hypocretin-initiated inhibitory signals from REM-off cells and direct projections from the hypocretin neurons themselves, and therefore must decide on how to respond to this push–pull pressure in different scenarios.

The midline and intralaminar thalamic neurons coordinate activity levels broadly across the cerebral cortex and support attention and awareness. Hcrt1 and Hcrt2 selectively depolarize midline-intralaminar thalamic neurons and not sensory thalamic neurons (Bayer et al., 2002a). Midline-intralaminar nuclei

express hcrtr2 (Trivedi et al., 1998; Marcus et al., 2001). The hypocretins excite arousal-related cholinergic neurons in the basal forebrain (Eggermann et al., 2001).

Hcrt neurons project directly to the cerebral cortex in addition to projecting onto subcortical relay neurons. In the cortex, Hcrt fibers are distributed through all layers, although most densely in the deeper layers. In layers 1 through 6a, hypocretins have no direct effect upon cortical neurons, although there are indirect effects through presynaptic terminals of inputs (Bayer et al., 2004). Hcrt has a direct postsynaptic depolarizing action upon cortical neurons located exclusively in layer 6b that is mediated by Hcrtr2. Layer 6b neurons project diffusely to layer 1 of surrounding cortical areas (Clancy and Cauller, 1999), allowing cortical activation to propagate widely.

## 9 Integrating What We Know

Hypocretin peptides excite LC and dorsal raphe neurons to elevate muscle tone and excite TMN neurons to promote wakefulness. These components of the ascending reticular activating system, and the Hcrt neurons themselves, project to and stimulate thalamic and basal forebrain neurons, and all of these groups contribute to the depolarization of layer 6b neurons of the cerebral cortex. Arousal-related signaling occurs through both Hcrtr1 and Hcrtr2. The involvement of both receptors is consistent with the more severe phenotype of the double receptor knockout mice compared with either single receptor knockout (Willie et al., 2003). It is also consistent with the observation that sporadic cases of canine narcolepsy associated with lower or undetectable CSF hypocretin are more severe than are cases with the Hcrtr2 deficiencies alone (Hungs and Mignot, 2001). Nevertheless, Hcrtr2 plays the more prominent role in raising arousal levels.

These peptides also have diverse effects on brain reward systems and autonomic systems related to stress that serve to increase motivated behaviors, for example, feeding. The relation to feeding is a complex one. Acute administration of Hcrt to sleeping rats increases food consumption. However, patients with narcolepsy have chronically low concentrations of the hypocretins, but have an increased likelihood of being obese despite reduced daily calorie intake (Schuld et al., 2000; Nishino et al., 2001). Similarly, although hypocretin knockout mice are hypophagic, they do not have lower weights than the unaffected controls. Mice that have been genetically engineered to lack hypocretin neurons by expressing a toxic gene from the hypocretin transcriptional promoter exhibit a phenotype similar to that of humans with narcolepsy, not only with respect to sleep/REM measures, but also in demonstrating late-onset obesity despite eating less than their nonaffected littermates (Hara et al., 2001). Thus, chronic hypocretin underactivity does not reduce body weight but increases it. Rather than considering the hypocretins to be orexigenic, they appear to serve as a counter-regulatory response to obesity.

## 10 Diagnosis and Potential Therapeutics for Sleep Disorders

Measurement of Hcrt1 in CSF provides a reliable diagnostic for sporadic narcolepsy. Although local release of Hcrt at its targets within the brain varies during the 24-h day, CSF Hcrt1 levels are relatively stable (Salomon et al., 2003). In a study of 274 patients with various sleep disorders (171 with narcolepsy) and 296 controls, a cutoff value of 110 pg/mL (30% of the mean control values) was the most predictive of narcolepsy (Mignot et al., 2002). Most narcolepsy patients had undetectable levels, while a few had detectable, but very reduced levels. The assay was 99% specific for narcolepsy.

Hcrt1 has also been detected in plasma, although its origin remains to be demonstrated and high nonspecific background immunoreactivities partially mask its detection. Decreased levels of plasma Hcrt1 were measured in narcoleptic patients using high-performance liquid chromatography separation to confirm that the signal included genuine Hcrt1 (Higuchi et al., 2002).

Given that most human narcolepsy is sporadic and results from depletion of Hcrt-producing neurons, replacement therapies can be envisioned. Small molecule agonists of the hypocretin receptors might have therapeutic potential for human sleep disorders and might be preferable to the traditionally prescribed amphetamines. ICV administration of Hcrt1 to normal mice and dogs strongly promotes wakefulness

(Nishino et al., 2003). The effect is predominantly mediated by Hcrtr2, because the same dose of Hcrt1 has no effect in Hcrtr2-mutated narcoleptic dogs (Yoshida et al., 2003). Hcrt1 has low penetrance of the blood–brain barrier, so a centrally penetrable agonist will need to be devised.

## 11 Questions to be Answered

Clearly there is still much to be learned about the hypocretin system. Do Hcrt neurons represent a single, homogeneous population or do subpopulations exist? Do they all have the same function, or do subsets project to different targets? What is the nature of the selective vulnerability of Hcrt neurons that results in their degeneration and consequent narcolepsy in humans with particular histocompatibility types? Is Hcrt itself an antigenic target or is there another precipitating antigen? Is the degeneration truly autoimmune, and if so, why is immune privilege of the brain compromised? What are the roles of the two Hcrt receptors at the various target nuclei? What are the signals that affect Hcrt neuronal activity? What is the natural activity profile of these neurons? Is it state dependent?

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## References

Abrahamson EE, Leak RK, Moore RY. 2001. The suprachiasmatic nucleus projects to posterior hypothalamic arousal systems. Neuroreport 12: 435-440.

Alam MN, Gong H, Alam T, Jaganath R, McGinty D, et al. 2002. Sleep-waking discharge patterns of neurons recorded in the rat perifornical lateral hypothalamic area. J Physiol 538: 619-631.

Al Barazanji KA, Wilson S, Baker J, Jessop DS, Harbuz MS. 2001. Central orexin-A activates hypothalamic–pituitary–adrenal axis and stimulates hypothalamic corticotropin releasing factor and arginine vasopressin neurones in conscious rats. J Neuroendocrinol 13: 421-424.

Alvarez CE, Sutcliffe JG. 2002. Hypocretin is an early member of the incretin gene family. Neurosci Lett 324: 169-172.

Anand BK, Brobeck JR. 1951a. Hypothalamic control of food intake in rats and cats. Yale J Biol Med 24: 123-140.

Anand BK, Brobeck JR. 1951b. Localization of a "feeding center" in the hypothalamus of the rat. Proc Soc Exp Biol Med 77: 323-324.

Asahi S, Egashira S, Matsuda M, Iwaasa H, Kanatani A, et al. 2000. Structure–activity relationship studies on the novel neuropeptide orexin. Peptide science. Fujii N, editor. The Japanese Peptide Society, Tokyo, pp. 37-40.

Aston-Jones G, Bloom FF. 1981. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. J Neurosci 1: 876-886.

Bayer L, Colard C, Nguyen NU, Risold PY, Fellmann D, et al. 2000. Alteration of the expression of the hypocretin (orexin) gene by 2-deoxyglucose in the rat lateral hypothalamic area. Neuroreport 11: 531-533.

Bayer L, Eggermann E, Saint-Mleux B, Machard D, Jones BE, et al. 2002a. Selective action of orexin (hypocretin) on nonspecific thalamocortical projection neurons. J Neurosci 22: 7835-7839.

Bayer L, Eggermann E, Serafin M, Saint-Mleux B, Machard D, et al. 2001. Orexins (hypocretins) directly excite tuberomammillary neurons. Eur J Neurosci 14: 1571-1575.

Bayer L, Mairet-Coello G, Risold PY, Griffond B. 2002b. Orexin/hypocretin neurons: chemical phenotype and possible interactions with melanin-concentrating hormone neurons. Regul Pept 104: 33-39.

Bayer L, Saint-Mleux B, Serafin M, Eggermann E, Machard D, et al. 2004. Exclusive postsynaptic action of hypocretin-orexin on sublayer 6b cortical neurons. J Neurosci 24: 6760-6764.

Beck B, Richy S. 1999. Hypothalamic hypocretin/orexin and neuropeptide Y: divergent interaction with energy depletion and leptin. Biochem Biophys Res Commun 258: 119-122.

Bingham S, Davey PT, Babbs AJ, Irving EA, Sammons MJ, et al. 2001. Orexin-A, an hypothalamic peptide with analgesic properties. Pain 92: 81-90.

Bourgin P, Huitron S, Spier AD, Morte B, Criado JR, et al. 2000. Hypocretin-1 modulates REM sleep through activation of locus coeruleus neurons. J Neurosci 20: 7760-7765.

Broberger C, de Lecea L, Sutcliffe JG, Hökfelt T. 1998. Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and Agouti gene-related protein systems. J Comp Neurol 402: 460-474.

Brown RE, Sergeeva OA, Eriksson KS Haas HL. 2002. Convergent excitation of dorsal raphe serotonin neurons by multiple arousal systems (orexin/hypocretin, histamine and noradrenaline). J Neurosci 22: 8850-8859.

Burlet S, Tyler CJ, Leonard CS. 2002. Direct and indirect excitation of laterodorsal tegmental neurons by hypocretin/orexin peptides: implications for wakefulness and narcolepsy. J Neurosci 22: 2862-2872.

Cai XJ, Evans ML, Lister CA, Leslie RA, Arch JR, et al. 2001. Hypoglycemia activates orexin neurons and selectively increases hypothalamic orexin-B levels: responses inhibited by feeding and possibly mediated by the nucleus of the solitary tract. Diabetes 50: 105-112.

Campbell RE, Smith MS, Allen SE, Grayson BE, Ffrench-Mullen JM, et al. 2003. Orexin neurons express a functional pancreatic polypeptide Y4 receptor. J Neurosci 23: 1487-1497.

Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, et al. 1999. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98: 437-451.

Chen CT, Hwang LL, Chang JK, Dun NJ. 2000. Pressor effects of orexins injected intracisternally and to rostral ventrolateral medulla of anesthetized rats. Am J Physiol Regul Integr Comp Physiol 278: R692-R697.

Chou TC, Lee CE, Lu J, Elmquist JK, Hara J, et al. 2001. Orexin (hypocretin) neurons contain dynorphin. J Neurosci 21: RC168 (1-6).

Chou TC, Scammell TE, Gooley JJ, Gaus SE, Saper CB, et al. 2003. Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. J Neurosci 23: 10691-10702.

Clancy B, Cauller L. 1999. Widespread projections from subgriseal neurons (layer VII) to layer I in adult rat cortex. J Comp Neurol 407: 275-286.

Collin M, Backberg M, Ovesjo ML, Fisone G, Edwards RH, et al. 2003. Plasma membrane and vesicular glutamate transporter mRNAs/proteins in hypothalamic neurons that regulate body weight. Eur J Neurosci 18: 1265-1278.

Corrigall W. 1987. Heroin self-administration: effects of antagonist treatment in lateral hypothalamus. Pharmacol Biochem Behav 27: 693-700.

Dampney RAL. 1994. Functional organization of central pathways regulating the cardiovascular system. Physiol Rev 74: 323-364.

Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsujura S, et al. 1999. Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci USA 96: 748-753.

de Lecea L, Criado JR, Prospero-Garcia O, Gautvik KM, Schweitzer P, et al. 1996. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. Nature 381: 242-245.

de Lecea L, Kilduff TS, Peyron C, Gao X-B, Foye PE, et al. 1998. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci USA 95: 322-327.

Dube MG, Kalra SP, Kalra P. 1999. Food intake elicited by central administration of orexins/hypocretins: identification of hypothalamic sites of action. Brain Res 842: 473-477.

Duxon MS, Stretton J, Starr K, Jones DNC, Holland V, et al. 2001. Evidence that orexin-A-evoked grooming in the rat is mediated by orexin-1 ($OX_1$) receptors, with downstream 5-$HT_{2C}$ receptor involvement. Psychopharmacology (Berl) 153: 203-209.

Edwards CM, Abusnana S, Sunter D, Murphy KG, Ghatei MA, et al. 1999. The effect of the orexins on food intake: comparison with neuropeptide Y, melanin-concentrating hormone and galanin. J Endocrinol 160: R7-R12.

Eggermann E, Serafin M, Bayer L, Machard D, Saint-Mleux B, et al. 2001. Orexins/hypocretins excite basal forebrain cholinergic neurons. Neuroscience 108: 177-181.

Elias CF, Saper CB, Maratos-Flier E, Tritos NA, Lee C, et al. 1998. Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. J Comp Neurol 402: 442-459.

Ennis M, Aston-Jones G. 1989. GABA-mediated inhibition of locus coeruleus from the dorsomedial rostral medulla. J Neurosci 9: 2973-2981.

Eriksson KS, Sergeeva O, Brown RE, Haas HL. 2001. Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. J Neurosci 21: 9273-9279.

Espana RA, Baldo BA, Kelley AE, Berridge CW. 2001. Wake-promoting and sleep-suppressing actions of hypocretin (orexin): basal forebrain sites of action. Neuroscience 106: 699-715.

Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, et al. 2001. Fos expression in orexin neurons varies with behavioral state. J Neurosci 21: 1656-1662.

Fadel J, Deutch AY. 2002. Anatomical substrates of orexin-dopamine interactions: lateral hypothalamic projections to the ventral tegmental area. Neuroscience 111: 379-387.

Follwell MJ, Ferguson AV. 2002. Cellular mechanisms of orexin actions on paraventricular nucleus neurones in rat hypothalamus. J Physiol 545: 855-867.

Forster GL, Blaha CD. 2000. Laterodorsal tegmental stimulation elicits dopamine efflux in the rat nucleus accumbens by activation of acetylcholine and glutamate receptors in the ventral tegmental area. Eur J Neurosci 12: 3596-3604.

Fujiki N, Yoshida Y, Ripley B, Honda K, Mignot E, et al. 2001. Changes in CSF hypocretin-1 (orexin A) levels in rats across 24 hours and in response to food deprivation. Neuroreport 12: 993-997.

Gautvik KM, de Lecea L, Gautvik VT, Danielson PE, Tranque P, et al. 1996. Overview of the most prevalent hypothalamus-specific mRNAs, as identified by directional tag PCR subtraction. Proc Natl Acad Sci USA 93: 8733-8738.

Georgescu D, Zachariou V, Barrot M, Mieda M, Willie JT, et al. 2003. Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. J Neurosci 23: 3106-3111.

Gerashchenko D, Kohls MD, Greco M, Waleh NS, Salin-Pascual R, et al. 2001. Hypocretin-2-saporin lesions of the lateral hypothalamus produce narcoleptic-like sleep behavior in the rat. J Neurosci 21 : 7273-7283.

Griffond B, Risold PY, Jacquemard C, Colard C, Fellmann D. 1999. Insulin-induced hypoglycemia increases preprohypocretin (orexin) mRNA in the rat lateral hypothalamic area. Neurosci Lett 262: 77-80.

Gronenborn AM, Bovermann G, Clore GM. 1987. A 1H-NMR study of the solution conformation of secretin. Resonance assignment and secondary structure. FEBS Lett 215: 88-94.

Grudt TJ, van den Pol AN, Perl ER. 2002. Hypocretin-2 (orexin-B) modulation of superficial dorsal horn activity in rat. J Physiol 538: 517-525.

Guan JL, Uehara K, Lu S, Wang QP, Funahashi H, et al. 2002. Reciprocal synaptic relationships between orexin- and melanin-concentrating hormone-containing neurons in the rat lateral hypothalamus: a novel circuit implicated in feeding regulation. Int J Obes Relat Metab Disord 26: 1523-1532.

Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, et al. 1999. Orexin A activates locus coeruleus cell firing and increases arousal in the rat. Proc Natl Acad Sci USA 96: 10911-10916.

Hakansson M, de Lecea L, Sutcliffe JG, Yanagisawa M, Meister B. 1999. Leptin receptor-and STAT3-immunoreactivities in hypocretin/orexin neurons of the lateral hypothalamus. J Neuroendocrinol 11: 653-663.

Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, et al. 2001. Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. Neuron 30: 345-354.

Haynes AC, Chapman H, Taylor C, Moore GBT, Cawthorne MA, et al. 2002. Anorectic, thermogenic and anti-obesity activity of a selective orexin-1 receptor antagonist in ob/ob mice. Regul Pept 104: 153-159.

Hervieu GJ, Cluderay JE, Harrison DC, Roberts JC, Leslie RA. 2001. Gene expression and protein distribution of the orexin-1 receptor in the rat brain and spinal cord. Neuroscience 103: 777-797.

Hetherington AW, Ranson SW. 1940. Hypothalamic lesions and adiposity in the rat. Anat Rec 78: 149-172.

Higuchi S, Usui A, Murasaki M, Matsushita S, Nishioka N, et al. 2002. Plasma orexin-A lower is lower in patients with narcolepsy. Neurosci Lett 318: 61-64.

Hirose M, Egashira S, Goto Y, Hashihayata T, Ohtake N, et al. 2003. *N*-acyl 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline: the first orexin-2 receptor selective non-peptidic antagonist. Bioorg Med Chem Lett 13: 4497-4499.

Hobson JA, McCarley RW, Wyzinski PW. 1975. Sleep cycle oscillation: reciprocal discharge by two brainstem neuronal groups. Science 189: 55-58.

Hobson JA, Pace-Schott EF. 2002. The cognitive neuroscience of sleep: neuronal systems, consciousness and learning. Nat Rev Neurosci 3: 679-693.

Horvath TL, Diano S, van den Pol A. 1999a. Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. J Neurosci 19: 1072-1087.

Horvath TL, Peyron C, Diano S, Ivanov A, Aston-Jones, G, et al. 1999b. Hypocretin (orexin) activation and synaptic innervation of the locus coeruleus noradrenergic system. J Comp Neurol 415: 145-159.

Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, et al. 2001. Arousal effect of orexin A depends on activation of the histaminergic system. Proc Natl Acad Sci USA 98: 9965-9970.

Hungs M, Fan J, Lin L, Lin X, Maki RA, et al. 2001. Identification and functional analysis of mutations in the hypocretin (orexin) genes of narcoleptic canines. Genome Res 11: 531-539.

Hungs M, Mignot E. 2001. Hypocretin/orexin, sleep and narcolepsy. Bioessays 23: 397-408.

Hwang LL, Chen CT, Dun NJ. 2001. Mechanisms of orexin-induced depolarizations in rat dorsal motor nucleus of vagus neurones in vitro. J Physiol 537: 511-520.

Ida T, Nakahara K, Katayama T, Murakami N, Nakazato M. 1999. Effect of lateral cerebroventricular injection of the appetite-stimulating neuropeptide, orexin and neuropeptide Y, on the various behavioral activities of rats. Brain Res 821: 526-529.

Ida T, Nakahara K, Murakami T, Hanada R, Nakazato M, et al. 2000. Possible involvement of orexin in the stress reaction in rats. Biochem Biophys Res Commun 270: 318-323.

Iqbal J, Pompolo S, Sakurai T, Clarke IJ. 2001. Evidence that orexin-containing neurones provide direct input to gonadotropin-releasing hormone neurones in the ovine hypothalamus. J Neuroendocrinol 13: 1033-1041.

Ishii Y, Blundell JE, Halford JCG, Upton N, Porter R, et al. 2004. Differential effects of the selective orexin-1 receptor

antagonist SB-334867 and lithium chloride on the behavioural satiety sequence in rats. Physiol Behav 81: 129-140.

Ivanov A, Aston-Jones G. 2000. Hypocretin/orexin depolarizes and decreases potassium conductance in locus coeruleus neurons. Neuroreport 11: 1755-1758.

Jain MR, Horvath TL, Kalra PS, Kalra SP. 2000. Evidence that NPY Y1 receptors are involved in stimulation of feeding by orexins (hypocretins) in sated rats. Regul Pept 87: 19-24.

John J, Wu MF, Boehmer LN, Siegel JM. 2004. Cataplexy-active neurons in the hypothalamus: implications for the role of histamine in sleep and waking behavior. Neuron 42: 619-634.

Jones B. 1994. Basic mechanisms of sleep-wake states. Principles and practice of sleep medicine. Kryger M, Roth T, Dement WC, editors. Philadelphia, Pennsylvania: WB Saunders, pp. 145-162.

Jones DN, Gartlon J, Parker F, Taylor SG, Routledge C, et al. 2001. Effects of centrally administered orexin-B and orexin-A: a role for orexin-1 receptors in orexin-B-induced hyperactivity. Psychopharmacology 153: 210-218.

Karteris E, Randeva HS, Grammatopoulos DK, Jaffe RB, Hillhouse EW. 2001. Expression and coupling characteristics of the CRH and orexin type 2 receptors in human fetal adrenals. J Clin Endocrinol Metab 86: 4512-4519.

Kilduff TS, de Lecea L. 2001. Mapping of the mRNAs for the hypocretin/orexin and melanin-concentrating hormone receptors: networks of overlapping peptide systems. J Comp Neurol 435: 1-5.

Kirchgessner AL, Liu M. 1999. Orexin synthesis and response in the gut. Neuron 24: 941-951.

Korotkova TM, Eriksson KS, Haas HL, Brown RE. 2002. Selective excitation of GABAergic neurons in the substantia nigra of the rat by orexin/hypocretin in vitro. Regul Pept 104: 83-89.

Kukkonen JP, Akerman KE. 2001. Orexin receptors couple to $Ca^{2+}$ channels different from store-operated $Ca^{2+}$ channels. Neuroreport 12: 2017-2020.

Kunii K, Yamanaka A, Nambu T, Matsuzaki I, Goto K, et al. 1999. Orexins/ hypocretins regulate drinking behaviour. Brain Res 842: 256-261.

Kuru M, Ueta Y, Serino R, Nakazato M, Yamamoto Y, et al. 2000. Centrally administered orexin/hypocretin activates HPA axis in rats. Neuroreport 11: 1977-1980.

Kushikata T, Hirota K, Yoshida H, Kudo M, Lambert DG, et al. 2003. Orexinergic neurons and barbiturate anesthesia. Neuroscience 121: 855-863.

Lang M, Söll R, Dürrenberger F, Dautzenberg FM, Beck-Sickinger AG. 2004. Structure-activity studies of orexin A and orexin B at the human orexin 1 and orexin 2 receptors led to orexin 2 receptor selective and orexin 1 receptor preferring ligands. J Med Chem 47: 1153-1160.

Lee JH, Bang E, Chae KJ, Kim JY, Lee DW, et al. 1999. Solution structure of a new hypothalamic neuropeptide, human hypocretin-2/orexin-B. Eur J Biochem 299: 831-839.

Li Y, Gao XB, Sakurai T, van den Pol AN. 2002. Hypocretin/orexin excites hypocretin neurons via a local glutamate neuron-A potential mechanism for orchestrating the hypothalamic arousal system. Neuron 36: 1169-1181.

Lin L, Faraco J, Li R, Kadotani H, Rogers W, et al. 1999. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 98: 365-376.

Lin L, Hungs M, Mignot E. 2001. Narcolepsy and the HLA region. J Neuroimmunol 117: 9-20.

Liu RJ, van den Pol AN, Aghajanian GK. 2002. Hypocretins (orexins) regulate serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions. J Neurosci 22: 9453-9464.

Lopez M, Seoane L, Garcia MC, Lago F, Casanueva FF, et al. 2000. Leptin regulation of prepro-orexin and orexin receptor mRNA levels in the hypothalamus. Biochem Biophys Res Commun 269: 41-45.

Malendowicz LK, Tortorella C, Nussdorfer GG. 1999. Orexins stimulate corticosterone secretion of rat adrenocortical cells, through the activation of the adenylate cyclase-dependent signaling cascade. J Steroid Biochem Mol Biol 70: 185-188.

Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, et al. 2001. Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol 435: 6-25.

Martin G, Fabre V, Siggins GR, de Lecea L. 2002. Interaction of the hypocretins with neurotransmitters in the nucleus accumbens. Regul Pept 104: 111-117.

McCormick DA, Bal T. 1997. Sleep and arousal: thalamocortical mechanisms. Annu Rev Neurosci 20: 185-215.

Mignot E, Lammers G, Ripley B, Okun M, Nevsimalova S, et al. 2002. The role of CSF hypocretin measurements in the diagnosis of narcolepsy and other hypersomnias. Arch Neurol 59: 1553-1562.

Mignot E, Lin L, Rogers W, Honda Y, Qiu X, et al. 2001. Complex HLA-DR and -DQ interactions confer risk of narcolepsy–cataplexy in three ethnic groups. Am J Hum Genet 68: 686-699.

Mikkelsen JD, Hauser F, de Lecea L, Sutcliffe JG, Kilduff TS, et al. 2001. Hypocretin (orexin) in the rat pineal gland: a central transmitter with effects on noradrenaline-induced release of melatonin. Eur J Neurosci 14: 419-425.

Mondal MS, Nakazato M, Date Y, Murakami N, Yanagisawa M, et al. 1999. Widespread distribution of orexin in rat brain and its regulation upon fasting. Biochem Biophys Res Commun 256: 495-499.

Moore RY, Abrahamson EA, van den Pol A. 2001. The hypocretin neuron system: an arousal system in the human brain. Arch Ital Biol 139: 195-205.

Moragues N, Ciofi P, Lafon P, Tramu G, Garret M. 2003. GABAA receptor epsilon subunit expression in identified peptidergic neurons of the rat hypothalamus. Brain Res 967: 285-289.

Moriguchi T, Sakurai T, Nambu T, Yanagisawa M, Goto K. 1999. Neurons containing orexin in the lateral hypothalamic area of the adult rat brain are activated by insulin-induced acute hypoglycemia. Neurosci Lett 264: 101-104.

Muraki Y, Yamanaka A, Tsujino N, Kilduff TS, Goto K, et al. 2004. Serotonergic regulation of the orexin/hypocretin neurons through the 5-HT1A receptor. J Neurosci 24: 7159-7166.

Muroya S, Uramura K, Sakurai T, Takigawa M, Yada T. 2001. Lowering glucose concentrations increases cytosolic $Ca^{2+}$ in orexin neurons of the rat lateral hypothalamus. Neurosci Lett 309: 165-168.

Niimi M, Sato M, Taminato TJ. 2001. Neuropeptide Y in central control of feeding and interactions with orexin and leptin. Endocrine 14: 269-273.

Nilaweera KN, Barrett P, Mercer JG, Morgan PJ. 2003. Precursor-protein convertase 1 gene expression in the mouse hypothalamus: differential regulation by ob gene mutation, energy deficit and administration of leptin, and coexpression with prepro-orexin. Neuroscience 119: 713-720.

Nishino S, Fujiki N, Yoshida Y, Mignot E. 2003. The effects of hypocretin-1 in hypocretin receptor-2 mutated and hypocretin deficient narcoleptic dogs. Sleep 26: A287.

Nishino S, Ripley B, Overeem S, Lammers JG, Mignot E. 2000. Hypocretin (orexin) deficiency in human narcolepsy. Lancet 355: 39-40.

Nishino S, Ripley B, Overeem S, Nevsimalova S, Lammers GJ, et al. 2001. Low cerebrospinal fluid hypocretin (orexin) and altered energy homeostasis in human narcolepsy. Ann Neurol 50: 381-388.

Nowak KW, Mackowiak P, Switonska MM, Fabis M, Malendowicz LK. 2000. Acute orexin effects on insulin secretion in the rat: in vivo and in vitro studies. Life Sci 66: 449-454.

Olds J. 1962. Hypothalamic substrates of reward. Physiol Rev 42: 554-604.

Peever JH, Lai YY, Siegel JM. 2003. Excitatory effects of hypocretin-1 (orexin-A) in the trigeminal motor nucleus are reversed by NMDA antagonism. J Neurophysiol 89: 2591-2600.

Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, et al. 2000. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med 6: 991-997.

Peyron C, Tighe DK, van den Pol A, de Lecea L, Heller HC, et al. 1998. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 18: 9996-10015.

Piper DC, Upton N, Smith MI, Hunter AJ. 2000. The novel brain neuropeptide, orexin-A, modulates the sleep-wake cycle of rats. Eur J Neurosci 12: 726-730.

Porter RA, Chan WN, Coulton S, Johns A, Hadley MS, et al. 2001. 1,3-Biarylureas as selective non-peptide antagonists of the orexin-1 receptor. Bioorg Med Chem Lett 11: 1907-1910.

Pu S, Jain MR, Kalra PS, Kalra SP. 1998. Orexins, a novel family of hypothalamic neuropeptides, modulate pituitary luteinizing hormone secretion in an ovarian steroid-dependent manner. Regul Pept 78: 133-136.

Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, et al. 1996. A role for melanin-concentrating hormone in the central regulation of feeding behaviour. Nature 380: 243-247.

Randeva HS, Karteris E, Grammatopoulos D, Hillhouse EW. 2001. Expression of orexin-A and functional orexin type 2 receptors in the human adult adrenals: implications for adrenal function and energy homeostasis. J Clin Endocrinol Metab 86: 4808-4813.

Ranson SW. 1939. Somnolence caused by hypothalamic lesions in the monkey. Arch Neurol Psychiatr 41: 1-23.

Reti IM, Reddy R, Worley PF, Baraban JM. 2002. Selective expression of Narp, a secreted neuronal pentraxin, in orexin neurons. J Neurochem 82: 1561-1565.

Ripley B, Overeem S, Fujiki N, Nevsimalova S, Uchino M, et al. 2001. CSF hypocretin/orexin levels in narcolepsy and other neurological conditions. Neurology 57: 2253-2258.

Rodgers RJ, Halford JCG, Nunes de Souza RL, Canto de Souza AL, Piper DC, et al. 2001. SB-334867, a selective orexin-1 receptor antagonist, enhances behavioural satiety and blocks the hyperphagic effect of orexin-A in rats. Eur J Neurosci 13: 1444-1452.

Rosin DL, Weston MC, Sevigny CP, Stornetta RL, Guyenet PG. 2003. Hypothalamic orexin (hypocretin) neurons express vesicular glutamate transporters VGLUT1 or VGLUT2. J Comp Neurol 465: 593-603.

Russell SH, Small CJ, Dakin CL, Abbott CR, Morgan DG, et al. 2001a. The central effects of orexin-A in the hypothalamic–pituitary–adrenal axis in vivo and in vitro in male rats. J Neuroendocrinol 13: 561-566.

Russell SH, Small CJ, Kennedy AR, Stanley SA, Seth A, et al. 2001b. Orexin A interactions in the hypothalamo-pituitary gonadal axis. Endocrinology 142: 5294-5302.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, et al. 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled Receptors that regulate feeding behavior. Cell 92: 573-585.

Salomon R, Ripley B, Kennedy J, Johnson B, Schmidt D, et al. 2003. Diurnal variation of cerebrospinal fluid hypocretin-1 (orexin-A) levels in control and depressed subjects. Biol Psychiatry 54: 96-104.

Samson WK, Gosnell B, Chang JK, Resch ZT, Murphy TC. 1999. Cardiovascular regulatory actions of the hypocretins in brain. Brain Res 831: 248-253.

Samson WK, Taylor MM. 2001. Hypocretin/orexin suppresses corticotroph responsiveness in vitro. Am J Physiol Regul Integr Comp Physiol 281: R1140-R1145.

Samson WK, Taylor MM, Follwell M, Ferguson AV. 2002. Orexin actions in hypothalamic paraventricular nucleus: physiological consequences and cellular correlates. Regul Pept 104: 97-103.

Saper CB, Chou TC, Scammell TE. 2001. The sleep switch: hypothalamic control of sleep and wakefulness. Trends Neurosci 24: 726-731.

Sartin JL, Dyer C, Matteri R, Buxton D, Buonomo F, et al. 2001. Effect of intracerebroventricular orexin-B on food intake in sheep. J Anim Sci 79: 1573-1577.

Schuld A, Hebebrand J, Geller Fand Pollmacher T. 2000. Increased body-mass index in patients with narcolepsy. Lancet 355: 1274-1275.

Sherin JE, Shiromani PJ, McCarley RW, Saper CB. 1996. Activation of ventrolateral preoptic neurons during sleep. Science 271: 216-219.

Shirasaka T, Nakazato M, Matsukura S, Takasaki M, Kannan H. 1999. Sympathetic and cardiovascular actions of orexins in conscious rats. Am J Physiol 277: R1780-R1785.

Shirasaka T, Miyahara S, Kunitake T, Jin QH, Kato K, et al. 2001. Orexin depolarizes rat hypothalamic paraventricular nucleus neurons. Am J Physiol Regul Integr Comp Physiol 281: R1114-R1118.

Smart D, Jerman JC, Brough SJ, Rushton SL, Murdock PR, et al. 1999. Characterization of recombinant human orexin receptor pharmacology in a Chinese hamster ovary cell-line using FLIPR. Br J Pharmacol 128: 1-3.

Smart D, Sabido-David C, Brough SJ, Jewitt F, Johns A, et al. 2001. SB-334867-A: the first selective orexin-1 receptor antagonist. Br J Pharmacol 132: 1179-1182.

Smith BN, Davis SF, van den Pol AN, Xu W. 2002. Selective enhancement of excitatory synaptic activity in the rat nucleus tractus solitarius by hypocretin 2. Neuroscience 115: 707-714.

Steriade M, McCormick DA, Sejnowski TJ. 1993. Thalamocortical oscillations in the sleeping and aroused brain. Science 262: 679-685.

Sutcliffe JG, de Lecea L. 2002. The hypocretins: setting the arousal threshold. Nat Rev Neurosci 3: 339-349.

Swart I, Michael Overton J, Houpt TA. 2001. The effect of food deprivation and experimental diabetes on orexin and NPY mRNA levels. Peptides 22: 2175-2179.

Taheri S, Mahmoodi M, Opacka-Juffry J, Ghatei MA, Bloom SR. 1999. Distribution and quantification of immunoreactive orexin A in rat tissues. FEBS Lett 457: 157-161.

Takahashi N, Okumura T, Yamada H, Kohgo Y. 1999. Stimulation of gastric acid secretion by centrally administered orexin-A in conscious rats. Biochem Biophys Res Commun 254: 623-627.

Thakkar MM, Ramesh V, Strecker RE, McCarley RW. 2001. Microdialysis perfusion of orexin-A in the basal forebrain increases wakefulness in freely behaving rats. Arch Ital Biol 139: 313-28.

Thakkar MM, Winston S, McCarley RW. 2002. Orexin neurons of the hypothalamus express adenosine A1 receptors. Brain Res 944: 190-194.

Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, et al. 2000. Reduced number of hypocretin neurons in human narcolepsy. Neuron 27: 469-474.

Torrealba F, Yanagisawa M, Saper CB. 2003. Colocalization of orexin A and glutamate immunoreactivity in axon terminals in the tuberomammillary nucleus in rats. Neuroscience 119: 1033-1044.

Tritos NA, Mastaitis JW, Kokkotou E, Maratos-Flier E. 2001. Characterization of melanin concentrating hormone and preproorexin expression in the murine hypothalamus. Brain Res 895: 160-166.

Trivedi P, Yu H, MacNeil DJ, Van der Ploeg LHT, Guan X-M. 1998. Distribution of orexin receptor mRNA in the rat brain. FEBS Lett 438: 71-75.

Upton N. 2005. In vivo pharmacology of orexins (hypocretin) receptors. The hypocretins as integrators of physiological signals. de Lecea L, Sutcliffe JG, editor. New York: Springer; pp. 203-218.

Uramura K, Funahashi H, Muroya S, Shioda S, Takigawa M, et al. 2001. Orexin-a activates phospholipase C- and protein kinase C-mediated $Ca^{2+}$ signaling in dopamine neurons of the ventral tegmental area. Neuroreport 12: 1885-1889.

van den Pol AN. 1999. Hypothalamic hypocretin (orexin): robust innervation of the spinal cord. J Neurosci 19: 3171-3182.

van den Pol AN, Gao XB, Obrietan K, Kilduff TS, Belousov AB. 1998. Presynaptic and postsynaptic actions and modulation of neuroendocrine neurons by a new hypothalamic peptide, hypocretin/orexin. J Neurosci 18: 7962-7971.

von Economo C. 1930. Sleep as a problem of localization. J Nerv Ment Dis 71: 249-259.

Wang J, Osaka T, Inoue S. 2001. Energy expenditure by intracerebroventricular administration of orexin to anesthetized rats. Neurosci Lett 315: 49-52.

Willie JT, Chemelli RM, Sinton CM, Tokita S, Williams SC, et al. 2003. Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. Neuron 38: 715-730.

Winsky-Sommerer R, Yamanaka A, Diano S, Roberts AJ, Sakurai T, et al. 2004. Interaction between the corticotropin-releasing factor system and hypocretins (orexins): a novel circuit mediating stress response. J Neurosci 24: 11439-11448.

Wu M, Zaborszky L, Hajszan T, van den Pol AN, Alreja M. 2004. Hypocretin/orexin innervation and excitation of identified septohippocampal cholinergic neurons. J Neurosci 24: 3527-3536.

Xi MC, Morales FR, Chase MH. 2001. Effects on sleep and wakefulness of the injection of hypocretin-1 (orexin-A) into the laterodorsal tegmental nucleus of the cat. Brain Res 901: 259-264.

Yamamoto Y, Ueta Y, Nakazato M, Hara Y, Serino R, et al. 1999. Down regulation of the prepro-orexin gene expression in genetically obese mice. Brain Res Mol Brain Res 65: 14-22.

Yamanaka A, Kunii K, Nambu T, Tsujino N, Sakai A, et al. 2000. Orexin-induced food intake involves neuropeptide Y pathway. Brain Res 859: 404-409.

Yamanaka A, Muraki Y, Tsujino N, Goto K, Sakurai T. 2003. Regulation of orexin neurons by the monoaminergic and cholinergic systems. Biochem Biophys Res Commun 303: 120-129.

Yamanaka A, Sakurai T, Katsumoto T, Yanagisawa M, Goto K. 1999. Chronic intracerebroventricular administration of orexin-A to rats increases food intake in daytime, but has no effect on body weight. Brain Res 849: 248-252.

Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JL, et al. 2002. Orexins activate histaminergic neurons via the orexin 2 receptor. Biochem Biophys Res Commun 290: 1237-1245.

Yamuy J, Fung SJ, Xi M, Chase MH. 2004. Hypocretinergic control of spinal cord motoneurons. J Neurosci 24: 5336-5345.

Yasuda Y, Takeda A, Fukuda S, Suzuki H, Ishimoto M, Mori Y, et al. 2003. Orexin a elicits arousal electroencephalography without sympathetic cardiovascular activation in isoflurane-anesthetized rats. Anesth Analg 97: 1663-1666.

Yoshida Y, Fujiki N, Maki R, Schwarz D, Nishino S. 2003. Differential kinetics of hypocretins in the cerebrospinal fluid after intracerebroventricular administration in rats. Neurosci Lett 346: 182-186.

Yoshida Y, Fujiki N, Nakajima T, Ripley B, Matsumura H, et al. 2001. Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light-dark cycle and sleep-wake activities. Eur J Neurosci 14: 1075-1081.

Yoshimichi G, Yoshimatsu H, Masaki T, Sakata T. 2001. Orexin-A regulates body temperature in coordination with arousal status. Exp Biol Med 226: 468-476.

Zhu Y, Card JP, Aston-Jones G. 2003. Frequent hypocretin innervation of GABA neurons in the peri-locus coeruleus region: ultrastructural studies. Soc Neurosci Abstract 29: 931.15.

Zhu Y, Yamanaka A, Kunii K, Tsujino N, Goto K, et al. 2002. Orexin-mediated feeding behavior involves both leptin-sensitive and -insensitive pathways. Physiol Behav 77: 251-257.

# 23 Neuropeptide Y in Brain Function

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**Abstract:** Neuropeptide Y (NPY), a 36-amino-acid peptide is abundantly expressed throughout the mammalian nervous system including: neocortex, hippocampus, striatum, amygdala, hypothalamus, thalamus, and brain stem. NPY has been implicated in the regulation of a number of different behaviors and neurophysiological functions, many of them part of the complex system(s) involved in maintaining and regulating homeostasis. These include, among others, anxiety and stress related responses, feeding, learning and memory, endocrine function, and circadian rhythms. The functions are mediated via different receptor subtype populations (Y1–y6), all belonging to the G-protein-coupled receptor super-family. The Y1 subtype has been shown to mediate the anxiolytic effects of NPY, while the Y2 subtype is involved in regulation of circadian rhythms and neuronal excitability in the hippocampus, and may thus be the receptor subtype involved in the peptide's effects on memory function. Stimulation of food intake by NPY has been proposed to be mediated by Y5 and/or Y1 receptors within the hypothalamus. Here, we present a summary of current findings concerning the central nervous system (CNS) functions of NPY in the context of homeostasis and reaction to the environment.

**List of Abbreviations:** AA, amino acids; bp, base pair; CNS, central nervous system; CPON, C-flanking peptide of NPY; CRF, corticotropin-releasing factor; CSF, cerebrospinal fluid; GABA, gamma-aminobutyric acid; HPA, hypothalamic–pituitary–adrenal (axis); ICV, intracerebroventricular; IV, intravenously; NPY, neuropeptide Y; NPY-LI, NPY-like immunoreactivity; PCR, polymerase chain reaction; PP, pancreatic polypeptide; PVN, paraventricular nucleus; PYY, peptide YY; SCN, suprachiasmatic nucleus; SNP, single nucleotide polymorphism

## 1 Introduction

This chapter examines the function of neuropeptide Y (NPY) within the central nervous system (CNS). Information is provided on the discovery of NPY, how it is processed in neurons, where it is located within the CNS, and its receptor subtypes. Additional information on a range of behaviors NPY affects through its actions within the CNS is summarized.

In 1935, Sir Henry Dale postulated that information is exchanged between neurons as chemical signals, which spurred a plethora of work aimed at identifying the substances mediating these signals. In addition to the first characterized "classical" neurotransmitters, which include among others the catecholamines and acetylcholine, a number of neuroactive peptides have been isolated. These peptides have been grouped into families depending on common precursors and sequence homologies. One such family is the so-called pancreatic polypeptide family, which includes neuropeptide Y (NPY), pancreatic polypeptide (PP), peptide YY (PYY), and related peptides. Since NPY is phylogenetically the ancestor of this family, a more correct designation is probably the family of NPY-related peptides.

The first peptide to be isolated in the family of NPY-related peptides was PP, from avian pancreas, which therefore gave name to the family (Kimmel et al., 1975). Later, PP-like immunoreactivity was reported within the mammalian CNS, but attempts to extract the PP protein from the mammalian brain were unsuccessful pointing to the existence of "PP-like" peptides. PYY, and then NPY, were isolated in the beginning of the 1980s by Tatemoto, Mutt and coworkers using a method designed to detect peptides having a C-terminal amide group, a feature common to members of the PP-family together with a length of 36 amino acids (AA) (Tatemoto, 1982; Tatemoto et al., 1982). Subsequent work showed that NPY accounted for almost all the PP-like immunoreactivity in the brain, while PYY was mainly found in the intestine.

Phylogenetic studies have revealed NPY to be well conserved during evolution, implying an important functional role of the peptide (Cerda-Reverter and Larhammar, 2000; Conlon, 2002). The homology between species is high for gene organization, cDNA, and peptide sequences (*Table 23-1*). The organization of the gene is highly conserved between human and rat, and the peptide sequence is identical between the two species. The peptide precursor for NPY is 98 AA in both species. The precursor consists of a signal peptide of 28 AA (29 AA in rat), which is necessary for the nascent peptide chain to enter the lumen of the endoplasmic reticulum, and is cleaved upon entry into the lumen leaving a propeptide of 69 AA. The propeptide consists of the 36 AA of NPY, a three-amino-acid motif (glycine–lysine–arginine) necessary for

**Table 23-1**
**Comparison of sequences between the members of the NPY-family of peptides**

| | Sequence |
|---|---|
| hNPY | YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY- amide |
| cNPY | YPSKPD**S**PGEDAPAEDMARYYSALRHYINLITRQRY- amide |
| tNPY | YPSKPDNPGE**G**APAED**LA**K**K**YYSALRHYINLITRQRY- amide |
| pNPY | YPSKPDNPGEDAPAED**L**ARYYSALRHYINLITRQRY- amide |
| pPYY | YP**A**KP**EA**PGEDA**SP**E**E**L**S**RYY**AS**LRHY**L**NLVTRQRY- amide |
| hPYY | YP**I**KPEAPGEDASPEEL**N**RYYASLRHYLNLVTRQRY- amide |
| pPP | APLEPUYPGDDATPEQMAQYAAELRRYINMLTRPRY- amide |
| rPP | APLEP**M**YPGD**Y**AT**H**EQ**R**AQY**ETQ**LRRYIN**T**LTRPRY- amide |
| cPP | **G**P**SQ**P**T**YPGDDA**PV**E**DLIRFYND**L**QQ**YL**N**VV**TR**H**RY- amide |

Sequence homologies between NPY from human (hNPY), chicken (cNPY), fish (*Torpedo marmorata*; tNPY), and pig (pNPY). Porcine PYY and human PYY, as well as human PP, rat PP (rPP), and chicken PP (cPP) are also displayed. *Bold* letters indicate sequence differences compared with the top human sequence (for NPY), or with pig NPY (for pig PYY), or compared with pig PYY (for human PYY), or compared with human PP (for rPP and cPP). The sequences for human and rat NPY are identical, as are the sequences for pig and rat PYY. NPY peptide sequences have also been obtained from guinea pig and rabbit (identical to hNPY), as well as cow (1 AA different from hNPY), frog (1 AA different from hNPY), and goldfish (5 AA different from hNPY) among other species. The sequences for human PP and pig PP are identical. Peptide sequences for PP from dog, cat, guinea pig, goose, ostrich, and a number of other species have also been determined

generation of mature NPY from the propeptide, and a 30 AA peptide named C-flanking peptide of NPY (CPON) (*Figure 23-1*). The NPY propeptide, generated in the endoplasmic reticulum, migrates into specific large, dense core vesicles present in dendrites, cell bodies, and axons. Expression of the NPY gene is tightly regulated, and NPY is primarily expressed in cells derived from the neural crest. Several factors are involved in the regulation of expression indicated by the presence of a number of consensus sequences for DNA-binding proteins within the NPY gene. These include: five potential, GC-rich SP-1 sites, two CCCCTC sites, a partial CAAT-box, and one AP-1 binding site (Minth et al., 1986; Minth and Dixon, 1990). Additionally, NPY expression is controlled by activators of cAMP and calcium- or phospholipid-dependent protein kinases. Phosphorylated cAMP responsive element-binding protein (CREB) regulates expression of NPY, and a decrease in p-CREB has been speculated to result in decreased NPY levels (Pandey, 2003; Pandey et al., 2003).

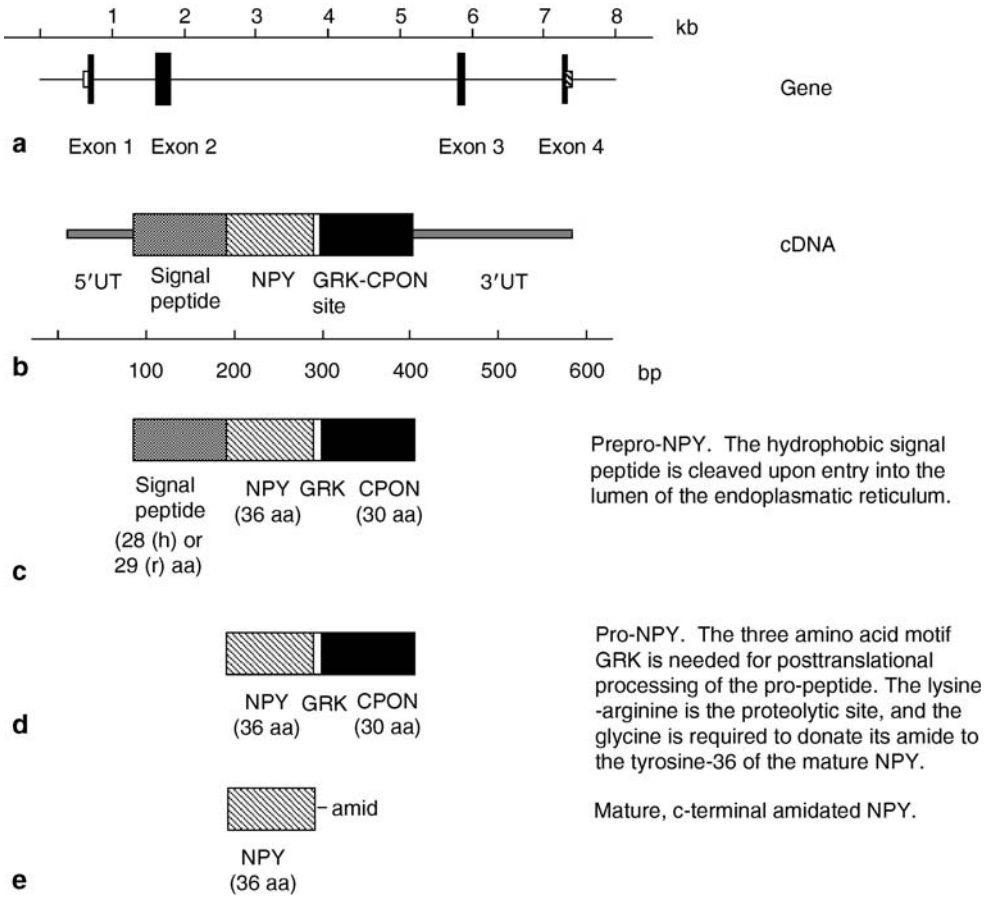
Within the human NPY sequence, there exists a number of single nucleotide polymorphisms (SNPs). The first one identified (1128 T/C) causes an AA change from leucine to proline at codon 7 in the signal peptide of NPY (Karvonen et al., 1998). A recent study further examined the human NPY gene and determined the existence of eight additional SNPs (Ding et al., 2005). The significance of these SNPs with regard to peptide processing and expression remains to be elucidated, although some studies examining SNP frequency and behavioral correlates have been performed (See Sect. 4).

## 2 Distribution of NPY Neurons in the CNS

The distribution of NPY neurons has been extensively studied using immunocytochemical methods, radioimmunoassay (RIA), and in situ hybridization. Early studies demonstrated that NPY is the most abundant and widely distributed neuropeptide in mammalian brain. In the rat, it is expressed at particularly high levels in hypothalamic areas, nucleus accumbens, septum, and the periaqueductal gray matter. Moderate expression levels have been shown in the hippocampus, the amygdala, the thalamus, and in the basal ganglia, while expression is almost absent in the pons and the cerebellum. In humans, the highest density of NPY mRNA-positive cells was seen in nucleus accumbens, caudate nucleus, putamen, and

**Figure 23-1**

**A summary of the processing steps preceding generation of the mature (neuropeptide Y) NPY-peptide. The organization of rat and human NPY gene, mRNA, and protein are very similar. (a) General organization of the NPY gene. The overall homology between rat and human NPY gene is 60%, a number that rises to 85% when nucleotides in the open reading frame are compared. (b) The complementary DNA (cDNA) in rat is 559 bp while the human NPY cDNA is 591 bp. This is mainly due to a difference in length of the poly(A) tail. (c) The cDNA in rat predicts an open reading frame encoding a 98 amino acid precursor of NPY. In humans, the NPY precursor is 97 AA. The difference is due to the presence of two contiguous initiation codons (ATG) in rat. (d) The signal peptide is cleaved upon entry into the lumen of the endoplasmic reticulum. (e) The mature peptide consists of 36 AA and is amidated at the C-terminal**




substantia innominata. Fewer NPY mRNA-containing neurons were found in frontal and parietal cortex, the amygdala, and dentate gyrus. No NPY mRNA-containing cells were found in substantia nigra (Brene et al., 1989; Caberlotto et al., 1997). Tissue concentrations of NPY seem to correlate well with fiber density and terminal networks, but not with density of cell bodies. NPY has been found in two basic types of neurons; interneurons (Chronwall et al., 1985; De Quidt and Emson, 1986) and long projection cells (De Quidt and Emson, 1986). In the forebrain NPY is mainly found in interneurons thought to constitute elements of local inhibitory circuits. NPY containing long projection neurons are mainly found in the brainstem where they project a considerable distance to a number of sites in the neural axis.

## 2.1 Neocortex

NPY neurons in the cortex are organized in a way that correlates with areal, laminar, and columnar borders of the cerebral cortex. Areal differences are apparent from RIA studies where the concentration of NPY in rat and human cortex varies with as much as a factor of three between cortical regions. The highest concentrations are detected in the cingulate gyrus and in association areas of the temporal lobe. Areas with the lowest NPY expression include frontal and occipital lobes. For NPY mRNA, similar results have been reported in the human brain. The presence of NPY-positive somata has been demonstrated throughout the cerebral cortex with the highest concentrations observed in layers II, III, V, and VI (Chan-Palay et al., 1985; Brene et al., 1989; Caberlotto et al., 2000). Unlike the somata, the distribution of NPY-LI fiber plexuses varies across cortical layers and areas. The NPY immunoreactive cortical neurons are mainly nonpyramidal cells.

Most NPY-positive neurons also contain other neurotransmitter/neuromodulatory substances. Many contain somatostatin-like immunoreactivity and, in the cortex, this subpopulation also displays immunoreactivity for NADPH-diaphorase/nitric oxide synthase (Chronwall et al., 1984; Hendry et al., 1984b; Unger and Lange, 1992; Huh et al., 1997). There is a large group of inhibitory interneurons, where NPY and GABA coexist. These GABA-NPY-positive neurons influence cortical output via synapses formed mainly on the processes of pyramidal projection neurons. Only a small subpopulation of the total cortical NPY cell population is not immunoreactive for GABA or its synthetic enzyme glutamic acid decarboxylase (Hendry et al., 1984a; Aoki and Pickel, 1990; Demeulemeester et al., 1991). This subpopulation of neurons display immunoreactivity for two members of the tachykinin peptide family: substance P and neurokinin A (Jones et al., 1988). NPY-LI cell bodies and processes are also found in subcortical white matter (Hendry et al., 1984b).

## 2.2 Hippocampus

Evolutionarily, the hippocampal formation is an old part of the cerebral cortex. As in the neocortex, hippocampal NPY-immunoreactive neurons are mainly nonpyramidal cells. There is colocalization of NPY with somatostatin and also GABA, but to lesser and varying degrees across the hippocampal formation (Chan-Palay et al., 1986; Kohler et al., 1986a, b). Somata and processes displaying NPY-LI are unevenly distributed in the parts of the hippocampus. Cell bodies containing NPY-LI are found in the stratum pyramidale, radiatum, and oriens of the CA region. The highest density of NPY neurons is found in the hilus of the dentate gyrus. The majority of NPY innervation form local projections, but some evidence exists for extrahippocampal origin of NPY-terminals (Chan-Palay et al., 1986).

## 2.3 Striatum

The striatum is a chemically heterogeneous structure consisting of patches, or striosomes, containing neuronal populations with certain chemical signatures and a matrix surrounding the patches displaying another set of chemical characteristics.

The majority of NPY-LI-positive somata are located in the matrix; however, there is a great deal of species specificity. In cat, somata have been demonstrated in both compartments, while only the matrix contains fibers that stain for NPY. In contrast no clustering of NPY somata is seen in human or monkey striatum (Dawbarn et al., 1984; Smith and Parent, 1986). In rat, the majority of the NPY-LI-positive somata are located in the matrix, but some neurons are also present in the patches. The primary dendrites of NPY cells extend only within the compartment of origin (Kubota and Kawaguchi, 1993). As is seen in cortical areas, the majority of NPY-LI-positive cells in the striatum are interneurons.

Colocalization of NPY with both somatostatin and NADPH-diaphorase is found in the majority of striatal NPY neurons (Vincent et al., 1983; Gaspar et al., 1987). There is evidence that dopamine afferents from the substantia nigra regulate expression of NPY in the striatum. The nature of that regulation appears to be a partial suppression under normal conditions (Kerkerian et al., 1986; Brene et al., 1989; Vuillet et al., 1989).

## 2.4 Amygdala

The amygdala is a structure consisting of a number of nuclei with different neurochemistry and cytoarchitecture. The expression of NPY-LI is not homogenous throughout the structure. In primates, the NPY-LI perikarya are denser in the medial and basal nuclei, and more scattered in the central and cortical nuclei (Gustafson et al., 1986; Schwartzberg et al., 1990).

There are three morphologically different types of NPY neurons within the amygdala; one group consisting of small and bipolar neurons, a second group possessing large somata and being present at the base of the stria terminalis, and a third group of very large, multipolar cells (Gustafson et al., 1986). A fraction of the NPY neurons in rat amygdala are positive for somatostatin, and these are mainly the small, bipolar neurons (McDonald, 1989; McDonald et al., 1995). Amygdaloid NPY-containing cells are mainly interneurons, but the existence of an amygdalofugal projection has also been suggested (Allen et al., 1984).

Within the central nucleus of the amygdala a dense fiber plexus has been located with a distribution similar to that of noradrenergic axons rising from the brainstem (Fallon et al., 1978). However, 6-hydroxydopamine lesions indicate that the two networks are not identical (Gustafson et al., 1986) since complete loss of noradrenergic fibers is not found to alter NPY neurons. This has also been demonstrated in pharmacological studies (Smialowska et al., 2001).

## 2.5 Hypothalamus and Thalamus

Hypothalamus has one of the highest expression levels of NPY in the CNS. NPY-LI-positive cell bodies or preproNPY mRNA-containing cell bodies are mainly found in the arcuate nucleus and in the lateral hypothalamus (Gehlert et al., 1987; Morris, 1989), while fibers and terminals are spread throughout the region. In the rat hypothalamus, the suprachiasmatic nuclei (SCN), supraoptic, and paraventricular nuclei (PVN) are densely innervated, but the density of fibers is uneven within the individual nuclei (Gustafson and Moore, 1987; Card and Moore, 1988). The SCN is innervated by NPY axons arising from the ventral lateral geniculate nucleus of the thalamus (Gustafson et al., 1986; Harrington et al., 1987). NPY innervation of the PVN arises from the brainstem (Sawchenko and Pfeiffer, 1988) as well as from a shorter projection system originating in the arcuate nucleus (Bai et al., 1985). In the arcuate nucleus, NPY is colocalized with agouti gene-related protein (Broberger et al., 1998; Hahn et al., 1998). The NPY-LI axons innervating the parvocellular and magnocellular divisions of the PVN arise principally from adrenergic cell groups in the medulla as well as from noradrenergic somata in the A1 cell group (Sawchenko et al., 1985). In the parvocellular division, the innervation is the densest for neurons positive for corticotropin-releasing factor (CRF) and thyrotropin-releasing factor (Sawchenko et al., 1985). Within the magnocellular division innervation appears to be equally dense for vasopressin and oxytocin neurons.

The thalamus has low concentrations of NPY in the CNS except in discrete nuclei (Dawbarn et al., 1984; Chronwall et al., 1985). Very few somata and only scattered fibers are found in the dorsal thalamus. NPY fibers are present in the anterior thalamic nuclei of the rat (Nakagawa et al., 1985) and within certain midline thalamic nuclei. There is a mismatch between the distribution of preproNPY mRNA and NPY-LI within the reticular nucleus. Most somata are found to express preproNPY mRNA, but very few fibers and no somata display NPY-LI. A plausible explanation for this discrepancy is the transient expression of NPY-LI during ontogeny in this nucleus, and the NPY mRNA present in the adult animal may be a remnant of this expression (Foster et al., 1984).

## 2.6 Brain Stem

In the rat, NPY somata have been demonstrated in the central grey, interpeduncular nucleus, and inferior colliculus (De Quidt and Emson, 1986; Morris, 1989), however, distribution of NPY fibers and somata is sparse in other areas of the midbrain. NPY-LI has been shown in some cranial nerve nuclei and monamine-containing nuclei in the lower brainstem. NPY-positive neurons are found in the respiratory nuclei in cat

(Aguirre, 1989). Other populations of NPY-LI-positive neurons are found in the spinal trigeminal nucleus of the rat (De Quidt and Emson, 1986) and the dorsal motor nucleus of the vagus of the rabbit (Blessing et al., 1986). Noradrenergic cells containing NPY are located in the A1 group in the ventrolateral medulla and in the locus coeruleus (Everitt et al., 1984; Smialowska, 1988). Adrenergic C1 and C2 cell groups, as well as serotonergic cells in the nucleus raphe pallidus, have also been shown to contain NPY (Everitt et al., 1984; Blessing et al., 1986).

## 3 Neuropeptide Y Receptors

To date no less than six different receptor subtypes for NPY have been discovered (Y1, Y2, Y4, Y5, and y6), each with a unique pharmacological profile and anatomical distribution. The Y4 receptor has higher affinity for another member of the NPY family of peptides, PP, and the y6 receptor is present in mouse but not in human or rat, and thus has limited generalizability. The molecular evolution of the NPY receptor subtypes has been recently reviewed (Larhammar and Salaneck, 2004).

The presence of more than one receptor subtype was first demonstrated in a study where C-terminal fragments of NPY/PYY were found to be active at prejunctional sites, but not postjunctional sites, at the sympathetic neuroeffector junction (Wahlestedt et al., 1986). The post- and prejunctional receptors were referred to as Y1 and Y2, respectively. All receptors for NPY belong to the superfamily of G-protein-coupled receptors and share the common feature of seven transmembrane spanning regions. NPY receptor subtypes also differ in their ligand-binding profiles.

NPY receptors mediate the inhibition of adenylate cyclase resulting in reduced cAMP accumulation and/or elevated intracellular $Ca^{2+}$ concentrations (Wahlestedt et al., 1986; Herzog et al., 1992). An accumulation of inositol phosphatases has also been observed in blood vessels and in the cerebral cortex as a result of NPY receptor activation (Hinson et al., 1988).

The Y1 receptor (Eva et al., 1992; Larhammar et al., 1992) requires the entire NPY peptide for activation (Wahlestedt et al., 1986). Truncation of the first N-terminal residue results in a reduction of biological activity at the Y1 receptor. Thus, both the N- and C-terminal portions of NPY are required for recognition and activation at the Y1 receptor. In the periphery, the Y1 receptor is present postjunctionally at the vascular sympathetic neuroeffector junction and mediates pressor responses to NPY (Grundemar and Hakanson, 1993). In the brain, Y1 receptor sites are concentrated in distinct layers of the cerebral cortex, the anterior olfactory nucleus, dentate gyrus of the hippocampus, amygdala, and a few thalamic and hypothalamic nuclei (Dumont et al., 1998b).

The Y2 receptor subtype (Gerald et al., 1995) is activated both by full-length NPY peptide and by C-terminal fragments such as NPY13-36. In the periphery, the Y2 receptor is located at prejunctional sites of autonomic fibers, where it suppresses release of transmitters (Wahlestedt et al., 1986). In the CNS, presynaptic Y2 receptors have been shown to decrease postsynaptic excitability through a $Ca^{2+}$-mediated downregulation of glutamate release at the synapse (Colmers et al., 1991). The majority of NPY receptors in the CNS are most likely of the Y2 subtype, with especially high densities in the hippocampal formation (Aicher et al., 1991; Dumont et al., 2000). Y2-specific binding sites have been found to be prominent in the lateral septum, piriform cortex, bed nucleus of stria terminalis, dorsal hippocampus, substantia nigra, dorsal raphe nucleus, and the cerebellum (for review see Kaga et al., 2001).

The Y5 receptor was discovered in 1996 (Gerald et al., 1996). Y5 mRNA can be detected in brain by in situ hybridization in the dentate gyrus and CA3 area of the hippocampus, cingulate cortex, in a number of hypothalamic nuclei including the lateral hypothalamus, the PVN, and the supraoptic nucleus, as well as the in the central and anterior cortical amygdaloid nuclei (Gerald et al., 1996; Fetissov et al., 2004). Competitive binding studies have indicated presence of "Y5-like" sites, determined as [$^{125}$I][Leu31Pro34]PYY/BIBP3226-insensitive sites, in the olfactory bulb, the lateral septum, the anteroventral thalamic nucleus, the CA3 subfield of the ventral hippocampus, the nucleus tractus solitarius, and the area postrema (Dumont et al., 1998a). However, unlike in situ hybridization studies, only a limited number of Y5 receptor sites were found in hypothalamic nuclei using this methodology. The use of transgenic and knockout models has also helped to unravel some of the different functions of these Y receptors (see Lin et al., 2004 for review).

## 4 Central Nervous System Functions of NPY

NPY has a number of actions within the CNS that are important in homeostatic function. These actions which include processes such as locomotion, cardiovascular regulation, circadian rhythms and sleep, feeding, endocrine regulation, brain excitability, anxiety and depression, which appear at first to represent a highly diverse set of functions. Using an integrative view, however, one can view NPY neural networks as acting as inhibitory brain systems that dominate during times of "safety in the environment." Thus, NPY systems generally reduce locomotion, promote sleep, stimulate ovulation, enhance feeding responses, reduce cardiovascular tone, lower overall brain excitability, and suppress anxious and depressive-like behaviors. These responses are counteracted by the effects of CRF and other stress hormones that promote the opposite behavioral profile during times of "unsafety in the environment." Therefore NPY and CRF/stress systems can be envisioned as acting in concert to promote homeostasis in the face of changing environmental conditions. These systems have also been suggested to regulate allostatic processes occurring during chronic stress, drug addiction, and psychiatric disorders. Keeping this framework in mind, behavioral responses to NPY are summarized in the next sections of this review. Due to space limitations, each section only outlines a few key findings and then guides the reader to recent comprehensive reviews in each area (for instance see Chronwall and Zukowska, 2004). A summary of some important functions of NPY within the CNS grouped by association with different receptor subtypes is given in *Table 23-2*.

### 4.1 Locomotor Activity, Sedation, and Sleep

Central administration of NPY into the cerebral ventricles has been shown to produce a series of behaviors. One obvious result is a dose-dependent suppression of locomotor activity in both the home cage environment and in the open field test of responding in a novel environment (Heilig and Murison, 1987a). This behavior most likely reflects sedation, since lower doses of NPY induce EEG synchronization typical of anxiolytic-like sedation (Fuxe et al., 1983; Ehlers et al., 1997a), and central administration of high doses of NPY causes frank behavioral sedation. However, in spontaneously hypertensive rats, NPY paradoxically increases locomotor activity. This is most likely due to differential Y1 vs. Y2 receptor distribution in the brain of the different strains (Heilig et al., 1989b), as injection of NPY directly into the frontal cortex appears to increase locomotor activity (Smialowski et al., 1992).

It has been suggested based on these and other data that the suppression of locomotor activity is mediated via Y1 receptors in hypothalamic sites, while the Y2 receptor subtype may be responsible for increased locomotor activity (Heilig et al., 1988b; Naveilhan et al., 2001). For instance, ICV injection of Y1 receptor antisense oligodeoxynucleotides does not affect locomotor activity (Heilig, 1995), whereas intrahypothalamic injections cause a decrease in locomotor activity (Lopez-Valpuesta et al., 1996). Recent studies using Y1, Y2, and Y5 receptor agonists further suggest that Y5 receptors may have a larger role in sedation than Y1 and Y2 types (Sorensen et al., 2004). Thus, endogenous NPY may have differential effects on locomotor activity depending on the brain system activated.

NPY has also been implicated in the regulation of sleep. In animal models NPY has been demonstrated to shorten the latency to the onset of sleep and antagonize the insomnia producing effects of CRF (Ehlers et al., 1997b). NPY has also been demonstrated to shorten the latency to sleep onset when given IV in young men as well as increase the amount of sleep and reduce cortisol and ACTH secretion (Antonijevic et al., 2000). These studies suggest that NPY participates in sleep regulation and may be important in the timing of sleep onset (for review see Steiger, 2003).

### 4.2 Cardiovascular Regulation

ICV administration of NPY produces lowered mean arterial blood pressure, heart rate, and respiratory rate (Fuxe et al., 1983) and has been suggested as an important regulator in both cardiovascular and pulmonary

**Table 23-2**
**Important functions of neuropeptide Y associated with the Y1-, Y2-, and Y5-receptor subtypes in the brain**

| Receptor | Function | Location | Reference |
|---|---|---|---|
| Y1 | Locomotor activity suppression | Hypothalamus | Heilig et al. (1988b), Naveillhan et al. (2001) |
| | Cardiovascular function (lower heart rate, mean arterial blood pressure, respiratory rate) | (Nucleus tractus solitarius); (posterior hypothalamus) | Fuxe et al. (1983), Barraco et al. (1990), Martin (2004) |
| | Seizure modulation | Hippocampus | Gariboldi et al. (1998) |
| | Anxiety-related behavior | Amygdala | Britton et al. (1997), Heilig (1995) |
| | Antidepressant-like effects | | Redrobe et al. (2003), Tschenett et al. (2003) |
| Y2 | Locomotor activation | Hypothalamus | Heilig et al. (1988b), Naveilhan et al. (2001) |
| | Cardiovascular function | Nucleus tractus solitarius (posterior hypothalamus | Aguirre et al. (1990) |
| | Circadian rhythm | Suprachiasmatic nucleus (SCN) | Golombek et al. (1996), Soscia and Harrington (2005) |
| | Feeding behavior | | Naveilhan et al. (1999) |
| | Memory function | Hippocampus | Flood et al. (1989), Redrobe et al. (2004) |
| | Seizure modulation | Hippocampus | Colmers et al. (1991), Schwarzer et al. (1998) |
| | Antidepressant-like effect | | Redrobe et al. (2003), Tschenett et al. (2003) |
| Y5 | Sedation | | Sorensen et al. (2004) |
| | Circadian rhythm regulation | SCN? | Yannielli et al. (2004) |
| | Feeding behavior | Hypothalamus | Gerald et al. (1996), Schaffhauser et al. (1997) |
| | Seizure modulation | Hippocampus | Benmaamar et al. (2005), Vezzani et al. (2000) |

disorders (Groneberg et al., 2004). Central NPY regulation of cardiovascular functions was initially suggested to be mediated by the nucleus tractus solitarius, as direct injections of the peptide into that nucleus produced a reduction in blood pressure and heart rate (Barraco et al., 1990). Central regulation of cardiovascular function by NPY may be mediated by Y2 (Aguirre et al., 1990) and/or Y1 receptors in this structure. For instance, microinjections of [Leu34Pro31]-NPY caused dose-dependent vasodepressor and bradycardic responses (Narvaez et al., 1993). More recent studies suggest that the Y1 receptor subtype mediates the cardiovascular changes evoked by NPY central administration into posterior hypothalamic sites (Martin, 2004). In addition to its role in vasoconstriction through Y1 receptors, it has been suggested that NPY may have trophic properties that promote cardiac and vascular remodeling through a number of Y-type receptor systems (see Pedrazzini et al., 2003; Pons et al., 2004 for reviews).

## 4.3 Circadian Rhythms

The importance of NPY in the regulation of circadian rhythms has been verified in a number of different studies and in a variety of species. NPY was localized in the rat suprachiasmatic nucleus (Card and

Moore, 1988), and a pathway of NPY afferents from the intergeniculate leaflet to the SCN identified (Card and Moore, 1989). Lesions or electrical stimulation of the thalamic intergeniculate leaflet (IGL) are known to alter circadian rhythms (Harrington and Rusak, 1986; Rusak et al., 1989). NPY neurons in the geniculohypothalamic tract, which links IGL to the SCN, are thought to relay photic stimuli as well as nonphotic stimuli such as novelty-induced arousal (see Yannielli and Harrington, 2001, 2004; Lall and Biello, 2002 for reviews). The expression levels of NPY and mRNA in the SCN have been shown to have a circadian rhythm (Calza et al., 1990), with NPY levels increasing during the light phase of the cycle.

Microinjections of NPY directly into the SCN can generate a shift in the circadian rhythms, the direction of which is dependent on the time of injection without an alteration in phase length (Albers and Ferris, 1984). When NPY is injected during the subjective night, no phase-shift is reported, although it may inhibit the phase-shifting effect of light. Injections made during the active day-phase gave rise to a dose-dependent phase shift in both in vivo and in vitro systems (Weber and Rea, 1997; Harrington and Schak, 2000). The time of maximal effect of endogenous NPY on circadian systems appears to be a time when exogenous administration of NPY has little effect (Yannielli and Harrington, 2001).

The shift of circadian rhythm by NPY is suggested to be mediated via Y2 receptors (Golombek et al., 1996; Huhman et al., 1996; Soscia and Harrington, 2005) through period 1 (per1) and period 2 (per2) gene expression and/or glutamate mechanisms in SCN (Fukuhara et al., 2001; Maywood et al., 2002; Gamble et al., 2004). Although there is also recent data to suggest that blockade of the Y5 receptor can potentiate circadian responses to light in vivo and in vitro (Yannielli et al., 2004).

## 4.4 Feeding Behavior

Stimulation of food intake is one of the peptide's most prominent effects when administered into the CNS, and may be one of NPY's most important actions in homeostasis. Acutely, the peptide stimulates food intake for hours following central administration (Levine and Morley, 1984; Stanley and Leibowitz, 1985). When administered chronically, NPY induces a state that mimics hormonal and metabolic changes seen in obesity (Zarjevski et al., 1993; Vettor et al., 1994). NPY preferentially stimulates carbohydrate intake, having little or no effect on intake of protein or fat (Stanley et al., 1985). It has been suggested that NPY-induced food consumption is due to an increased motivation to eat, since NPY-treated animals will perform activities, such as lever-pressing, and will endure electric shocks in order to obtain food, without increasing their passive food consumption (Flood and Morley, 1991) (see Kalra and Kalra, 2004b; Levine et al., 2004) for reviews).

The site of action of NPY on feeding was found to be a pathway from the arcuate nucleus to the paraventricular nucleus of the hypothalamus Sahu et al., 1988 (see (Kalra and Kalra, 2004a) for review). It is presently not clear whether the profound effects of NPY on feeding are mediated by Y5 receptors, or a combination of different Y receptors within the hypothalamus (Gerald et al., 1996; Kanatani et al., 2000). A Y5 receptor antisense oligodeoxynucleotide was shown to inhibit food intake (Schaffhauser et al., 1997), while inactivation of the Y5 receptor in mice did not cause any alteration in feeding behavior (Marsh et al., 1998). Thus, even though the Y5 subtype may be involved in mediating feeding effects for NPY, it may not be critical in overall regulation of food intake and related physiological factors. Indeed, Y5 mRNA expression has been demonstrated to always be colocalized with expression of Y1 mRNA (but not vice versa) (Parker and Herzog, 1999).

Involvement of the Y2 receptor subtype in mediating normal feeding behavior and leptin response has also been suggested (Naveilhan et al., 1999). Mice lacking the Y2 receptor display increased body weight, food intake, and fat deposition, as well as an attenuated response to leptin administration. Response to central NPY administration was normal, as was refeeding following starvation. The Y2 receptor may thus have an inhibitory role in the central regulation of feeding behavior.

In addition to increased feeding, NPY also affects energy expenditure (see Williams et al., 2004; Kishi and Elmquist, 2005 for reviews). ICV administration of NPY has effects on energy metabolism such as decreased brown-fat thermogenesis and increased white-fat lipoprotein lipase (LPL) enzymatic activity

(Billington et al., 1991, 1994). A leucine(7)-to-proline(7) polymorphism in the signal peptide of NPY has been associated with high serum total cholesterol and LDL cholesterol levels (Karvonen et al., 1998, 2000). NPY neurons in the arcuate nucleus are found to be overactive in animals that are in negative energy balance due to factors such as starvation, lactation, or diabetes (Frankish et al., 1995). Thus, it appears that NPY neurons are responsive to numerous metabolic and hormonal signals including insulin and glucocorticoids (see Herzog, 2003; Williams et al., 2004 for reviews).

## 4.5 Endocrine Regulation and Stress Responses

NPY appears to be important in many aspects of endocrine function including regulation of ovulation, control of metabolism, and modulation of the stress response. NPY stimulates corticotropin-releasing hormone (CRH) gene expression through actions in the PVN both in vitro and in vivo (Haas and George, 1987; Tsagarakis et al., 1989). Injections of NPY into the PVN increase serum levels of ACTH, corticosterone, and aldosterone (Harfstrand et al., 1987; Wahlestedt et al., 1987), in addition to influencing CRH gene expression.

NPY also appears to play a significant role in the modulation of stress responses. For instance, ICV administration of NPY largely prevents gastric ulceration induced by a strong stressor (Heilig and Murison, 1987b). Transgenic rats overexpressing NPY in hippocampus were shown to be resistant to stress-induced increases in anxiety-like behavior (Thorsell et al., 2000; Carvajal et al., 2004). The physiological involvement of endogenous NPY in mediation of stress responses was also demonstrated in two studies showing that NPY gene expression is affected by stress. Acute stress was found to downregulate NPY-IR and NPY mRNA expression in amygdala and cortex, while repeated exposure to the stressor lead to an upregulation of NPY in the amygdala (Thorsell et al., 1998, 1999). On the basis of these and other pharmacological and expression studies, it was proposed that an upregulation of NPY expression may contribute to successful behavioral adaptation to stress through its "buffering" of stress-promoting signals such as CRF (Heilig and Thorsell, 2002). NPY has also been proposed to be involved in the integration of stress and feeding behavior through hypothalamic–pituitary–adrenal (HPA) axis activity (Hanson and Dallman, 1995).

Central administration of NPY also affects secretion of prolactin, growth hormone, and thyrotropin, among others (Harfstrand et al., 1987). Depending on the hormonal state of the animal, NPY has also been reported to modulate the release of luteinizing hormone from the pituitary gland (McDonald et al., 1985) (see Evans, 1999; Magni, 2003 for reviews).

## 4.6 Memory Function

NPY has also been implicated in modulation of memory function as central administration of NPY has been shown to improve memory retention and to reverse scopolamine-induced amnesia in mice (Flood et al., 1987). Central injection of NPY results in differential effects of NPY on memory retention depending on brain site. When injected into the rostral hippocampus and septum memory retention was enhanced, while injection into the amygdala or caudal hippocampus impaired retention (Flood et al., 1989). In a model of short-term or working memory, the delayed matching to sample test (DMTS), low doses of NPY enhanced, while high doses were found to impair working memory (Thomas and Ahlers, 1991). Learning deficits have been reported in younger but not older transgenic rats that overexpressed NPY (Thorsell et al., 2000; Carvajal et al., 2004). The Y2 receptor subtype has been suggested to be involved in learning and memory, perhaps through sites in hippocampus. NPY genes have been found to be upregulated following long-term potentiation, an electrophysiological model of short-term memory, in the hippocampus (Thompson et al., 2003). Furthermore NPY Y2 receptor knockout mice have been demonstrated to show deficits in the Morris maze and in an object recognition test (Redrobe et al., 2004). However, it remains important to dissect out whether these cognitive effects of NPY may be confounded by its actions on emotionality and response to stress.

## 4.7 Modulation of Brain Excitability and Seizures

There is ample data to suggest that NPY neuronal systems are important in the regulation of brain excitability. The generation of epileptic seizures in the brain is largely thought of as resulting from an imbalance between excitatory systems, the bulk of which are glutaminergic, and GABAergic inhibitory systems. However, over the last two decades data have emerged underscoring the idea that peptides can exert important modulatory effects on the generation of seizures (Ehlers et al., 1983). NPY has been demonstrated to produce anticonvulsant activity in a number of animal models of epilepsy both in vivo and in vitro (Klemp and Woldbye, 2001; Stroud et al., 2005; Tu et al., 2005; for reviews see Colmers and El, 2003; Woldbye and Kokaia, 2004). Further data has been provided by NPY null mutants, which have been shown to be more susceptible to seizures induced by a GABA antagonist (Erickson et al., 1996). NPY null mutant mice have also been demonstrated to have lower seizure thresholds and more severe seizures to kindling and chemically induced stimulation (Shannon and Yang, 2004), whereas rats overexpressing the NPY gene have been found to be less susceptible to seizures (Vezzani et al., 2002).

A possible involvement of the Y1 receptor in seizure phenomena is supported by data showing that the Y1 antagonist BIBP3226 has anticonvulsant properties (Gariboldi et al., 1998). Other studies, however, have indicated that this role may be exerted by Y2 (Colmers et al., 1991; Colmers and Bleakman, 1994; Schwarzer et al., 1998) or Y5 receptors (Woldbye et al., 1997; Vezzani et al., 2000; Reibel et al., 2001; Benmaamar et al., 2005). Additionally, overexpression of NPY and/or its receptors has been demonstrated following the induction of seizures in animals produced by electroshock or kainic acid (Schwarzer et al., 1998) as well as in alcohol withdrawal (Bison and Crews, 2003). Overexpression of NPY and Y2 receptors have also been found in human epileptic brain, and are suggested to be the result of mechanisms elicited to counter the hyperexcitability underlying epileptic seizure activity (Vezzani and Sperk, 2004).

## 4.8 NPY in Anxiety and Depression

Exogenously administered NPY has been shown to have anxiolytic effects in a wide range of animal models of experimental anxiety such as: the elevated plus maze (Heilig et al., 1989a; Broqua et al., 1995), the Geller-Seifer test of operant responding (Heilig et al., 1992), the Vogel punished drinking test (Heilig et al., 1989a), the social interaction test (Sajdyk et al., 1999), and fear-potentiated startle test (Broqua et al., 1995). Additionally, mutant mice lacking NPY show increased anxiety-like behavior (Bannon et al., 2000).

Antianxiety effects of NPY have been shown to rely in part on activation of Y1 receptors in the amygdala (Heilig et al., 1993). ICV injections of NPY or NPY Y1 receptor agonists, but not Y2 receptor agonists, produce anxiolysis in behavioral models of experimental anxiety (Broqua et al., 1995; Britton et al., 1997). Antisense oligodeoxynucleotides targeting the Y1 receptor sequence block the anxiolytic action of NPY (Heilig, 1995). Also, an anxiogenic-like effect has been reported after injection of the nonspecific Y1 antagonist BIBP3226 (Kask et al., 1996, 1998) further supporting this hypothesis. Recently, a Y1 mediation of the anxiolytic effects of NPY in rat amygdala was confirmed using the highly selective and soluble compound BIBO3304 (Sajdyk et al., 1999).

NPY has also been shown to have antidepressant-like actions in some animal models. For instance, NPY has been reported to produce an antidepressant effect in the Porsolt test (Stogner and Holmes, 2000). A differential NPY expression has also been detected in a genetic animal model of depression, the Flinders Sensitive Line (FSL) rats (Caberlotto et al., 1998, 1999; Jimenez-Vasquez et al., 2000). Treatment with clinically effective antidepressants has been reported to increase NPY expression in several brain regions in rats, with frontal cortex being the most consistent region (Heilig et al., 1988a). Electroconvulsive shock (ECS) has been much more consistent in upregulating brain NPY levels, with hippocampus as a seemingly central target (Wahlestedt et al., 1990; Mathe et al., 1997, 1998).

The antidepressive actions of NPY appear to be predominantly mediated via the Y1 and Y2 receptor systems. This has been shown in several animal models of depression (Redrobe et al., 2003; Tschenett et al., 2003), and the results are consistent with the anxiogenic-like effects of intraamygdala treatment of

Y2-preferring agonists in the rat social interaction test (Sajdyk et al., 2002a, b). Additionally NPY may mediate some of the effects of some standard psychotropic drugs (Obuchowicz et al., 2004).

### 4.9 NPY in Alcohol Dependence

A direct link between NPY signaling and regulation of alcohol consumption was first shown in a study where mice with a transgenic overexpression of NPY were found to consume less alcohol, while mice with an NPY-null-mutation had an increased consumption of alcohol (Thiele et al., 1998). NPY and NPY receptor expression patterns have been examined in "genetic models of alcohol dependence" (HAD/LAD, P/NP, AA/ANA). For example, alcohol preferring rats (P rats) have been shown to have low levels of NPY in amygdala, frontal cortex, and hippocampus compared with the nonpreferring (NP) line, but higher levels in the hypothalamus (Ehlers et al., 1998a; Murphy et al., 2002). In the HAD line NPY-IR was decreased in central nucleus of the amygdala, paraventricular nucleus of the hypothalamus, and the arcuate nucleus compared with LAD rats (Hwang et al., 1999). In the AA/ANA, a different pattern was seen, with lower hippocampal NPY mRNA expression compared with the nonpreferring line (Caberlotto et al., 2001). The NPY Y2 receptor subtype was also found to be reduced in the medial amygdala of the AA line compared with the ANA line.

The effect of exogenous application of NPY on alcohol consumption appears to be in part dependent on the individual's history of alcohol exposure. In animal studies, central administration of NPY into the lateral ventricles, central nucleus of the amygdala, or the third ventricle does not affect ethanol intake in normal, outbred rat strains (Slawecki et al., 2000; Badia-Elder et al., 2001; Katner et al., 2002a, b). However, a significant suppression of alcohol intake was found in the P-line compared with NP and normal Wistar rats, and in the HAD rat line (Badia-Elder et al., 2001, 2003). Additionally in animals in which alcohol "dependence" was produced by chronic alcohol exposure, NPY was also found to reduce alcohol drinking (Thorsell et al., 2005).

The fact that NPY has similar behavioral actions to that of ethanol such as anxiolysis, mild sedation, and stress buffering has led to the hypothesis that some of the effects of ethanol may be mediated through NPY receptor systems. NPY and alcohol have a very similar electrophysiological profile and have additive pharmacological effects (Ehlers et al., 1998b). Additionally, chronic exposure to alcohol causes upregulation of NPY in the hypothalamus (Ehlers et al., 1998a). It has been suggested that chronic alcohol exposure can cause a disruption of the homeostatic balance of both NPY and CRF leading to an allostatic state, i.e., a stable state maintained outside the normal homeostatic range of a parameter in order to adapt to a chronic environmental demand such as alcohol (Valdez and Koob, 2004). This theory also posits that these peptide systems might represent molecular targets for treatment of alcohol dependence.

## 5 Concluding Remarks

Since its isolation and identification around 25 years ago, NPY has been demonstrated to be involved in a number of centrally regulated processes and behaviors. NPY acts on a number of brain sites, including cortex, hippocampus, amygdala, hypothalamus, and brain stem, through a series of receptors (Y1–y6). Activation of NPY systems generally reduces locomotion, promotes sleep, stimulates ovulation, enhances feeding responses, reduces cardiovascular tone, lowers overall brain excitability, buffers stress responses, and suppresses anxious and depressive-like behaviors. These responses are counteracted by the effects of CRF and other stress hormones that promote the opposite behavioral profile. Therefore NPY and CRF/stress systems can be envisioned as acting in concert to promote homeostasis in the face of changing environmental conditions. These systems have also been suggested to regulate allostatic processes occurring during chronic stress. Dysfunctions and changes in the NPY system may underlie some aspects of psychiatric disorders and alcoholism, as well as endocrine syndromes, hypertension, and obesity. Thus NPY may be a successful target for drugs aimed at alleviating these disorders.

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## References

Aguirre JA, Covenas R, Dawid-Milner MS, Alonso JR, Garcia-Herdugo G, et al. 1989. Neuropeptide Y-like immunoreactivity in the brain stem respiratory nuclei of the cat. Brain Res Bull 23: 201-207.

Aguirre JA, Fuxe K, Agnati LF, von EG. 1990. Centrally injected neuropeptide Y (13–36) produces vasopressor effects and antagonizes the vasodepressor action of neuropeptide Y (1–36) in the awake male rat. Neurosci Lett 118: 5-8.

Aicher SA, Springston M, Berger SB, Reis DJ, Wahlestedt C. 1991. Receptor-selective analogs demonstrate NPY/PYY receptor heterogeneity in rat brain. Neurosci Lett 130: 32-36.

Albers HE, Ferris CF. 1984. Neuropeptide Y: role in light-dark cycle entrainment of hamster circadian rhythms. Neurosci Lett 50: 163-168.

Allen YS, Roberts GW, Bloom SR, Crow TJ, Polak JM. 1984. Neuropeptide Y in the stria terminalis: evidence for an amygdalofugal projection. Brain Res 321: 357-362.

Antonijevic IA, Murck H, Bohlhalter S, Frieboes RM, Holsboer F, et al. 2000. Neuropeptide Y promotes sleep and inhibits ACTH and cortisol release in young men. Neuropharmacology 39: 1474-1481.

Aoki C, Pickel VM. 1990. Neuropeptide Y in cortex and striatum. Ultrastructural distribution and coexistence with classical neurotransmitters and neuropeptides. Ann N Y Acad Sci 611: 186-205.

Badia-Elder NE, Stewart RB, Powrozek TA, Murphy JM, Li TK. 2003. Effects of neuropeptide Y on sucrose and ethanol intake and on anxiety-like behavior in high alcohol drinking (HAD) and low alcohol drinking (LAD) rats. Alcohol Clin Exp Res 27: 894-899.

Badia-Elder NE, Stewart RB, Powrozek TA, Roy KF, Murphy JM, et al. 2001. Effect of neuropeptide Y (NPY) on oral ethanol intake in Wistar, alcohol-preferring (P), and -non-preferring (NP) rats. Alcohol Clin Exp Res 25: 386-390.

Bai FL, Yamano M, Shiotani Y, Emson PC, Smith AD, et al. 1985. An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. Brain Res 331: 172-175.

Bannon AW, Seda J, Carmouche M, Francis JM, Norman MH, et al. 2000. Behavioral characterization of neuropeptide Y knockout mice. Brain Res 868: 79-87.

Barraco RA, Ergene E, Dunbar JC, el-Ridi MR. 1990. Cardiorespiratory response patterns elicited by microinjections of neuropeptide Y in the nucleus tractus solitarius. Brain Res Bull 24: 465-485.

Benmaamar R, Richichi C, Gobbi M, Daniels AJ, Beck-Sickinger AG, et al. 2005. Neuropeptide Y Y5 receptors inhibit kindling acquisition in rats. Regul Pept 125: 79-83.

Billington CJ, Briggs JE, Grace M, Levine AS. 1991. Effects of intracerebroventricular injection of neuropeptide Y on energy metabolism. Am J Physiol 260: R321-R327.

Billington CJ, Briggs JE, Harker S, Grace M, Levine AS. 1994. Neuropeptide Y in hypothalamic paraventricular nucleus: a center coordinating energy metabolism. Am J Physiol 266: R1765-R1770.

Bison S, Crews F. 2003. Alcohol withdrawal increases neuropeptide Y immunoreactivity in rat brain. Alcohol Clin Exp Res 27: 1173-1183.

Blessing WW, Howe PR, Joh TH, Oliver JR, Willoughby JO. 1986. Distribution of tyrosine hydroxylase and neuropeptide Y-like immunoreactive neurons in rabbit medulla oblongata, with attention to colocalization studies, presumptive adrenaline-synthesizing perikarya, and vagal preganglionic cells. J Comp Neurol 248: 285-300.

Brene S, Lindefors N, Kopp J, Sedvall G, Persson H. 1989. Regional distribution of neuropeptide Y mRNA in postmortem human brain. Brain Res Mol Brain Res 6: 241-249.

Britton KT, Southerland S, Van UE, Kirby D, Rivier J, et al. 1997. Anxiolytic activity of NPY receptor agonists in the conflict test. Psychopharmacology (Berl)132: 6-13.

Broberger C, Johansen J, Johansson C, Schalling M, Hokfelt T. 1998. The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proc Natl Acad Sci USA 95: 15043-15048.

Broqua P, Wettstein JG, Rocher MN, Gauthier-Martin B, Junien JL. 1995. Behavioral effects of neuropeptide Y receptor agonists in the elevated plus-maze and fear-potentiated startle procedures. Behav Pharmacol 6: 215-222.

Caberlotto L, Fuxe K, Hurd YL. 2000. Characterization of NPY mRNA-expressing cells in the human brain: co-localization with Y2 but not Y1 mRNA in the cerebral cortex, hippocampus, amygdala, and striatum. J Chem Neuroanat 20: 327-337.

Caberlotto L, Fuxe K, Overstreet DH, Gerrard P, Hurd YL. 1998. Alterations in neuropeptide Y and Y1 receptor mRNA expression in brains from an animal model of depression: region specific adaptation after fluoxetine treatment. Brain Res Mol Brain Res 59: 58-65.

Caberlotto L, Fuxe K, Sedvall G, Hurd YL. 1997. Localization of neuropeptide Y Y1 mRNA in the human brain: abundant expression in cerebral cortex and striatum. Eur J Neurosci 9: 1212-1225.

Caberlotto L, Jimenez P, Overstreet DH, Hurd YL, Mathe AA, et al. 1999. Alterations in neuropeptide Y levels and Y1 binding sites in the flinders sensitive line rats, a genetic animal model of depression. Neurosci Lett 265: 191-194.

Caberlotto L, Thorsell A, Rimondini R, Sommer W, Hyytia P, et al. 2001. Differential expression of NPY and its receptors in alcohol-preferring AA and alcohol-avoiding ANA rats. Alcohol Clin Exp Res 25: 1564-1569.

Calza L, Giardino L, Zanni M, Velardo A, Parchi P, et al. 1990. Daily changes of neuropeptide Y-like immunoreactivity in the suprachiasmatic nucleus of the rat. Regul Pept 27: 127-137.

Card JP, Moore RY. 1988. Neuropeptide Y localization in the rat suprachiasmatic nucleus and periventricular hypothalamus. Neurosci Lett 88: 241-246.

Card JP, Moore RY. 1989. Organization of lateral geniculate-hypothalamic connections in the rat. J Comp Neurol 284: 135-147.

Carvajal CC, Vercauteren F, Dumont Y, Michalkiewicz M, Quirion R. 2004. Aged neuropeptide Y transgenic rats are resistant to acute stress but maintain spatial and non-spatial learning. Behav Brain Res 153: 471-480.

Cerda-Reverter JM, Larhammar D. 2000. Neuropeptide Y family of peptides: structure, anatomical expression, function, and molecular evolution. Biochem Cell Biol 78: 371-392.

Chan-Palay V, Allen YS, Lang W, Haesler U, Polak JM. 1985. Cytology and distribution in normal human cerebral cortex of neurons immunoreactive with antisera against neuropeptide Y. J Comp Neurol 238: 382-389.

Chan-Palay V, Kohler C, Haesler U, Lang W, Yasargil G. 1986. Distribution of neurons and axons immunoreactive with antisera against neuropeptide Y in the normal human hippocampus. J Comp Neurol 248: 360-375.

Chronwall BM, Chase TN, O'Donohue TL. 1984. Coexistence of neuropeptide Y and somatostatin in rat and human cortical and rat hypothalamic neurons. Neurosci Lett 52: 213-217.

Chronwall BM, DiMaggio DA, Massari VJ, Pickel VM, Ruggiero DA, et al. 1985. The anatomy of neuropeptide-Y-containing neurons in rat brain. Neuroscience 15: 1159-1181.

Chronwall BM, Zukowska Z. 2004. Neuropeptide Y, ubiquitous and elusive. Peptides 25: 359-363.

Colmers WF, Bleakman D. 1994. Effects of neuropeptide Y on the electrical properties of neurons. Trends Neurosci 17: 373-379.

Colmers WF, El BB. 2003. Neuropeptide Y and epilepsy. Epilepsy Curr 3: 53-58.

Colmers WF, Klapstein GJ, Fournier A, St-Pierre S, Treherne KA. 1991. Presynaptic inhibition by neuropeptide Y in rat hippocampal slice in vitro is mediated by a Y2 receptor. Br J Pharmacol 102: 41-44.

Conlon JM. 2002. The origin and evolution of peptide YY (PYY) and pancreatic polypeptide (PP). Peptides 23: 269-278.

Dawbarn D, Hunt SP, Emson PC. 1984. Neuropeptide Y: regional distribution chromatographic characterization and immunohistochemical demonstration in post-mortem human brain. Brain Res 296: 168-173.

De Quidt ME, Emson PC. 1986. Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system—II. Immunohistochemical analysis. Neuroscience 18: 545-618.

Demeulemeester H, Arckens L, Vandesande F, Orban GA, Heizmann CW, et al. 1991. Calcium binding proteins and neuropeptides as molecular markers of GABAergic interneurons in the cat visual cortex. Exp Brain Res 84: 538-544.

Ding B, Kull B, Liu Z, Mottagui-Tabar S, Thonberg H, et al. 2005. Human neuropeptide Y signal peptide gain-of-function polymorphism is associated with increased body mass index: possible mode of function. Regul Pept 127: 45-53.

Dumont Y, Cadieux A, Doods H, Pheng LH, Abounader R, et al. 2000. BIIE0246, a potent and highly selective non-peptide neuropeptide Y Y(2) receptor antagonist. Br J Pharmacol 129: 1075-1088.

Dumont Y, Fournier A, Quirion R. 1998a. Expression and characterization of the neuropeptide Y Y5 receptor subtype in the rat brain. J Neurosci 18: 5565-5574.

Dumont Y, Jacques D, Bouchard P, Quirion R. 1998b. Species differences in the expression and distribution of the neuropeptide Y Y1, Y2, Y4, and Y5 receptors in rodents, guinea pig, and primates brains. J Comp Neurol 402: 372-384.

Ehlers CL, Henriksen SJ, Wang M, Rivier J, Vale W, et al. 1983. Corticotropin releasing factor produces increases in brain excitability and convulsive seizures in rats. Brain Res 278: 332-336.

Ehlers CL, Li TK, Lumeng L, Hwang BH, Somes C, et al. 1998a. Neuropeptide Y levels in ethanol-naive alcohol-preferring and nonpreferring rats and in Wistar rats after ethanol exposure. Alcohol Clin Exp Res 22: 1778-1782.

Ehlers CL, Somes C, Cloutier D. 1998b. Are some of the effects of ethanol mediated through NPY? Psychopharmacology (Berl) 139: 136-144.

Ehlers CL, Somes C, Lopez A, Kirby D, Rivier JE. 1997a. Electrophysiological actions of neuropeptide Y and its analogs: new measures for anxiolytic therapy? Neuropsychopharmacology 17: 34-43.

Ehlers CL, Somes C, Seifritz E, Rivier JE. 1997b. CRF/NPY interactions: a potential role in sleep dysregulation in depression and anxiety Depress. Anxiety 6: 1-9.

Erickson JC, Clegg KE, Palmiter RD. 1996. Sensitivity to leptin and susceptibility to seizures of mice lacking neuropeptide Y. Nature 381: 415-421.

Eva C, Oberto A, Sprengel R, Genazzani E. 1992. The murine NPY-1 receptor gene. Structure and delineation of tissue-specific expression. FEBS Lett 314: 285-288.

Evans JJ. 1999. Modulation of gonadotropin levels by peptides acting at the anterior pituitary gland. Endocr Rev 20: 46-67.

Everitt BJ, Hokfelt T, Terenius L, Tatemoto K, Mutt V, et al. 1984. Differential co-existence of neuropeptide Y (NPY)-like immunoreactivity with catecholamines in the central nervous system of the rat. Neuroscience 11: 443-462.

Fallon JH, Koziell DA, Moore RY. 1978. Catecholamine innervation of the basal forebrain. II. Amygdala, suprarhinal cortex and entorhinal cortex. J Comp Neurol 180: 509-532.

Fetissov SO, Byrne LC, Hassani H, Ernfors P, Hokfelt T. 2004. Characterization of neuropeptide Y Y2 and Y5 receptor expression in the mouse hypothalamus. J Comp Neurol 470: 256-265.

Flood JF, Baker ML, Hernandez EN, Morley JE. 1989. Modulation of memory processing by neuropeptide Y varies with brain injection site. Brain Res 503: 73-82.

Flood JF, Hernandez EN, Morley JE. 1987. Modulation of memory processing by neuropeptide Y. Brain Res 421: 280-290.

Flood JF, Morley JE. 1991. Increased food intake by neuropeptide Y is due to an increased motivation to eat. Peptides 12: 1329-1332.

Foster GA, Schultzberg M, Goldstein M, Hokfelt T. 1984. Delayed appearance of tyrosine hydroxylase-like immunoreactivity in PNMT-immunoreactive neurones of the foetal rat brain. Acta Physiol Scand 122: 429-432.

Frankish HM, Dryden S, Hopkins D, Wang Q, Williams G. 1995. Neuropeptide Y, the hypothalamus, and diabetes: insights into the central control of metabolism. Peptides 16: 757-771.

Fukuhara C, Brewer JM, Dirden JC, Bittman EL, Tosini G, et al. 2001. Neuropeptide Y rapidly reduces Period 1 and Period 2 mRNA levels in the hamster suprachiasmatic nucleus. Neurosci Lett 314: 119-122.

Fuxe K, Agnati LF, Harfstrand A, Zini I, Tatemoto K, et al. 1983. Central administration of neuropeptide Y induces hypotension bradypnea and EEG synchronization in the rat. Acta Physiol Scand 118: 189-192.

Gamble KL, Novak CM, Albers HE. 2004. Neuropeptide Y and N-methyl-D-aspartic acid interact within the suprachiasmatic nuclei to alter circadian phase. Neuroscience 126: 559-565.

Gariboldi M, Conti M, Cavaleri D, Samanin R, Vezzani A. 1998. Anticonvulsant properties of BIBP3226, a non-peptide selective antagonist at neuropeptide Y Y1 receptors. Eur J Neurosci 10: 757-759.

Gaspar P, Berger B, Lesur A, Borsotti JP, Febvret A. 1987. Somatostatin 28 and neuropeptide Y innervation in the septal area and related cortical and subcortical structures of the human brain. Distribution, relationships and evidence for differential coexistence. Neuroscience 22: 49-73.

Gehlert DR, Chronwall BM, Schafer MP, O'Donohue TL. 1987. Localization of neuropeptide Y messenger ribonucleic acid in rat and mouse brain by in situ hybridization. Synapse 1: 25-31.

Gerald C, Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, et al. 1996. A receptor subtype involved in neuropeptide-Y-induced food intake. Nature 382: 168-171.

Gerald C, Walker MW, Vaysse PJ, He C, Branchek TA, et al. 1995. Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY Y2 receptor subtype. J Biol Chem 270: 26758-26761.

Golombek DA, Biello SM, Rendon RA, Harrington ME. 1996. Neuropeptide Y phase shifts the circadian clock in vitro via a Y2 receptor. Neuroreport 7: 1315-1319.

Groneberg DA, Folkerts G, Peiser C, Chung KF, Fischer A. 2004. Neuropeptide Y (NPY) Pulm. Pharmacol Ther 17: 173-180.

Grundemar L, Hakanson R. 1993. Multiple neuropeptide Y receptors are involved in cardiovascular regulation. Peripheral and central mechanisms. Gen Pharmacol 24: 785-796.

Gustafson EL, Card JP, Moore RY. 1986. Neuropeptide Y localization in the rat amygdaloid complex. J Comp Neurol 251: 349-362.

Gustafson EL, Moore RY. 1987. Noradrenaline and neuropeptide Y innervation of the rat hypothalamus are differentially affected by 6-hydroxydopamine. Neurosci Lett 83: 53-58.

Haas DA, George SR. 1987. Neuropeptide Y administration acutely increases hypothalamic corticotropin-releasing factor immunoreactivity: lack of effect in other rat brain regions. Life Sci 41: 2725-2731.

Hahn TM, Breininger JF, Baskin DG, Schwartz MW. 1998. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. Nat Neurosci 1: 271-272.

Hanson ES, Dallman MF. 1995. Neuropeptide Y (NPY) may integrate responses of hypothalamic feeding systems and the hypothalamo-pituitary-adrenal axis. J Neuroendocrinol 7: 273-279.

Harfstrand A, Eneroth P, Agnati L, Fuxe K. 1987. Further studies on the effects of central administration of

neuropeptide Y on neuroendocrine function in the male rat: relationship to hypothalamic catecholamines. Regul Pept 17: 167-179.

Harrington ME, Nance DM, Rusak B. 1987. Double-labeling of neuropeptide Y-immunoreactive neurons which project from the geniculate to the suprachiasmatic nuclei. Brain Res 410: 275-282.

Harrington ME, Rusak B. 1986. Lesions of the thalamic intergeniculate leaflet alter hamster circadian rhythms. J Biol Rhythms 1: 309-325.

Harrington ME, Schak KM. 2000. Neuropeptide Y phase advances the in vitro hamster circadian clock during the subjective day with no effect on phase during the subjective night Can. J Physiol Pharmacol 78: 87-92.

Heilig M. 1995. Antisense inhibition of neuropeptide Y (NPY)-Y1 receptor expression blocks the anxiolytic-like action of NPY in amygdala and paradoxically increases feeding. Regul Pept 59: 201-205.

Heilig M, McLeod S, Brot M, Heinrichs SC, Menzaghi F, et al. 1993. Anxiolytic-like action of neuropeptide Y: mediation by Y1 receptors in amygdala, and dissociation from food intake effects. Neuropsychopharmacology 8: 357-363.

Heilig M, McLeod S, Koob GK, Britton KT. 1992. Anxiolytic-like effect of neuropeptide Y (NPY), but not other peptides in an operant conflict test. Regul Pept 41: 61-69.

Heilig M, Murison R. 1987a. Intracerebroventricular neuropeptide Y suppresses open field and home cage activity in the rat. Regul Pept 19: 221-231.

Heilig M, Murison R. 1987b. Intracerebroventricular neuropeptide Y protects against stress-induced gastric erosion in the rat. Eur J Pharmacol 137: 127-129.

Heilig M, Soderpalm B, Engel JA, Widerlov E. 1989a. Centrally administered neuropeptide Y (NPY) produces anxiolytic-like effects in animal anxiety models. Psychopharmacology (Berl) 98: 524-529.

Heilig M, Thorsell A. 2002. Brain neuropeptide Y (NPY) in stress and alcohol dependence. Rev Neurosci 13: 85-94.

Heilig M, Vecsei L, Widerlov E. 1989b. Opposite effects of centrally administered neuropeptide Y (NPY) on locomotor activity of spontaneously hypertensive (SH) and normal rats. Acta Physiol Scand 137: 243-248.

Heilig M, Wahlestedt C, Ekman R, Widerlov E. 1988a. Antidepressant drugs increase the concentration of neuropeptide Y (NPY)-like immunoreactivity in the rat brain. Eur J Pharmacol 147: 465-467.

Heilig M, Wahlestedt C, Widerlov E. 1988b. Neuropeptide Y (NPY)-induced suppression of activity in the rat: evidence for NPY receptor heterogeneity and for interaction with alpha-adrenoceptors. Eur J Pharmacol 157: 205-213.

Hendry SH, Jones EG, DeFelipe J, Schmechel D, Brandon C, et al. 1984a. Neuropeptide-containing neurons of the cerebral cortex are also GABAergic. Proc Natl Acad Sci USA 81: 6526-6530.

Hendry SH, Jones EG, Emson PC. 1984b. Morphology, distribution, and synaptic relations of somatostatin- and neuropeptide Y-immunoreactive neurons in rat and monkey neocortex. J Neurosci 4: 2497-2517.

Herzog H. 2003. Neuropeptide Y and energy homeostasis: insights from Y receptor knockout models. Eur J Pharmacol 480: 21-29.

Herzog H, Hort YJ, Ball HJ, Hayes G, Shine J, et al. 1992. Cloned human neuropeptide Y receptor couples to two different second messenger systems. Proc Natl Acad Sci USA 89: 5794-5798.

Hinson J, Rauh C, Coupet J. 1988. Neuropeptide Y stimulates inositol phospholipid hydrolysis in rat brain miniprisms. Brain Res 446: 379-382.

Huh Y, Kim C, Lee W, Kim J, Ahn H. 1997. Age-related change in the neuropeptide Y and NADPH-diaphorase-positive neurons in the cerebral cortex and striatum of aged rats. Neurosci Lett 223: 157-160.

Huhman KL, Gillespie CF, Marvel CL, Albers HE. 1996. Neuropeptide Y phase shifts circadian rhythms in vivo via a Y2 receptor. Neuroreport 7: 1249-1252.

Hwang BH, Zhang JK, Ehlers CL, Lumeng L, Li TK. 1999. Innate differences of neuropeptide Y (NPY) in hypothalamic nuclei and central nucleus of the amygdala between selectively bred rats with high and low alcohol preference. Alcohol Clin Exp Res 23: 1023-1030.

Jimenez-Vasquez PA, Overstreet DH, Mathe AA. 2000. Neuropeptide Y in male and female brains of flinders sensitive line, a rat model of depression. Effects of electroconvulsive stimuli. J Psychiatr Res 34: 405-412.

Jones EG, DeFelipe J, Hendry SH, Maggio JE. 1988. A study of tachykinin-immunoreactive neurons in monkey cerebral cortex. J Neurosci 8: 1206-1224.

Kaga T, Fujimiya M, Inui A. 2001. Emerging functions of neuropeptide Y Y(2) receptors in the brain. Peptides 22: 501-506.

Kalra SP, Kalra PS. 2004a. Overlapping and interactive pathways regulating appetite and craving. J Addict Dis 23: 5-21.

Kalra SP, Kalra PS. 2004b. NPY and cohorts in regulating appetite, obesity and metabolic syndrome: beneficial effects of gene therapy. Neuropeptides 38: 201-211.

Kanatani A, Mashiko S, Murai N, Sugimoto N, Ito J, et al. 2000. Role of the Y1 receptor in the regulation of neuropeptide Y-mediated feeding: comparison of wild-type, Y1 receptor-deficient, and Y5 receptor-deficient mice. Endocrinology 141: 1011-1016.

Karvonen MK, Koulu M, Pesonen U, Uusitupa MI, Tammi A, et al. 2000. Leucine 7 to proline 7 polymorphism in the preproneuropeptide Y is associated with birth weight and

serum triglyceride concentration in preschool aged children. J Clin Endocrinol Metab 85: 1455-1460.

Karvonen MK, Pesonen U, Koulu M, Niskanen L, Laakso M, et al. 1998. Association of a leucine(7)-to-proline(7) polymorphism in the signal peptide of neuropeptide Y with high serum cholesterol and LDL cholesterol levels. Nat Med 4: 1434-1437.

Kask A, Rago L, Harro J. 1996. Anxiogenic-like effect of the neuropeptide Y Y1 receptor antagonist BIBP3226: antagonism with diazepam. Eur J Pharmacol 317: R3-R4.

Kask A, Rago L, Harro J. 1998. Anxiogenic-like effect of the NPY Y1 receptor antagonist BIBP3226 administered into the dorsal periaqueductal gray matter in rats. Regul Pept 75–76: 255-262.

Katner SN, Slawecki CJ, Ehlers CL. 2002a. Neuropeptide Y administration into the amygdala does not affect ethanol consumption. Alcohol 28: 29-38.

Katner SN, Slawecki CJ, Ehlers CL. 2002b. Neuropeptide Y administration into the third ventricle does not increase sucrose or ethanol self-administration but does affect the cortical EEG and increases food intake. Psychopharmacology (Berl)160: 146-154.

Kerkerian L, Bosler O, Pelletier G, Nieoullon A. 1986. Striatal neuropeptide Y neurones are under the influence of the nigrostriatal dopaminergic pathway: immunohistochemical evidence. Neurosci Lett 66: 106-112.

Kimmel JR, Hayden LJ, Pollock HG. 1975. Isolation and characterization of a new pancreatic polypeptide hormone. J Biol Chem 250: 9369-9376.

Kishi T, Elmquist JK. 2005. Body weight is regulated by the brain: a link between feeding and emotion. Mol Psychiatry 10: 132-146.

Klemp K, Woldbye DP. 2001. Repeated inhibitory effects of NPY on hippocampal CA3 seizures and wet dog shakes. Peptides 22: 523-527.

Kohler C, Eriksson L, Davies S, Chan-Palay V. 1986a. Neuropeptide Y innervation of the hippocampal region in the rat and monkey brain. J Comp Neurol 244: 384-400.

Kohler C, Smialowska M, Eriksson LG, Chanpalay V, Davies S. 1986b. Origin of the neuropeptide Y innervation of the rat retrohippocampal region. Neurosci Lett 65: 287-292.

Kubota Y, Kawaguchi Y. 1993. Spatial distributions of chemically identified intrinsic neurons in relation to patch and matrix compartments of rat neostriatum. J Comp Neurol 332: 499-513.

Lall GS, Biello SM. 2002. Attenuation of phase shifts to light by activity or neuropeptide Y: a time course study. Brain Res 957: 109-116.

Larhammar D, Blomqvist AG, Yee F, Jazin E, Yoo H, et al. 1992. Cloning and functional expression of a human neuropeptide Y/peptide YY receptor of the Y1 type. J Biol Chem 267: 10935-10938.

Larhammar D, Salaneck E. 2004. Molecular evolution of NPY receptor subtypes. Neuropeptides 38: 141-151.

Levine AS, Jewett DC, Cleary JP, Kotz CM, Billington CJ. 2004. Our journey with neuropeptide Y: effects on ingestive behaviors and energy expenditure. Peptides 25: 505-510.

Levine AS, Morley JE. 1984. Neuropeptide Y: a potent inducer of consummatory behavior in rats. Peptides 5: 1025-1029.

Lin S, Boey D, Herzog H. 2004. NPY and Y receptors: lessons from transgenic and knockout models. Neuropeptides 38: 189-200.

Lopez-Valpuesta FJ, Nyce JW, Griffin-Biggs TA, Ice JC, Myers RD. 1996. Antisense to NPY-Y1 demonstrates that Y1 receptors in the hypothalamus underlie NPY hypothermia and feeding in rats. Proc R Soc Lond B Biol Sci 263: 881-886.

Magni P. 2003. Hormonal control of the neuropeptide Y system. Curr Protein Pept Sci 4: 45-57.

Marsh DJ, Hollopeter G, Kafer KE, Palmiter RD. 1998. Role of the Y5 neuropeptide Y receptor in feeding and obesity. Nat Med 4: 718-721.

Martin JR. 2004. The Y1 receptor subtype mediates the cardiovascular changes evoked by NPY administered into the posterior hypothalamic nucleus of conscious rat. Brain Res 1002: 11-20.

Mathe AA, Gruber S, Jimenez PA, Theodorsson E, Stenfors C. 1997. Effects of electroconvulsive stimuli and MK-801 on neuropeptide Y, neurokinin A, and calcitonin gene-related peptide in rat brain. Neurochem Res 22: 629-636.

Mathe AA, Jimenez PA, Theodorsson E, Stenfors C. 1998. Neuropeptide Y, neurokinin A and neurotensin in brain regions of Fawn Hooded "depressed", Wistar, and Sprague Dawley rats. Effects of electroconvulsive stimuli. Prog Neuropsychopharmacol Biol Psychiatry 22: 529-546.

Maywood ES, Okamura H, Hastings MH. 2002. Opposing actions of neuropeptide Y and light on the expression of circadian clock genes in the mouse suprachiasmatic nuclei. Eur J Neurosci 15: 216-220.

McDonald AJ. 1989. Coexistence of somatostatin with neuropeptide Y, but not with cholecystokinin or vasoactive intestinal peptide, in neurons of the rat amygdala. Brain Res 500: 37-45.

McDonald AJ, Mascagni F, Augustine JR. 1995. Neuropeptide Y and somatostatin-like immunoreactivity in neurons of the monkey amygdala. Neuroscience 66: 959-982.

McDonald JK, Lumpkin MD, Samson WK, McCann SM. 1985. Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. Proc Natl Acad Sci USA 82: 561-564.

Minth CD, Andrews PC, Dixon JE. 1986. Characterization, sequence, and expression of the cloned human neuropeptide Y gene. J Biol Chem 261: 11974-11979.

Minth CD, Dixon JE. 1990. Expression of the human neuropeptide Y gene. J Biol Chem 265: 12933-12939.

Morris BJ. 1989. Neuronal localisation of neuropeptide Y gene expression in rat brain. J Comp Neurol 290: 358-368.

Murphy JM, Stewart RB, Bell RL, Badia-Elder NE, Carr LG, et al. 2002. Phenotypic and genotypic characterization of the Indiana University rat lines selectively bred for high and low alcohol preference. Behav Genet 32: 363-388.

Nakagawa Y, Shiosaka S, Emson PC, Tohyama M. 1985. Distribution of neuropeptide Y in the forebrain and diencephalon: an immunohistochemical analysis. Brain Res 361: 52-60.

Narvaez JA, Aguirre JA, Fuxe K. 1993. Subpicomolar amounts of NPY(13-36) injected into the nucleus tractus solitarius of the rat counteract the cardiovascular responses to L-glutamate. Neurosci Lett 151: 182-186.

Naveilhan P, Canals JM, Arenas E, Ernfors P. 2001. Distinct roles of the Y1 and Y2 receptors on neuropeptide Y-induced sensitization to sedation. J Neurochem 78: 1201-1207.

Naveilhan P, Hassani H, Canals JM, Ekstrand AJ, Larefalk A, et al. 1999. Normal feeding behavior, body weight and leptin response require the neuropeptide Y Y2 receptor. Nat Med 5: 1188-1193.

Obuchowicz E, Krysiak R, Herman ZS. 2004. Does neuropeptide Y (NPY) mediate the effects of psychotropic drugs? Neurosci Biobehav Rev 28: 595-610.

Pandey SC. 2003. Anxiety and alcohol abuse disorders: a common role for CREB and its target, the neuropeptide Y gene. Trends Pharmacol Sci 24: 456-460.

Pandey SC, Roy A, Zhang H. 2003. The decreased phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein in the central amygdala acts as a molecular substrate for anxiety related to ethanol withdrawal in rats. Alcohol Clin Exp Res 27: 396-409.

Parker RM, Herzog H. 1999. Regional distribution of Y-receptor subtype mRNAs in rat brain Eur J Neurosci 11: 1431-1448.

Pedrazzini T, Pralong F, Grouzmann E. 2003. Neuropeptide Y: the universal soldier. Cell Mol Life Sci 60: 350-377.

Pons J, Lee EW, Li L, Kitlinska J. 2004. Neuropeptide Y: multiple receptors and multiple roles in cardiovascular diseases. Curr Opin Investig Drugs 5: 957-962.

Redrobe JP, Dumont Y, Herzog H, Quirion R. 2003. Neuropeptide Y (NPY) Y2 receptors mediate behaviour in two animal models of anxiety: evidence from Y2 receptor knockout mice. Behav Brain Res 141: 251-255.

Redrobe JP, Dumont Y, Herzog H, Quirion R. 2004. Characterization of neuropeptide Y, Y(2) receptor knockout mice in two animal models of learning and memory processing. J Mol Neurosci 22: 159-166.

Reibel S, Nadi S, Benmaamar R, Larmet Y, Carnahan J, et al. 2001. Neuropeptide Y and epilepsy: varying effects according to seizure type and receptor activation. Peptides 22: 529-539.

Rusak B, Meijer JH, Harrington ME. 1989. Hamster circadian rhythms are phase-shifted by electrical stimulation of the geniculo-hypothalamic tract. Brain Res 493: 283-291.

Sahu A, Kalra PS, Kalra SP. 1988. Food deprivation and ingestion induce reciprocal changes in neuropeptide Y concentrations in the paraventricular nucleus. Peptides 9: 83-86.

Sajdyk TJ, Schober DA, Gehlert DR. 2002a. Neuropeptide Y receptor subtypes in the basolateral nucleus of the amygdala modulate anxiogenic responses in rats. Neuropharmacology 43: 1165-1172.

Sajdyk TJ, Schober DA, Smiley DL, Gehlert DR. 2002b. Neuropeptide Y–Y2 receptors mediate anxiety in the amygdala. Pharmacol Biochem Behav 71: 419-423.

Sajdyk TJ, Vandergriff MG, Gehlert DR. 1999. Amygdalar neuropeptide Y Y1 receptors mediate the anxiolytic-like actions of neuropeptide Y in the social interaction test. Eur J Pharmacol 368: 143-147.

Sawchenko PE, Pfeiffer SW. 1988. Ultrastructural localization of neuropeptide Y and galanin immunoreactivity in the paraventricular nucleus of the hypothalamus in the rat. Brain Res 474: 231-245.

Sawchenko PE, Swanson LW, Grzanna R, Howe PR, Bloom SR, et al. 1985. Colocalization of neuropeptide Y immunoreactivity in brainstem catecholaminergic neurons that project to the paraventricular nucleus of the hypothalamus. J Comp Neurol 241: 138-153.

Schaffhauser AO, Stricker-Krongrad A, Brunner L, Cumin F, Gerald C, et al. 1997. Inhibition of food intake by neuropeptide Y Y5 receptor antisense oligodeoxynucleotides. Diabetes 46: 1792-1798.

Schwartzberg M, Unger J, Weindl A, Lange W. 1990. Distribution of neuropeptide Y in the prosencephalon of man and cotton-head tamarin (Saguinus oedipus): colocalization with somatostatin in neurons of striatum and amygdala. Anat Embryol (Berl)181: 157-166.

Schwarzer C, Kirchmair E, Sperk G. 1998. Metabotropic glutamate receptors mediate activation of NPY-Y2 receptor expression in the rat dentate gyrus. Neuroreport 9: 2347-2351.

Shannon HE, Yang L. 2004. Seizure susceptibility of neuropeptide-Y null mutant mice in amygdala kindling and chemical-induced seizure models. Epilepsy Res 61: 49-62.

Slawecki CJ, Betancourt M, Walpole T, Ehlers CL. 2000. Increases in sucrose consumption, but not ethanol consumption, following ICV NPY administration. Pharmacol Biochem Behav 66: 591-594.

Smialowska M. 1988. Neuropeptide Y immunoreactivity in the locus coeruleus of the rat brain. Neuroscience 25: 123-131.

Smialowska M, Bajkowska M, Heilig M, Obuchowicz E, Turchan J, et al. 2001. Pharmacological studies on the monoaminergic influence on the synthesis and expression of neuropeptide Y and corticotropin releasing factor in rat brain amygdala. Neuropeptides 35: 82-91.

Smialowski A, Lewinska-Gastol L, Smialowska M. 1992. The behavioural effects of neuropeptide Y (NPY) injection into the rat brain frontal cortex. Neuropeptides 21: 153-156.

Smith Y, Parent A. 1986. Neuropeptide Y-immunoreactive neurons in the striatum of cat and monkey: morphological characteristics, intrinsic organization and co-localization with somatostatin. Brain Res 372: 241-252.

Sorensen G, Lindberg C, Wortwein G, Bolwig TG, Woldbye DP. 2004. Differential roles for neuropeptide Y Y1 and Y5 receptors in anxiety and sedation. J Neurosci Res 77: 723-729.

Soscia SJ, Harrington ME. 2005. Neuropeptide Y does not reset the circadian clock in NPY Y2-/- mice. Neurosci Lett 373: 175-178.

Stanley BG, Daniel DR, Chin AS, Leibowitz SF. 1985. Paraventricular nucleus injections of peptide YY and neuropeptide Y preferentially enhance carbohydrate ingestion. Peptides 6: 1205-1211.

Stanley BG, Leibowitz SF. 1985. Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. Proc Natl Acad Sci USA 82: 3940-3943.

Steiger A. 2003. Sleep and endocrine regulation. Front Biosci 8: s358-s376.

Stogner KA, Holmes PV. 2000. Neuropeptide-Y exerts antidepressant-like effects in the forced swim test in rats. Eur J Pharmacol 387: R9-R10.

Stroud LM, O'brien TJ, Jupp B, Wallengren C, Morris MJ. 2005. Neuropeptide Y suppresses absence seizures in a genetic rat model. Brain Res 1033: 151-156.

Tatemoto K. 1982. Neuropeptide Y: complete amino acid sequence of the brain peptide. Proc Natl Acad Sci USA 79: 5485-5489.

Tatemoto K, Carlquist M, Mutt V. 1982. Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296: 659-660.

Thiele TE, Marsh DJ, Ste ML, Bernstein IL, Palmiter RD. 1998. Ethanol consumption and resistance are inversely related to neuropeptide Y levels. Nature 396: 366-369.

Thomas JR, Ahlers ST. 1991. Neuropeptide-Y both improves and impairs delayed matching-to-sample performance in rats. Pharmacol Biochem Behav 40: 417-422.

Thompson KJ, Orfila JE, Achanta P, Martinez JL Jr. 2003. Gene expression associated with in vivo induction of early phase-long-term potentiation (LTP) in the hippocampal mossy fiber-Cornus Ammonis (CA)3 pathway. Cell Mol Biol (Noisy. -le-grand)49: 1281-1287.

Thorsell A, Carlsson K, Ekman R, Heilig M. 1999. Behavioral and endocrine adaptation, and up-regulation of NPY expression in rat amygdala following repeated restraint stress. Neuroreport 10: 3003-3007.

Thorsell A, Michalkiewicz M, Dumont Y, Quirion R, Caberlotto L, et al. 2000. Behavioral insensitivity to restraint stress, absent fear suppression of behavior and impaired spatial learning in transgenic rats with hippocampal neuropeptide Y overexpression. Proc Natl Acad Sci USA 97: 12852-12857.

Thorsell A, Slawecki CJ, Ehlers CL. 2005. Effects of neuropeptide Y and corticotropin releasing factor on ethanol intake in Wistar rats: Interaction with chronic ethanol exposure. Behav Brain Res 161(1): 133-140.

Thorsell A, Svensson P, Wiklund L, Sommer W, Ekman R, et al. 1998. Suppressed neuropeptide Y (NPY) mRNA in rat amygdala following restraint stress. Regul Pept 75–76: 247-254.

Tsagarakis S, Rees LH, Besser GM, Grossman A. 1989. Neuropeptide-Y stimulates CRF-41 release from rat hypothalami in vitro. Brain Res 502: 167-170.

Tschenett A, Singewald N, Carli M, Balducci C, Salchner P, et al. 2003. Reduced anxiety and improved stress coping ability in mice lacking NPY-Y2 receptors. Eur J Neurosci 18: 143-148.

Tu B, Timofeeva O, Jiao Y, Nadler JV. 2005. Spontaneous release of neuropeptide Y tonically inhibits recurrent mossy fiber synaptic transmission in epileptic brain. J Neurosci 25: 1718-1729.

Unger JW, Lange W. 1992. NADPH-diaphorase-positive cell populations in the human amygdala and temporal cortex: neuroanatomy, peptidergic characteristics and aspects of aging and Alzheimer's disease. Acta Neuropathol (Berl) 83: 636-646.

Valdez GR, Koob GF. 2004. Allostasis and dysregulation of corticotropin-releasing factor and neuropeptide Y systems: implications for the development of alcoholism. Pharmacol Biochem Behav 79: 671-689.

Vettor R, Zarjevski N, Cusin I, Rohner-Jeanrenaud F, Jeanrenaud B. 1994. Induction and reversibility of an obesity syndrome by intracerebroventricular neuropeptide Y administration to normal rats. Diabetologia 37: 1202-1208.

Vezzani A, Michalkiewicz M, Michalkiewicz T, Moneta D, Ravizza T, et al. 2002. Seizure susceptibility and epileptogenesis are decreased in transgenic rats overexpressing neuropeptide Y. Neuroscience 110: 237-243.

Vezzani A, Moneta D, Mule F, Ravizza T, Gobbi M, et al. 2000. Plastic changes in neuropeptide Y receptor subtypes in experimental models of limbic seizures. Epilepsia 41 Suppl. 6: S115-S121.

Vezzani A, Sperk G. 2004. Overexpression of NPY and Y2 receptors in epileptic brain tissue: an endogenous neuroprotective mechanism in temporal lobe epilepsy? Neuropeptides 38: 245-252.

Vincent SR, Johansson O, Hokfelt T, Skirboll L, Elde RP, et al. 1983. NADPH-diaphorase: a selective histochemical marker for striatal neurons containing both somatostatin- and avian pancreatic polypeptide (APP)-like immunoreactivities. J Comp Neurol 217: 252-263.

Vuillet J, Kerkerian L, Salin P, Nieoullon A. 1989. Ultrastructural features of NPY-containing neurons in the rat striatum. Brain Res 477: 241-251.

Wahlestedt C, Blendy JA, Kellar KJ, Heilig M, Widerlov E, et al. 1990. Electroconvulsive shocks increase the concentration of neocortical and hippocampal neuropeptide Y (NPY)-like immunoreactivity in the rat. Brain Res 507: 65-68.

Wahlestedt C, Skagerberg G, Ekman R, Heilig M, Sundler F, et al. 1987. Neuropeptide Y (NPY) in the area of the hypothalamic paraventricular nucleus activates the pituitary–adrenocortical axis in the rat. Brain Res 417: 33-38.

Wahlestedt C, Yanaihara N, Hakanson R. 1986. Evidence for different pre-and post-junctional receptors for neuropeptide Y and related peptides. Regul Pept 13: 307-318.

Weber ET, Rea MA. 1997. Neuropeptide Y blocks light-induced phase advances but not delays of the circadian activity rhythm in hamsters. Neurosci Lett 231: 159-162.

Williams G, Cai XJ, Elliott JC, Harrold JA. 2004. Anabolic neuropeptides. Physiol Behav 81: 211-222.

Woldbye DP, Kokaia M. 2004. Neuropeptide Y and seizures: effects of exogenously applied ligands. Neuropeptides 38: 253-260.

Woldbye DP, Larsen PJ, Mikkelsen JD, Klemp K, Madsen TM, et al. 1997. Powerful inhibition of kainic acid seizures by neuropeptide Y via Y5-like receptors. Nat Med 3: 761-764.

Yannielli P, Harrington ME. 2004. Let there be "more" light: enhancement of light actions on the circadian system through non-photic pathways. Prog Neurobiol 74: 59-76.

Yannielli PC, Brewer JM, Harrington ME. 2004. Blockade of the NPY Y5 receptor potentiates circadian responses to light: complementary in vivo and in vitro studies. Eur J Neurosci 19: 891-897.

Yannielli PC, Harrington ME. 2001. Neuropeptide Y in the mammalian circadian system: effects on light-induced circadian responses. Peptides 22: 547-556.

Zarjevski N, Cusin I, Vettor R, Rohner-Jeanrenaud F, Jeanrenaud B. 1993. Chronic intracerebroventricular neuropeptide-Y administration to normal rats mimics hormonal and metabolic changes of obesity. Endocrinology 133: 1753-1758.

# 24 Cholecystokinin Peptides in Brain Function

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**Abstract:** Cholecystokinin (CCK) is a peptide originally discovered in the gastrointestinal tract but is also found in high density in the mammalian brain. The C-terminal sulfated octapeptide fragment of CCK8 constitutes one of the major neuropeptides in the brain. CCK8, interacting with nanomolar affinities with two different receptors designated $CCK_1$ and $CCK_2$, has been shown to be involved in numerous physiological functions and is involved in the modulation and control of multiple central functions. In particular, CCK is involved in the neurobiology of anxiety, depression, psychosis, cognition, nociception, and feeding behavior. The functional role of CCK has been facilitated thanks to the development of potent and selective CCK receptor antagonists and agonists. In this chapter, the strategies followed to design these probes, and their use to study the anatomy of CCK pathways, the neurochemical and pharmacological properties of this peptide, and the clinical perspectives offered by manipulation of the CCK system are reported.

**List of Abbreviations:** ACC, anterior cingulate cortex; APA, aminopeptidase A; CCK, cholecystokinin; CCKLM, CCK-like material; CNS, central nervous system; GABA, gamma-aminobutyric acid; GPCR, G-protein-coupled receptor; IP3, inositol 1,4,5-triphosphate; JNK, c-Jun-$NH_2$-terminal kinases; LETO, Long–Evans Tokushima Otsuka; MAPK, mitogen-activated protein kinase; NTS, nucleus tractus solitarius; OLETF, Otsuka Long–Evans Tokushima fatty; PC, prohormone convertase; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; PLC, phospholipase C; PTX, pertussis toxin; TM, transmembrane-spanning domains; VTA, ventral tegmental area

## 1 Introduction

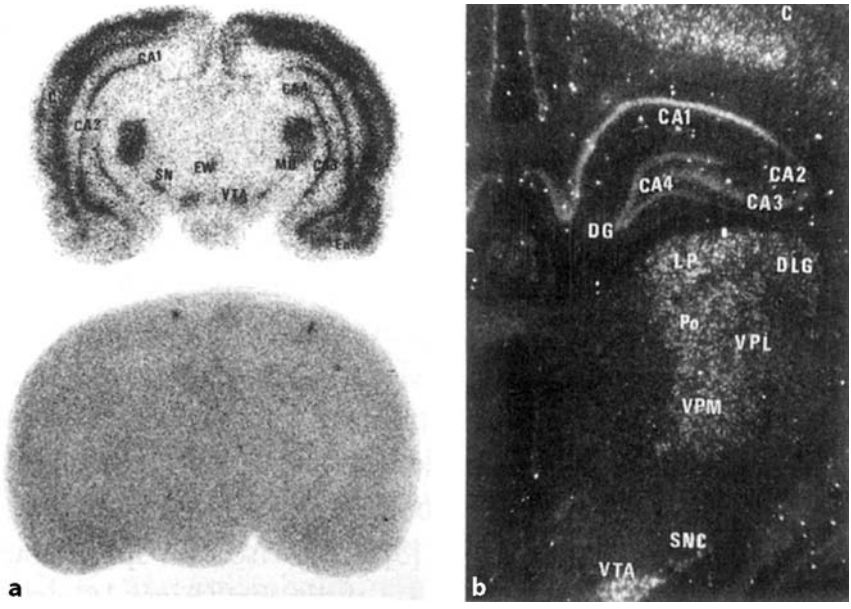
### 1.1 Discovery of CCK in the Brain

The peptide cholecystokinin (CCK) was originally discovered in the gastrointestinal tract (Ivy and Oldberg, 1928) and has been shown to be involved in the secretion of pancreatic enzymes, contraction of gallbladder, and gut motility. Then CCK was discovered in the mammalian central nervous system (CNS) as a gastrin-like immunoreactive material (Vanderhaegen et al., 1975). CCK is more abundant in the brain than in the periphery and is now generally believed to be the most widespread and abundant neuropeptide in the CNS (Crawley, 1985; Moran and Schwartz, 1994). CCK meets the criteria of neurotransmitters since it is synthesized de novo (Goltermann et al., 1981), is released via a calcium-dependent mechanism (Emson et al., 1980), is active at selective receptors ($CCK_1$ and $CCK_2$) (Moran et al., 1986), and its action interrupted by inactivating enzymes generating inactive metabolites (Roques, 2000).

CCK is colocalized in cell bodies and terminals with many other neurotransmitters such as gamma-aminobutyric acid (GABA) (Hendry et al., 1984), dopamine (Hokfelt et al., 1980), serotonin (van der Kooy et al., 1981), and opiates (Gall et al., 1987). There is also evidence of a major corticostriatal CCK-containing pathway, which is also thought to contain glutamate (Morino et al., 1994). In line with its different colocalization and with its wide distribution in the brain (Lanaud et al., 1989) (*Figure 24-1*), CCK is involved in the modulation and control of multiple central functions. In particular, as discussed further, numerous experimental and clinical studies have clearly shown that CCK, through its action at $CCK_1$ and $CCK_2$ receptors, participates in the neurobiology of anxiety, depression, psychosis, cognition, nociception, and food consumption.

CCK, initially characterized as a 33-amino-acid peptide (Mutt and Jorpes, 1968), is present in a variety of biologically active molecular forms (Rehfeld et al., 1982) derived from a 115-amino-acid precursor molecule (preprocholecystokinin, preproCCK) (Deschenes et al., 1984). Among these forms, small and large peptides have been characterized, such as CCK-58, CCK-39, CCK-33, CCK-22, sulfated CCK-8 and CCK-7, unsulfated CCK-8 and CCK-7, and CCK-5 and CCK-4 (Rehfeld and Nielsen, 1995) (*Figure 24-2*). The presence of CCK in both gut and brain raises the intriguing issue of the evolutionary significance of separate pools of a peptide in two systems originating from different embryonic areas (i.e., endoderm and ectoderm, respectively). Characteristically, however, the processing of procholecystokinin (proCCK) varies markedly between the brain and the gut. In neurons, CCK-8 is always the predominating form, whereas the endocrine cells contain a mixture of small and larger CCK peptides of which CCK-33 or CCK-22 often predominates.

**Figure 24-1**
**In situ hybridization of rat brain sections with a 35S-labeled CCK oligodeoxynucleotide probe. a, Positive prints of section exposed for 3 days in contact with an X-ray film. Sections were hybridized with a labeled oligonucleotide probe alone (*top*); nonspecific signal obtained after hybridization with a mixture of labeled and unlabeled CCK probes (*bottom*). b, Dark-field illumination of different brain regions after a 21-day exposure. *C* cortex, *CA1* *CA2*, *CA3*, *CA4* fields of Ammon's horn, *DG* dentate gyrus, *DLG* dorsal lateral geniculate nucleus, *LP* lateroposterior thalamus nucleus, *Po* posterior thalamus nuclear group, *VPL* posteroventral thalamus nucleus, *VPM* ventroposterior thalamus nucleus, *SNC* substantia nigra pars compacta, *VTA* ventral tegmental area, *EW* Edinger–Westphal nucleus**

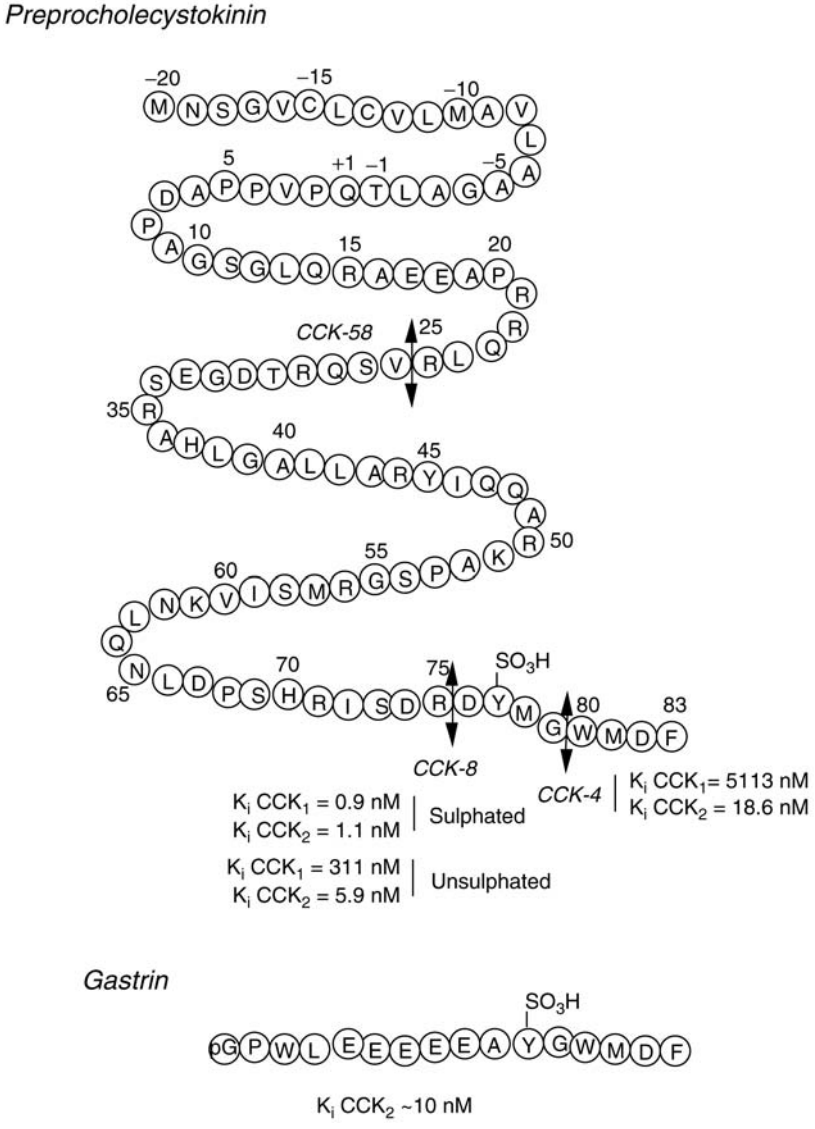



## 1.2 Processing of proCCK

During posttranslational processing, proCCK undergoes tyrosine sulfation, endoproteolytic cleavage, removal of carboxy-terminal arginines by carboxypeptidase E, and carboxy-terminal amidation (Beinfeld, 2003a). The endoproteolytic cleavages of proCCK occur almost exclusively at mono- and dibasic sites and are ensured by prohormone convertases (PCs), which are subtilisin- or kesin-like enzymes. These enzymes are found in cells that express CCK messenger RNA (mRNA), demonstrating that this class of enzymes has the correct distribution and catalytic activity to process proCCK to biologically active products. To date, eight mammalian PCs have been identified (Seidah and Chretien, 1999). The enzymes responsible for the endoproteolytic cleavages in CCK during its processing have not been completely established. The most likely candidates among this class are the prohormone convertases PC1, PC2, and PC5. In terms of catalytic activity the three enzymes are good candidates for proCCK endoproteolysis. Moreover, these enzymes are widely distributed in the brain, including in many areas that express high levels of CCK, as demonstrated using double-label in situ hybridization (Cain et al., 2003). PC2 is the most abundant of these enzymes in terms of the intensity and number of cells labeled, and is widely colocalized with CCK. PC1 and PC5 mRNA-positive cells are less abundant, but they are also widely distributed and strongly colocalized with CCK in different brain areas: cerebral cortex, hippocampus, amygdala, ventral tegmental area (VTA), and substantia nigra zona compacta. The degree of colocalization of the enzymes with CCK is region-specific. These three enzymes have their own unique distribution and, interestingly, many cells that express the

**Figure 24-2**

**Predicted structure of human preprocholecystokinin. The signal peptide consists of residues −20 to −1. The amino-terminal-flanking peptide consists of residues 1 to 25. The largest characterized form from brain and intestine, CCK-58, consists of residues 26 to 83. Other active molecular forms are derived from this precursor, such as CCK-39, CCK-33, CCK-22, CCK-7, and CCK-5**




enzymes did not express CCK, supporting the hypothesis that PC1, PC2, and PC5 are involved in the cleavage and processing of other prohormones or proteins in these neurons.

One way to establish the importance of these enzymes in proCCK processing in rodents is to examine enzyme knockout mice. However, the results reported in the literature are not clear. Thus, while Rehfeld et al. (2003) reported that the lack of PC1 was without effect on the cerebral maturation of proCCK, Cain et al. (2004) demonstrated that CCK levels were decreased in hippocampus, amygdala, and pons medulla in PC1 knockout mice as compared with wild-type animals. Regarding the role played by PC2, it has been

demonstrated that PC2-null mice displayed a ninefold increase in cerebral proCCK concentrations, whereas the concentrations of transmitter-active CCK peptides were reduced (Rehfeld et al., 2002). Thus, PC2 plays a major neuron-specific role in the processing of proCCK.

## 2 CCK Receptors

Receptors for CCK have been pharmacologically classified on the basis of their affinity for the endogenous peptide agonists. Receptors for CCK were first characterized on pancreatic acinar cells and identified as $CCK_1$ (also called CCK-A) receptors (Sankaran et al., 1980). Subsequently, a second receptor, the $CCK_2$ (also called CCK-B) receptor, was identified in the brain and was shown to exhibit a distinct pharmacology (Innis and Snyder, 1980).

### 2.1 Molecular Characterization

The $CCK_1$ and $CCK_2$ receptors have been cloned from several species (Wank et al., 1992; review in Noble et al., 1999). The $CCK_1$ receptor is highly conserved, with an overall amino acid homology of 80% and pairwise amino acid sequence identities of 87–92% in humans, guinea pigs, rats, and rabbits. Similarly, the $CCK_2$ receptor is highly conserved in humans, canines, guinea pigs, calves, rabbits, and rats, with an overall identity of 72% and pairwise amino acid sequence identities of 84–93% (review in Wank, 1995).

The deduced sequences of the rat $CCK_1$ and $CCK_2$ receptors correspond to 429- and 452-amino-acid proteins, respectively. Hydropathy analysis of the primary sequence of $CCK_1$ and $CCK_2$ receptors predicts seven transmembrane-spanning domains (TM), as expected for a member of the G-protein-coupled receptor (GPCR) superfamily (Dohlman et al., 1991).

In agreement with the heavy and variable degrees of glycosylation reported using ligand-affinity cross-linking techniques (De Weerth et al., 1993), at least three consensus sequence sites for N-linked glycosylation (Asn-X-Ser/Thr) have been identified in the $CCK_1$ and $CCK_2$ receptor sequences. There are multiple potential serine and threonine phosphorylation sites in the $CCK_2$ receptor: for protein kinase C (PKC) (serine 82 in the first intracellular loop) and for protein kinase A (PKA) (serine 154 in the second intracellular loop and serine 442 in the cytoplasmic tail). Like the $CCK_2$ receptor, the $CCK_1$ receptor has three consensus sequences for PKC phosphorylation in the third intracellular loop, and one site in the cytoplasmic tail of the rat pancreatic $CCK_1$ receptor (Ozcelebi and Miller, 1995).

Moreover, in both receptors there are two cysteines in the first and second extracellular loops, which may form a disulfide bridge required for stabilization of the tertiary structure as demonstrated for other receptors belonging to the GPCR superfamily (Silvente-Poirot et al., 1998), and a cysteine in the C terminus of the receptor that may serve as a membrane-anchoring palmitoylation site as demonstrated for rhodopsin and the β2-adrenergic receptors (O'Dowd et al., 1988; Ovchinikov et al., 1988).

Finally, on the basis of pharmacological and biochemical studies, the existence of subtypes of $CCK_1$ and $CCK_2$ receptors has been postulated (Durieux et al., 1986; Knapp et al., 1990; Talkad et al., 1994). Nevertheless, at this time only two genes have been cloned. Gastrin receptors in the stomach and $CCK_2$ receptors in the brain were earlier viewed as distinct CCK receptors on the basis of their difference in the affinity for CCK- and gastrin-like peptides (Menozzi et al., 1989). Endogenous peptide agonists CCK8 [Asp-Tyr($SO_3H$)-Met-Gly-trp-Met-Asp-Phe-$NH_2$] and gastrin [$H_2N$-Gln-Gly-Pro-Trp-Met-Glu-Glu-Glu-Glu-Glu-Ala-Tyr($SO_3H$)-Gly-Trp-Met-Asp-Phe-$NH_2$] share the same COOH-terminal pentapeptide amide sequence but differ in sulfation at the sixth (gastrin) or seventh (CCK) tyrosyl residue. Agonist-binding studies on brain membranes and parietal cells show a six- to tenfold and one- to twofold higher affinity for CCK than for gastrin, respectively (Jensen et al., 1990). These small differences in agonist binding have generated controversy regarding the existence of subtypes within this receptor class. The identification of a single $CCK_2$-receptor-encoding gene through low- and high-stringency hybridization of complementary DNA (cDNA) and genomic libraries and Northern and Southern blot analyses in numerous species indicates that gastrin receptors do correspond to $CCK_2$ receptors located in the gastrointestinal tract and do not constitute a third type of CCK receptor (Wank, 1995).

A third receptor has been pharmacologically identified, but not yet cloned. This receptor is a gastrin-preferring receptor, and has been described for the first time on an immortalized fibroblast cell line (Swiss 3T3 cells) that does not discriminate between iodinated and glycine-extended gastrins (Singh et al., 1995).

### 2.2 CCK Receptor Localization

$CCK_1$ receptors are found principally in the gastrointestinal tract and some brain structures, while $CCK_2$ receptors are widely distributed in the CNS and in particular regions of the gastrointestinal tract, and on pancreatic acinar and parietal cells (Moran et al., 1986; Pélaprat et al., 1987; Jensen et al., 1994).

Specific CCK-binding sites were characterized in membranes from brain homogenates in the 1980's (Innis and Snyder, 1980; Saito et al., 1980). Autoradiographic studies using CCK-related peptides in the brain that do not distinguish between the two CCK receptors in several species (e.g., rats, guinea pigs, monkeys, humans) showed high densities of CCK-binding sites in several areas, including the cerebral cortex, striatum, olfactory bulb and tubercle, and certain amygdaloid nuclei. Moderate levels were observed in the hippocampus, claustrum, substantia nigra, superior colliculus, periaqueductal gray matter, and pontine nuclei. Low densities were reported in several thalamic and hypothalamic nuclei and in the spinal cord. Nevertheless, it is important to note that species-specific heterogeneity in tissue expression is apparent in different structures and indicates that the results of studies performed in one species may not necessarily be generalized to other species (Niehoff, 1989). For example, in the cerebellum, high densities of CCK-binding sites were present in guinea pigs, humans, and mice, whereas only low levels were detected in rats (Zarbin et al., 1983; Gaudreau et al., 1985).

With the development of specific radioligands, both $CCK_1$ and $CCK_2$ receptors were found in some brain structures. However, the vast majority of CCK receptors in the CNS are of the $CCK_2$ type, while $CCK_1$ receptors are restricted to rather discrete regions (Moran et al., 1986). Nevertheless, a recent report on the immunohistochemical distribution of $CCK_1$ receptors in rat CNS, using antiserum, described numerous brain regions displaying $CCK_1$-receptor-like immunoreactivity (Mercer and Beart, 1997). Thus, $CCK_1$ receptors were found in the interpeduncular nucleus, area postrema, medial nucleus tractus solitarius, and, with additional areas of binding, in the habenular nuclei, dorsomedial nucleus of the hypothalamus, central amygdala, nucleus accumbens, superior colliculus, periaqueductal gray matter, olivary nuclei, and anteroventral thalamic nuclei (see references cited in Noble et al., 1999). The precise anatomical localization of the two CCK receptor types serves to provide morphological substrates for many of the diverse functions attributed to neural CCK, including involvement in feeding, satiety, cardiovascular regulation, anxiety, pain, analgesia, memory, neuroendocrine control, osmotic stress, dopamine-related behaviors, and neurodegenerative and neuropsychiatric disorders (see Crawley and Corwin, 1994).

In the rat, $CCK_2$ mRNA was shown to be widely distributed in areas such as the cerebral cortex, the olfactory regions, the hippocampal formation, the septum, the amygdala, the basal ganglia and related regions (nucleus accumbens, caudate putamen, substantia nigra), the interpeduncular nucleus, and the cerebellum (Honda et al., 1993). This mRNA localization is largely consistent with previously reported histochemical binding studies (Pélaprat et al., 1987; Niehoff, 1989), except for regions such as the cerebellum, where neither $CCK_2$ receptors (Pélaprat et al., 1987) nor preproCCK mRNA (Lanaud et al., 1989) were previously detected. This is consistent with the absence of the mRNA encoding the complete sequence of the $CCK_2$ receptor (Jagerschmidt et al., 1994).

## 3 Ligands of CCK Receptors

### 3.1 Endogenous Ligands

At $CCK_1$ receptors, sulfated CCK8 was the minimal sequence for high-affinity binding, whereas CCK5, CCK4, gastrin, and unsulfated CCK8 interact with $CCK_2$ receptors; albeit, their affinities are lower than that for sulfated CCK8.

CCK8 was shown to be efficiently cleaved by aminopeptidase A (APA) in vivo (Migaud et al., 1996; Khaznadar et al., 1997), leading to formation of CCK7, and by the ubiquitously distributed peptidase II (TPPII, EC 3.4.14.10), which is mainly enriched in the cytosol but is also present as an internal and/or external component of the cell membrane (Rose et al., 1996). In contrast to various neuropeptides, the degradation of CCK8 is much slower in brain membranes, which suggests that peptidases could be associated with other clearing mechanisms. Indeed, an uptake system has been identified for CCK8 (Migaud et al., 1995), which could function to accumulate CCK8 and/or CCK7 for subsequent degradation by TPPII inside cells, although we cannot exclude a role for the dipeptidylaminopeptidase (DAP, EC 3.4.13.11) in CCK8 and CCK7 degradation (review in Roques, 2000).

CCK regularly colocalizes with classic transmitters and is most likely stored in morphologically distinct vesicles. These vesicles have a larger diameter and are dense-cored when viewed under the electron microscope due to the accumulation of soluble peptides (Zhu et al., 1986; Verhage et al., 1991). As in the case of a classic neurotransmitter, presynaptic CCK release is regulated by depolarization of the terminal plasma membrane upon arrival of a train of action potentials, which leads to $Ca^{2+}$ entry through high-voltage-activated Ca channels. CCK release has been studied in vivo by microdialysis, as well as in vitro from slices and purified nerve terminals. It appears that this release is subject to modulation by a variety of transmitters, including effects of dopamine, serotonin, opioids, glutamate, and GABA receptor activation (review in Ghijsen et al., 2001).

## 3.2 Synthetic Ligands

### 3.2.1 Agonists

Only a few compounds have been reported to be $CCK_1$-selective agonists; most of them are peptides, such as A-71378 [des-$NH_2$-Tyr($SO_3H$)-Nle-Gly-Trp-Nle-(NMe)Asp-Phe-$NH_2$], or A-71623 and A-70874. In order to overcome the number of difficulties that limit the use of peptides as drugs, efforts have been devoted toward the development of nonpeptide ligands. Among the nonpeptide $CCK_1$ receptor agonists, two main families may be highlighted. The first family constitutes the series of 1,5-benzodiazepines acting in vitro and in vivo, developed by Glaxo-Wellcome. Potency within this series was modulated by a substituent on the N1-anilinoacetamide moiety (Aquino et al., 1996), and by substitution and/or replacement of phenylurea moiety in the C3 position (GW 5823, GW 7854; Henke et al., 1997). These modifications were also successfully adapted to a series of 1,4-benzodiazepines (Sherrill et al., 2001). The second family constitutes dipeptoid hybrids mainly discovered by Parke-Davis. PD-170,292 behaves as a full agonist at the high-affinity sites and as an antagonist at the low-affinity sites of the $CCK_1$ receptor (Bernad et al., 2000). Finally, a new potent and selective nonpeptide agonist of the $CCK_1$ receptor, SR146131, has been characterized (Bignon et al., 1999). This compound is chemically related to the selective $CCK_1$ receptor antagonist SR27897B (❯ *Table 24-1*).

Different strategies have been followed to design potent and selective agonists of $CCK_2$ receptors. In spite of its intrinsic flexibility, $CCK_8$ was found by nuclear magnetic resonance (NMR) to exist preferentially in folded form in aqueous solution (Fournié-Zaluski et al., 1986), with a proximity between Asp1 and Gly4. This property was used to synthesize cyclic peptides such as BC 254 and BC 197, which were found to be highly potent and selective $CCK_2$ agonists (Charpentier et al., 1988a, 1989).

Another approach toward $CCK_2$ agonists was to protect $CCK_8$ from degrading enzymes such as APA (Migaud et al., 1996), and a thiol/serine protease cleaving this peptide at the Met-Gly bond (Rose et al., 1996).

Biologically active Boc[$Nle^{28,31}$]$CCK_{27–33}$ (BDNL; Ruiz-Gayo et al., 1985) was used as the parent compound to design enzyme-resistant analogs. In this compound, the major sites of cleavage are at the $Trp^{30}$/$Nle^{31}$ and $Nle^{28}$/$Gly^{29}$ bonds. Consequently, several enzyme-resistant BDNL analogs containing either a retro–inverso 28–29 amide bond or an (NMe)$Nle^{31}$ residue, or a combination of these two modifications have been synthesized (Charpentier et al., 1988b). This led to BC 264, a highly potent $CCK_2$ agonist that exhibits about the same affinity ($K_D = 0.1–0.5$ nM) in all species (Charpentier et al., 1988b; Durieux et al., 1991).

**Table 24-1**
**Ligands and their action**

| $CCK_1$ | | $CCK_2$ | |
|---|---|---|---|
| **Agonists** | **Antagonists** | **Agonists** | **Antagonists** |
| A-71378 | SR-27897 | BC254 | L-365,260 |
| A-71623 | Loxiglumide (CR-1505) | BC197 | L-740,093 |
| A-70874 | Lorglumide (CR-1409) | BC264 | YM-022 |
| GW-5823 | 2-NAP | RB 400 | YF-476 |
| GW-7854 | PD-140,548 | BBL454 | PD-134,308 (CI-988) |
| PD-170,292 | IQM-95,333 | | CI-1015 |
| SR-146131 | L-364,718 (MK329, devazepide) | | RB 211 |
| | | | CR-2194 |
| | | | LY-262,691 |
| | | | RP-73,870 |

RB 101, dual inhibitor of enkephalin-degrading enzymes; BUBU, selective delta opioid receptor agonist; naltrindole, selective delta opioid receptor antagonist

The behavioral results obtained with BC 264, such as increase in memory processes and increase in dopamine release in mesolimbic and nigrostrial pathways (review in Daugé and Roques, 1995), suggest that the development of nonpeptide $CCK_2$-selective agonists endowed with good stability and bioavailability should provide useful pharmacological tools and possibly interesting therapeutic agents. In order to design such derivatives, the C-terminal tetrapeptide showing significant $CCK_2$ affinity and selectivity was used as a scaffold, although it has been shown to trigger panic attacks in humans (De Montigny, 1989; Bradwejn et al., 1991). Several modifications of CCK4, such as N-terminal protection of the tetrapeptide in Boc-$CCK_4$ (Harhammer et al., 1991), and modifications of the different constituting amino acids with replacement of Met by Nle or (NMe)Nle increased $CCK_2$ selectivity of $CCK_4$ (Corringer et al., 1993). NMR and molecular dynamics studies indicated that the $CCK_2$-receptor-selective CCK4 analogs adopt an S-shaped conformation with a relatively well-defined orientation of the side chains (Goudreau et al., 1994). The same type of folded structures has been reported for several potent agonists derived from CCK4 and containing [*trans*-3-propyl-L-proline] (Nadzan et al., 1991), a diketopiperazine skeleton (Shiosaki et al., 1990), or an [(alkylthio) proline] residue (Kolodziej et al., 1995). Moreover, to stabilize the bioactive conformation of $CCK_2$ receptor agonists with the aim of designing nonpeptide ligands, macrocyclic constrained CCK4 analogs that are endowed with $CCK_2$ agonist properties and were found able to cross the blood–brain barrier have been developed (Blommaert et al., 1997). On the basis of these results, compounds combining the modifications introduced in BC 264 and CCK4 such as RB 400 (Million et al., 1997) and BBL454 (Bellier et al., 2004) were found to be highly potent.

### 3.2.2 Antagonists

Much of the early research regarding the physiological effects of CCK was hindered by the lack of selective antagonists. The first CCK antagonists were derived from a naturally occurring benzodiazepine, asperlicin, which has been isolated from the fungus *Aspergillus alliaceus* (Chang et al., 1985). The demonstrated high in vitro and in vivo potency of asperlicin at $CCK_1$ receptors conferred clear advantages over previously reported CCK antagonists as a tool for investigating the physiological and pharmacological actions of CCK. The 5-phenyl-1,4-benzodiazepine ring was used as a model to design improved CCK receptor antagonists (Evans et al., 1986), leading to several compounds such as L-364,718 (MK-329, devazepide), which remained for several years the most potent CCK antagonist with a good selectivity for $CCK_1$ receptors. Various benzodiazepine derivatives were developed, such as FK-480, a highly selective and potent

$CCK_1$ receptor antagonist (Ito et al., 1994). Several other potent and selective antagonists of the $CCK_1$ receptor have been described, including glutamic acid derivatives, such as loxiglumide (CR-1505) or lorglumide (CR-1409; Makovec et al., 1995). Several other dipeptoids were synthesized, such as 2-NAP [2-naphtalenesulfonyl 1-aspartyl-(2-phenethyl)amide] (Hull et al., 1993), or PD-140,548 (Boden et al., 1993).

Interest in nonpeptide CCK-receptor-selective ligands has directed efforts toward the incorporation of conformationally restricted structures as spacers between Trp and Phe residues in the sequence of the CCK receptor endogenous ligand CCK4. Thus, recently, a new series of CCK4-restricted analogs with a 3-oxoindolizidine ring was synthesized: IQM-95,333 (Martin-Martinez et al., 1997). Another $CCK_1$ receptor antagonist, SR-27,897, which is chemically unrelated to peptoids, benzodiazepine, or glutamic acid derivatives, has been developed (Gully et al., 1993).

The moderate affinity of L-364,718 for $CCK_2$ receptors suggested that the benzodiazepine nucleus might also hold a key to selective ligands for these receptors. The first compound of interest developed using this strategy was L-365,260 (Bock et al., 1989). Undoubtedly, the benzodiazepine template that is present in asperlicin, L-364,718 and L-365,260, has been the most exploited structure in the development of CCK receptor antagonists. This is particularly true for the $CCK_2$ receptor where substantial investment by the pharmaceutical industry has resulted in the generation of more potent and selective antagonists incorporating the benzodiazepine moiety, such as L-740,093 (Patel et al., 1994), YM022 (Nishida et al., 1994), and YF476 (Takinami et al., 1997).

A second approach in the development of nonpeptide antagonists of the $CCK_2$ receptor has been the synthesis of "dipeptoids" (Horwell et al., 1991). This led to tryptophan dipeptoid derivatives such as PD-134,308 (CI-988) with nanomolar affinity for $CCK_2$ receptors. A direct comparison of the structure of these compounds showed that their sizes could be reduced to increase their lipophilicity. Indeed, the clinical development of CI-988 was limited due to its poor bioavailability, which was attributed to poor absorption and efficient hepatic extraction. Several modifications have been performed, leading to compounds such as CI-1015 (Trivedi et al., 1998) or RB 211 (Blommaert et al., 1993).

Three other series have been described, leading to the synthesis of derivatives that have both excellent selectivity and high affinity for $CCK_2$ receptor: the 4-benzamido-5-oxopentanoic derivatives, the diphenylpyrazolidinone series, and the ureidoacetamides, of which CR-2194 (Revel et al., 1992), LY-262,691 (Howbert et al., 1992), and RP-73,870 (Pendley et al., 1995), respectively, are representative examples.

## 4 CCK Signaling

The signal transduction pathways linked to $CCK_1$ receptor activation have been extensively studied in pancreatic acinar cells, where CCK stimulates amylase secretion. In this system it has been well established that $CCK_1$ receptor is capable of coupling to both phospholipase C (PLC) and adenylyl cyclase at physiological concentrations. CCK activates the hydrolysis of polyphosphoinositides by PLC with subsequent formation of the second messengers inositol 1,4,5-triphosphate (IP3) (De Weerth et al., 1993) and 1,2-diacylglycerol, leading to the release of intracellular calcium (Yule et al., 1993; Dunlop et al., 1997) and the activation of PKC, respectively. In addition, stimulation of the $CCK_1$ receptor activates other intracellular events, such as the phospholipase A2 (PLA2). Moreover, it was shown that mitogen-activated protein kinase (MAPK) and c-Jun-$NH_2$-terminal kinases (JNK, which phosphorylate serine residues of c-Jun) are rapidly activated by the octapeptide CCK8 in rat pancreas both in vitro and in vivo (Dabrowski et al., 1996), and can also enhance the expression of immediate early genes (Day et al., 1994). Most of these signaling pathways have not yet been confirmed in the CNS, even if we could speculate that the intracellular effectors coupled to $CCK_1$ receptors are identical.

This lack of characterization of signal transduction for $CCK_2$ receptors in the brain is largely due to the difficulty of working with isolated neurons. Thus, for a long time, central $CCK_2$ receptors have not been proved to be linked to a well-characterized second-messenger system in the brain, including the phosphoinositide system, although phosphoinositide metabolism was shown to be affected by CCK

in neuroblastoma (Barrett et al., 1989) and in the embryonic pituitary cell line (Lo and Hughes, 1988). More recently, Zhang et al. (1992) showed that $CCK_8$ increased the turnover of phosphoinositides and IP3 labeling in dissociated neonatal rat brain cells, in which both $CCK_1$ and $CCK_2$ receptors were expressed. However, it has not been possible to demonstrate their possible coupling with adenylyl cyclase or PLC by using synaptoneurosomes from guinea pig cortex, although $Ca^{2+}$ release from intracellular stores, possibly via G-protein-independent mechanisms could be triggered by a CCK analog (Galas et al., 1992).

Moreover, in a mammalian expression system it has been shown that $CCK_2$ receptors are coupled to a phospholipase pathway, leading to the release of arachidonic acid via a pertussis toxin (PTX)-sensitive G protein (Pommier et al., 1999, 2003), and to an MAPK pathway (Taniguchi et al., 1994).

## 5 Potential Therapeutic Applications of CCK in Diseases

### 5.1 Food Intake

Numerous neuropeptides affect the food intake by either stimulating (ghrelin, orexins) or inhibiting (CCK, leptin, oxyntomodulin) the expression and release in the arcuate nucleus of hypothalamus of neuropeptide Y and agouti-related protein, which are the central (hypothalamic centers) orexigenic substances responsible for ingestive behavior in animals and humans.

The role of CCK and peripheral $CCK_1$ receptors in the regulation of feeding behavior is an area under intense investigation (review in Moran, 2004). $CCK_1$ receptors appear to mediate the transmission of sensory information from the gut to the brain. Peripherally administered CCK inhibits food consumption, even after fasting, in many species, including humans (for reviews see Smith and Gibbs, 1992; Crawley and Corwin, 1994). $CCK_1$ receptor agonists have been proposed as anorectics for the treatment of obesity (Simmons et al., 1994; Wettstein et al., 1994). Conversely, $CCK_1$ receptor antagonists have been proposed for the treatment of anorexia disorders (Wolkowitz et al., 1990). Recent work examining controls of food intake and energy balance in the hyperphagic and obese Otsuka Long–Evans Tokushima Fatty (OLETF) rats that lack $CCK_1$ receptors have both confirmed the role of CCK in limiting meal size and identified new actions of CCK in food intake control (Bi and Moran, 2002). The phenotype of OLETF rats appears to be different from that of $CCK_1$-receptor-deficient mice. Thus, a parallel analysis of the knockout animals revealed that these mice reached adult weights that were indistinguishable from the corresponding age- and sex-matched animals. The similarity in weights between knockout and wild-type animals persisted through the rapid growth phase and extended well into adulthood. Moreover, the mutant mice had normal pancreatic morphology and were normoglycemic. However, in contrast to wild-type animals, $CCK_1$-receptor-deficient mice showed no change in food consumption after administration of exogenous CCK8, supporting the hypothesis that CCK-induced inhibition of food intake is mediated through the $CCK_1$ receptors (Kopin et al., 1999).

The entry of food into the intestine triggers the release of endogenous CCK by the intestinal mucosa, thereby activating $CCK_1$ receptors located in the periphery to transmit, mainly through the vagus nerve, sensation of fullness to the brain, which subsequently terminates feeding and initiates the sequence of behaviors associated with satiety (Smith and Gibbs, 1992). Besides activating vagal afferent nerve fibers, CCK released in the plasma may also act on $CCK_1$ receptors in the area postrema, a region of the brain stem that has a leaky blood–brain barrier and monosynaptic connection to the nucleus tractus solitarus (NTS). Studies investigating the role of $CCK_1$ receptors in activating NTS neurons using highly selective $CCK_1$ receptor agonists and antagonists have yielded conflicting data. Thus, $CCK_1$ receptor blockade had no effect on the postprandial activation of NTS neurons in one study, whereas others have shown a significant reduction (Chen et al., 1993; Fraser and Davison, 1993; Zittel et al., 1999).

Controversial data regarding the role of $CCK_2$ receptor in feeding behavior have been reported (Corwin et al., 1991; Reidelberger et al., 1991), which led to the hypothesis that under certain conditions, the $CCK_2$ receptor may play a role in modulating food intake. As a possible mechanism it was postulated that during stress or anxiety, $CCK_2$-receptor-mediated pathways are activated and in turn may influence eating

behavior (review in Crawley and Corwin, 1994). However, when $CCK_2$-receptor-deficient mice were studied, the dose-dependent CCK8-induced inhibition of food intake was found similar to the pattern observed in wild-type animals, suggesting that the $CCK_2$ receptor does not play a major role in mediating the inhibitory effect of exogenous CCK (Kopin et al., 1999). Moreover, $CCK_2$-receptor-deficient mice had body weights comparable with the corresponding age- and sex-matched controls, suggesting that $CCK_2$ receptor is not essential for the maintenance of normal body weight.

## 5.2 CCK in Panic Attacks and Anxiety

Several lines of evidence point to a role for the peptide neurotransmitter CCK in the pathogenesis of panic disorder and in mechanisms related to anxiety. The initial suggestion that the CCK system might be involved in anxiety came from experiments of Bradwejn and de Montigny (1984, 1985) that showed that benzodiazepine receptor agonists could attenuate CCK-induced excitation of rat hippocampal neurons. Subsequent clinical studies demonstrated that bolus injections of the $CCK_2$ receptor agonists CCK4 or pentagastrin provoke panic attacks in patients with panic disorders (Bradwejn et al., 1991, 1992). Recent investigations have revealed that the panicogenic effects of $CCK_2$ receptor agonists are not limited to panic disorder, because individuals with social phobia, generalized anxiety disorder, obsessive compulsive disorder, and premenstrual dysphoric disorder also exhibit an augmented behavioral response to these ligands (Le Melledo et al., 1995; de Leeuw et al., 1996; van Vliet et al., 1997). Interestingly, a significant association exists between panic disorder and polymorphism of the $CCK_2$ receptor gene (Kennedy et al., 1999). The neurobiological mechanisms by which $CCK_2$ receptor agonists provoke panic and concomitant biological changes (robust increase in heart rate, blood pressure, hypothalamic–pituitary–adrenal axis activity, elevated blood levels of dopamine, epinephrine, norepinephrine, and neuropeptide Y) have been the subject of considerable research activity. Animal studies suggest that anxious behavior induced by various CCK fragments is associated with selective $CCK_2$ receptor stimulation (Harro et al., 1993). However, the anxiogenic effects of CCK peptides in animals have not been observed by all investigators, and the relevant negative findings should not be ignored (Shlik et al., 1997). The effect of CCK compounds could vary considerably because of existing differences in the distribution and binding characteristics of CCK receptor types and/or affinity states among species. $CCK_2$ sites in several structures, including the nucleus tractus solitaris, amygdala, nucleus accumbens, frontal cortex, hippocampus, and dorsal raphe nucleus have been implicated in the anxiogenic properties of CCK (Frankland et al., 1997; Becker et al., 2001; Wunderlich et al., 2002).

Recently, the effects of the selective $CCK_2$ receptor agonist BC 264 and BC 197 and of the nonselective CCK receptor agonist BDNL were investigated in rats subjected to the elevated plus maze (❯ *Figure 24-2*). Surprisingly, BDNL and BC 197 did induce anxiogenic-like effects, but BC 264 was devoid of any effect. Complementary works have demonstrated intrinsic anxiolytic actions of $CCK_2$ antagonists, principally employing models of exploratory behavior, notably the elevated plus maze (Wunderlich et al., 2002; reviews in Herranz, 2003; Millan, 2003). Contrariwise, anxiolytic actions of $CCK_2$ (and $CCK_1$) antagonists in conflict paradigms are weak, inconsistent, and rarely dose-dependent (Singh et al., 1991; Hendrie et al., 1993; Charrier et al., 1995; Dawson et al., 1995). Moreover, mice lacking $CCK_2$ receptors show modest alterations in anxious behavior, and may even reveal an anxiogenic phenotype (Daugé et al., 2001; Miyasaka et al., 2002; Abramov et al., 2004), as in OLETF rats (Kobayashi et al., 1996). These findings question the anxiolytic potential of $CCK_2$ (and $CCK_1$) antagonists in humans. Correspondingly, in clinical studies, despite their ability to block the panicogenic actions of CCK4, $CCK_2$ antagonists have not proven to be consistently efficacious as anxiolytic agents (review in Bradwejn and Koszycki, 2001; Radu et al., 2003).

Panic disorder, like other neuropsychiatric disorders, is believed to be caused by multiple psychosocial and biological factors. Moreover, several data are consistent with the notion that genetic variation in the CCK neurotransmitter system contributes to the pathogenesis of panic disorder (Hosing et al., 2004; Miyasaka et al., 2004), whereas other studies investigating the polymorphisms of the CCK and $CCK_2$ receptor genes yielded inconclusive results (Hattori et al., 2002; Ise et al., 2003).

## 5.3 Evidence of Regulatory Mechanisms Between Endogenous CCK and Enkephalin Systems in the Control of Pain

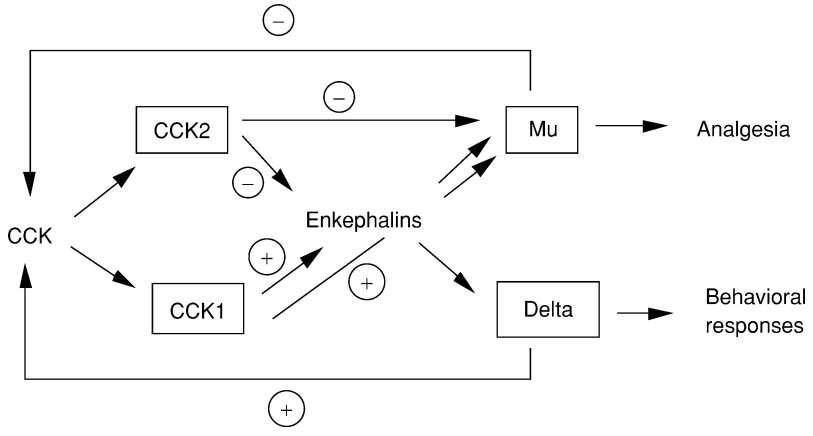
A large body of evidence has now been accumulated supporting physiological interaction between CCK and endogenous opioid peptides, enkephalins. Anatomical studies have shown that the distribution of CCK8 and CCK receptors parallels that of endogenous opioid peptides and opioid receptors in the pain-processing regions in both the brain and the spinal cord (Gall et al., 1987; Pohl et al., 1990). This overlapping distribution triggered numerous investigations on the role of CCK in nociception.

The opioid and CCK systems are critically involved, generally in an opposite manner in various physiological processes. It has been suggested that CCK8 has an antiopioid activity. Thus, Faris et al. (1983) found that CCK reduced the antinociceptive effects produced by the stress-induced release of endogenous opioids, and did not modify nonopiate responses induced by hind paw shock. In addition, it has been shown that peripherally administered CCK antagonists or active immunization against CCK potentiates exogenous opiate-produced antinociception (Faris et al., 1984; Baber et al., 1989). However, few studies have been performed on the possible physiological interactions between endogenous CCK and endogenous opioid systems. It is now well established that endogenous opioid peptides, enkephalins, are cleaved into inactive fragments by means of ectopeptidases (review in Roques, 2000). The development of efficient inhibitors of these metabolizing enzymes allows the extracellular levels of enkephalins to be monitored. The joint use of these inhibitors and CCK antagonists allowed the physiological responses of the two neuropeptide systems to be studied.

The existence of regulatory mechanisms between CCK and enkephalin systems in the control of pain has been proposed (*Figure 24-3*). Thus, activation of $CCK_1$ receptors potentiates the analgesic effects

**Figure 24-3**

**Hypothetical model of the supraspinal interactions between CCK, via $CCK_1$ and $CCK_2$ receptors, and the opioid system via delta and mu opioid receptors. CCK receptor agonists, endogenous or exogenous, stimulate $CCK_2$ and/or $CCK_1$ receptors, which can modulate the opioidergic (enkephalinergic) systems either directly (via the binding of opioid agonists or via C-fiber-evoked activity) or indirectly (via the release of endogenous enkephalins). In addition, activation of mu opioid receptors, which leads to antinociceptive responses, can negatively modulate the release of endogenous CCK, whereas delta opioid receptor activation may enhance it**

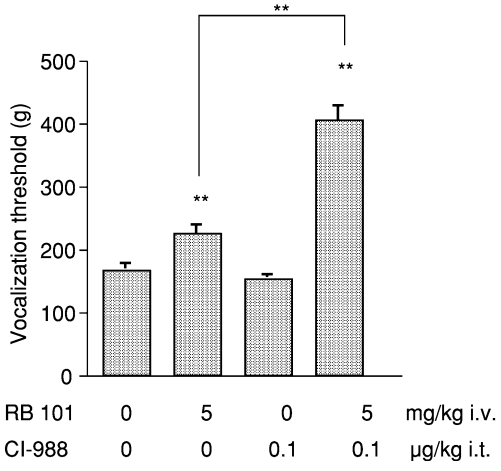



induced by the complete inhibitor of enkephalin-degrading enzymes able to cross the blood–brain barrier, i.e., RB 101 (Fournié-Zaluski et al., 1992), while activation of $CCK_2$ receptors reduces these effects (Derrien et al., 1993; Noble et al., 1993). Schematically, stimulation of $CCK_1$ receptors could enhance opioid release, and/or directly improve the efficacy of transduction processes occurring at the mu sites, which might be

allosterically evoked by $CCK_1$ site occupation (Magnuson et al., 1990). In contrast, $CCK_2$ receptor activation could negatively modulate the opioidergic system. Furthermore, the opioid system appears also to be able to regulate the release of CCK peptides. Thus, the stimulation of mu opioid receptors has an inhibitory influence on the $K^+$-evoked release of CCK-like material (CCKLM) at spinal and supraspinal levels (Rattray and De Belleroche, 1987; Rodriguez and Sacristan, 1989; Benoliel et al., 1991, 1992). On the other hand, in vitro studies have shown that delta opioid agonists enhance the $K^+$-evoked release of CCKLM from slices of rat substantia nigra (Benoliel et al., 1991, 1992). This result has been confirmed by in vivo binding studies. Thus, the binding of the CCK2-selective agonist [$^3$H]pBC 264 was found to be reduced by administration of the delta-selective agonist BUBU, as by RB 101, through activation of delta opioid receptors by endogenous enkephalins (Ruiz-Gayo et al., 1992). Consequently, activation of delta opioid receptors potentiates the release of CCK8, which could bind to $CCK_1$ and $CCK_2$ receptors. This increase is supported by the blockade of $CCK_2$-binding sites by selective antagonists (blocking the negative feedback control achieved by CCK8 via $CCK_2$ receptor activation), which strongly potentiates (200–800%) the antinociceptive effects induced by RB 101 in the rat tail flick test and the mouse hot plate test (Valverde et al., 1994) and prolongs the action of the inhibitor (Valverde et al., 1995). Moreover, selective $CCK_2$ receptor antagonists may potentiate the antiallodynic effects of morphine or endogenous enkephalins (Coudoré-Civiale et al., 2000, 2001) (*Figure 24-4*); these antagonists suppressed the development of autotomy behavior in a

**Figure 24-4**

**Antinociceptive effects on the paw pressure-induced vocalization threshold in diabetic rats due to the association between the dual inhibitor of enkephalin-degrading enzymes, RB 101, with the $CCK_2$ antagonist CI-988. $^{**}p < 0.01$ as compared with control group**




model of neuropathic pain in rat, and efficiently relieved the allodynia-like symptoms in spinally injured rats (review in Roques and Noble, 1996).

Several human and animal studies indicate that the anterior cingulate cortex (ACC), where CCK is especially abundant, plays an important role in the affective component of pain. In recent studies it has been reported that the releases of CCK in the rat ACC were enhanced following peripheral axotomy, a model of phantom limb pain (Gustafsson et al., 2000), and during carrageenan-induced arthritis (Erel et al., 2004). Because CCK has been implicated in anxiety, it could be suggested that an altered cholecystokinergic activity in the ACC may be of importance for the affective component of pain, as well as for an involvement in the modulation of nociception.

There is evidence that the action of endogenous or exogenous opioids leads to an enhancement of CCK activity, which in turn attenuates the acute antinociceptive effect of opioids, as previously described, and

may be one of the mechanisms of opioid tolerance. Thus, systemically administered CCK antagonists have been shown to prevent or revert tolerance to systemic exogenous opioids (Watkins et al., 1984; Dourish et al., 1990; Wiesenfeld-Hallin et al., 1999; Tortorici et al., 2003). In agreement with these observations, Zhou et al. (1992) and Pu et al. (1994) have shown that the development of morphine tolerance in the rat was associated with increased hybridization signals for the CCK mRNA in the brain.

## 5.4 CCK and Depression

One of the physiological actions of the neuropeptide CCK seems to involve modulation of the nigrostriatal and mesolimbic dopaminergic pathways. Taking into consideration that the mesolimbic dopaminergic pathways play a crucial role in motivation and rewarding processes, which are likely to be altered in depression (for review see Willner, 1990), a role of CCK in mood disorders cannot be excluded.

Several studies have shown that selective $CCK_2$ receptor agonists facilitate the suppression of motility test in mice, an animal model used to select antidepressant drugs. Moreover, this effect was inhibited by L-365,260, demonstrating the selective involvement of the $CCK_2$ receptors (Derrien et al., 1994a). However, the most interesting results were obtained with the $CCK_2$ antagonist, which alone decreased motor inhibition in shocked mice and induced antidepressant-like effect in the forced swim test in mice (Smadja et al., 1995). This could result from an increase of extracellular dopamine contents since this effect was suppressed both by $D_1$- and $D_2$-selective antagonists. Moreover, it has been shown that the association of ineffective doses of nomifensine (a blocker of dopamine reuptake) and L-365,260 leads to a significant decrease in the duration of immobility, suggesting that both drugs could act by a related mechanism (Hernando et al., 1994).

Furthermore, it has been suggested that the endogenous enkephalins might be involved in the etiology of depression. Accordingly, the "anxious" behavioral responses triggered by forced swimming, conditioned suppression of motility, and learned helplessness were attenuated by treatment with the enkephalin-degrading enzyme inhibitors (Ben Natan et al., 1984; Gibert-Rahola et al., 1990; Tejedor-Real et al., 1993, 1995; Baamonde et al., 1992; Smadja et al., 1995), suggesting a potential role of endogenous enkephalins in depressive syndromes. It has been demonstrated in these tests that the inhibitors modulated the functioning of the mesocorticolimbic and nigrostriatal dopaminergic systems, which are known to be implicated in mood control and have been shown to be connected with enkephalin pathways (review in Roques et al., 1993).

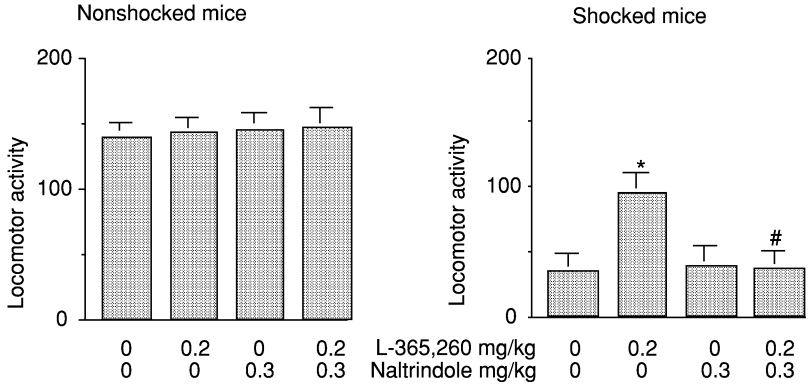
It was therefore of interest to investigate the possible modulation of RB 101-induced behavioral responses by CCK ligands. The results obtained showed that the antidepressant-like effects induced by the $CCK_2$ antagonist L-365,260 was suppressed by the selective antagonist for delta opioid receptors, suggesting the occurrence of physiologically adverse interactions between CCK and opioid systems (❯ *Figure 24-5*) (Derrien et al., 1994a; Hernando et al., 1996). This indicates that $CCK_2$ antagonists could block centrally located $CCK_2$ receptors, thus reinforcing the antidepressant-like effects induced by delta opioid receptor stimulation. Accordingly, the antidepressant-like effect of RB 101 was potentiated by L-365,260 and suppressed by BC 264. As expected, the facilitation induced by L-365,260 on RB 101 responses was blocked by naltrindole (Smadja et al., 1995). Furthermore, Smadja et al. (1997) have shown that the endogenous CCK system, through $CCK_2$ receptors, could modulate opioid behavioral responses by a mechanism directly involving two different mesolimbic structures, the anterior nucleus accumbens and the central amygdala. Taken together, these data suggest that the clinical use of $CCK_2$ antagonists, administered alone, or in association with classical treatments or inhibitors of enkephalin catabolism, could be extended to the treatment of depressive syndromes.

However, relatively little is known about the role of CCK in clinical depression. Several laboratories have demonstrated that patients with major depression display cerebrospinal fluid CCK concentrations comparable with those of control subjects (Geracioti et al. 1993). However, there is some evidence that an increase in cerebrospinal fluid CCK levels can occur in particularly severe depression (Löfberg et al., 1998).

On the other hand, postmortem studies have revealed that compared with healthy controls and patients with schizophrenia, suicide victims have elevated preproCCK mRNA levels and an increased density of

**Figure 24-5**

**Effects of the $CCK_2$ antagonist L-365,260 in shocked and nonshocked mice in the conditioned suppression of motility test, and reversal by naltrindole, a selective delta opioid receptor antagonist. *$p < 0.05$ as compared with control group; #$p < 0.05$ as compared with the same dose of L-365,260 without naltrindole**




CCK-containing neurons in the dorsolateral prefrontal cortex and a high density of CCK receptors in the frontal cortex (Ferrier et al., 1983; Harro et al., 1992).

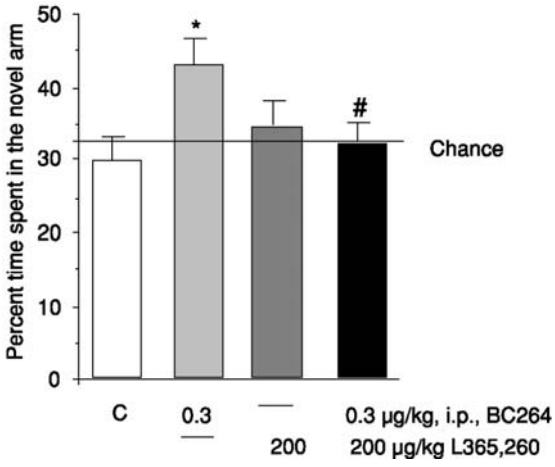
## 5.5 CCK in Learning and Memory Processes

During learning and memory processes, besides structural synaptic remodeling, changes are observed at molecular and metabolic levels with the alterations in neurotransmitter and neuropeptide synthesis and release (review in Gulpinar and Yegen, 2004). The peptide CCK has been proposed to facilitate memory processing, and CCK-like immunoreactivity in the hypothalamus was observed upon stress response and stress-induced memory dysfunction. Moreover, on the basis of anatomical data, studies of CCK on memory processes have constituted an important field of investigation (review in Hadjiivanova et al., 2003), as this neuropeptide is present in regions such as limbic structure and cortical areas, which are implicated in the control of cognitive processes, and motivational and emotional behaviors. It has been suggested that $CCK_1$ and $CCK_2$ receptors have different roles in learning and memory functions (Harro and Oreland, 1993). In particular, a balance between $CCK_1$-receptor-mediated facilitatory effects and $CCK_2$-receptor-mediated inhibitory effects on memory retention has been postulated (Lemaire et al., 1992). However, there are conflicting reports on the effects of $CCK_2$ receptor agonists in the animal model of memory. For instance, although some groups have reported that selective $CCK_2$ receptor agonists ($CCK_4$, BC 264) impair memory (Lemaire et al., 1992; Derrien et al., 1994b), others have found that these peptides enhance memory (Gerhardt et al., 1994; Léna et al., 1999; Taghzouti et al., 1999) (*Figure 24-6*). Treatment with BC 264 has also been described to elicit prominent hypervigilance in monkeys and to increase behavioral arousal in rats (Daugé and Roques, 1995). The latter findings suggest a possible role for $CCK_2$ receptor in attention activation that can facilitate learning. The apparent discrepancies observed with CCK agonists indicate that $CCK_2$ receptors could have different functions involving probably different neuronal pathways, according to the task carried out by the animal.

Different animal models, including spontaneous mutants and knockout mice, may be interesting to clarify the physiological role of specific receptors. Because OLETF rats lack both central and peripheral $CCK_1$ receptors, this model may be useful to clarify the functions of $CCK_1$ receptors in the CNS. Learning and memory in a Morris water maze learning task were significantly impaired in the OLETF rats compared with the Long–Evans Tokushima Otsuka (LETO) controls, which were not considered to be due to hypoactivity (Matsushita et al., 2003). On the other hand, mice lacking $CCK_2$ receptors showed a decrease in spontaneous alternation behavior as measured in the Y maze compared with that of wild-type animals, as

**Figure 24-6**

**Effects of L-365,260 (200 μg/kg) on the BC 264 induced improvement of performance. The $CCK_2$ antagonist was injected i.p. alone, 60 min before the experiment or 30 min before injection of the $CCK_2$ agonist. The results are expressed as means of the percentage of time spent in the novel arm ± SEM ($n = 8–15$). *$p < 0.05$ as compared with control groups; #$p < 0.05$ as compared with $CCK_2$ agonist group**




well as an impairment in retention of spatial recognition using a two-trial memory test (Sebret et al., 1999; Daugé et al., 2001). These results emphasize the physiological role of CCK improving, through $CCK_1$ and $CCK_2$ receptor stimulation, some memory and/or attention processes.

To date only few studies have been devoted to the effects of CCK receptor agonists on human memory. Recently, Shlik et al. (1998) found that the continuous administration of the selective $CCK_2$ receptor agonist, CCK4, had no effect on psychomotor performance, although it produced impairment in cognitive tests of free recall and recognition. The results of this study suggest that CCK4 may exert a negative influence on memory consolidation and retrieval.

## 5.6 Addiction

It is known that the dopaminergic projections from the VTA to the nucleus accumbens are involved in positively motivated behaviors such as feeding, sexual behavior, and locomotor activity. The mesoaccumbens dopamine pathway is also highly implicated in the primary rewarding effects of drugs of abuse and in the process of sensitization, in which subsequent presentations of a drug result in an increased neurochemical and behavioral response. Sensitization is thought to play an important role in the development of addictions. The colocalization of CCK with dopamine in this pathway suggests a functional role for CCK in reward behaviors. Furthermore, CCK-containing projections from the prefrontal cortex to the striatum, which also contain glutamate, may also play an important role in reward-related behaviors, given that glutamate has also been shown to be important in the induction and expression of sensitization (Vanderschuren and Kalivas, 2000).

Evidence suggests that $CCK_1$ receptors mediate dopamine-agonist-like effects in the caudal shell area of the nucleus accumbens. For example, $CCK_1$ agonists in this brain structure potentiate $K^+$-stimulated endogenous dopamine release (Marshall et al., 1991), and increase extracellular concentrations of dopamine and its metabolites, DOPAC and HVA (Voigt et al., 1985; Karia et al., 1994). Conversely, CCK stimulation in the rostral core of the nucleus accumbens has dopamine antagonistic effects, such as attenuated $K^+$-stimulated dopamine release and decreased extracellular dopamine concentrations and turnover, which appear to be mediated by $CCK_2$ receptors (Fuxe et al., 1980; Voigt et al., 1985). Recent

studies of the $CCK_2$ receptor knockout mice strongly support the role of $CCK_2$ receptor as a negative modulator of dopamine neurotransmission. In the absence of $CCK_2$ receptor, these mice behave as they have augmented dopamine neurotransmission compared with wild-type animals. The knockout mice displayed more locomotor activity than the wild type (Daugé et al., 2001), had increased sensitivity of dopamine D2 receptors (Koks et al., 2003), and displayed more locomotor activity in response to a high dose of amphetamine than did the wild type (Koks et al., 2001).

In drug self-administration, intra-accumbens administration of $CCK_2$ agonist pentagastrin, increases responding on a fixed-ratio schedule for intravenous amphetamine self-administration, consistent with the effects of dopamine antagonists (Bush et al., 1999; Epping-Jordan et al., 1998). $CCK_2$ antagonists have also been shown to decrease cocaine drinking in cocaine-preferring rats in a free-choice model (Crespi, 1998). $CCK_1$ ligands have not been thoroughly examined in drug self-administration paradigms, although there is a suggestion that $CCK_1$ antagonists may decrease alcohol consumption in alcohol-preferring rats, but have no effect on cocaine intake (Crespi, 1998).

CCK also plays an important role in psychostimulant sensitization, in which the locomotor response to the same dose of psychostimulant is increased after repeated drug administration. Endogenous CCK in the nucleus accumbens seems to play an important role in mediating psychostimulant-induced locomotor activity, mainly in animals that have previously experienced chronic psychostimulant exposure (Wunderlich et al., 2004). This is consistent with microdialysis studies, showing that systemic cocaine treatment of drug-naive and cocaine-sensitized rats caused a sustained increase in extracellular CCK levels in the nucleus accumbens that was more pronounced in sensitized rats. The increased basal levels of extracellular CCK in cocaine-sensitized rats suggest that the CCK system is upregulated by repeated cocaine injections (Beinfeld, 2003b). These studies provide a neurochemical basis for the role of endogenous CCK in the nucleus accumbens in the events following psychostimulant administration. $CCK_2$ receptors, but not $CCK_1$ receptors, have been shown to be involved in the development of sensitization (Wunderlich et al., 2000), which suggests an important role of $CCK_2$ receptors in the acute effects of psychostimulants. Conversely, $CCK_1$, but not $CCK_2$, antagonists attenuate the expression of amphetamine-induced sensitization, suggesting that $CCK_1$ receptors may interact with the long-term neurochemical consequences of psychostimulants (DeSousa et al., 1999; Wunderlich et al., 2000). The role of $CCK_1$ receptors in the expression of sensitization is consistent with the role of $CCK_1$ receptors in facilitating dopamine function in the caudal part of the nucleus accumbens.

It has been proposed that the craving and self-administration of opioid drugs could be explained either by a preexisting deficit in the endogenous opioid system or by a deficit that could occur after chronic administration of opiates. Thus, the use of a treatment increasing the level of endogenous opioid peptides could be an interesting new approach in the treatment of drug abuse. Indeed, it has been shown that mixed inhibitors such as RB 101 reduced the severity of the withdrawal syndrome in morphine-dependent rats after administration of naloxone (Maldonado et al., 1995). Moreover, as indicated above, several studies demonstrated that activation of $CCK_2$ receptors could modulate the opioid system negatively, suggesting that selective blockade of these receptors may increase the ability of mixed enkephalin-degrading enzyme inhibitors to reduce the opioid withdrawal syndrome precipitated by naloxone. This has been recently confirmed using RB 101 in association with the $CCK_2$ antagonist PD-134,308 (Maldonado et al., 1995).

Although early abstinence syndrome may be an important clinical problem in the treatment of addiction, the most difficult aspect is the protracted abstinence syndrome, one of the main factors contributing to relapse. Indeed, the first days after cessation of prolonged drug use leads to acute withdrawal syndrome, which consists of physiological changes (i.e., agitation, hyperalgesia, tachycardia, hypertension, diarrhea, and vomiting) and a variety of phenomena (i.e., cardiovascular, visceral, thermoregulatory, and subjective changes), or depressive states that may persist for months or more after the last dose of opiate. Thus, the main challenge in the management of opioid addiction is to develop a pharmacotherapy to minimize the short-term withdrawal syndrome and protracted opiate abstinence syndrome. The complete inhibitors of enkephalin-degrading enzymes could be administered alone or in combination with the selective $CCK_2$ antagonists to increase the endogenous opioid peptide levels, thus reducing the discomfort of the short-term withdrawal syndrome, as previously described (Maldonado et al., 1995). Moreover, the protracted abstinence syndrome also could be ameliorated owing to the antidepressant-like properties of

these compounds, and thus, the possibility of relapse, the most important problem in the management of opioid addiction, should be minimized (review in Roques and Noble, 1995).

### 5.7 Schizophrenia

Dysfunction of mesolimbic dopamine transmission is believed to be an important component underlying schizophrenia. As previously mentioned, it has been shown that the $CCK_1$ receptor modulates CCK-stimulated dopamine release in the posterior nucleus accumbens. Thus, it was speculated that alterations in $CCK_1$ receptor lead to an increase in dopamine release, which may in turn constitute a predisposition to schizophrenia. This was confirmed by an association analysis conducted between unrelated schizophrenic patients and healthy volunteers, which confirmed that the frequency of the 201A allele in the promoter region of the human $CCK_2$ receptor gene was higher in the schizophrenic group, especially in the paranoid type, than in the control group (Tachikawa et al., 2000). Interestingly, significant associations have also been reported between the polymorphic site of $CCK_1$ receptor gene located at 779 in the intron n1 and exon 2 boundary and schizophrenic patients with auditory hallucinations (Wei and Hemmings, 1999; Sanjuan et al., 2004) and between the promoter polymorphism located at −85 site and patients with hallucinations, particularly those with hallucinations accompanying delirium tremens (Okubo et al., 2002). The same type of study has been performed on the $CCK_2$ receptors. However, the results suggest that the $CCK_2$ receptor gene polymorphisms have no association with schizophrenia (Tachikawa et al. 1999).

The precise role of CCK in schizophrenia remains incompletely understood (review in Bourin et al., 1996; De Wied and Sigling, 2002). The most prominent finding relevant to this disorder is a reduction in postmortem CCK mRNA levels in different brain areas (frontal, cerebral and entorhinal cortices, and subiculum) of schizophrenic patients (Ferrier et al., 1983; Carruthers et al., 1984), especially those with predominantly negative symptoms. On the other hand, a lower density of CCK-receptor-binding sites has been found in the hippocampus and frontal cortex of schizophrenic patients compared with controls (Farmery et al., 1985). However, it should be noted that not all studies confirmed the decrease in CCK mRNA in schizophrenia. Indeed, in the postmortem study of Schalling et al. (1990), schizophrenic patients had even higher CCK mRNA levels in the VTA and substantia nigra than control subjects. Such a finding should suggest that elevated CCK synthesis in regions rich in dopaminergic neurons may be associated with schizophrenia. Methodological problems, small number of patients, and heterogeneity in studied individuals might have contributed to these inconsistent results. Nevertheless, on the whole, the available data suggest that negative symptoms of schizophrenia may be associated with reduced CCK activity (Ferrier et al., 1983; Carruthers et al., 1984). This reduction may be attributed to either a decreased processing of preproCCK in neurons or a reduction in synaptic levels of CCK due to activations in catabolic or reuptake processes (Migaud et al., 1995). Several open studies reported that administration of nonselective CCK receptor agonists in addition to ongoing neuroleptic treatment improved psychotic symptoms in schizophrenic patients, as CCK appears to be required for neuroleptic-induced depolarization inactivation of dopamine neurons and associated antipsychotic responses (Beinfeld and Garver, 1991). However, other placebo-controlled studies indicated that nonselective CCK receptor agonists or antagonists are ineffective in the treatment of schizophrenia (Whiteford et al., 1992).

## 6 Conclusions: Potential Therapeutic Applications

Since the original characterization of CCK by Ivy and Oldberg in 1928, followed by the isolation and sequencing of this hormone (Jorpes and Mutt, 1966), and its detection in the CNS (Vanderhaeghen et al., 1975), considerable advances have been made in the knowledge of the roles of this neuropeptide. The actions of CCK and related peptides have been extended to include regulation of satiety, anxiety, pain, and dopamine-mediated behavior in the central and peripheral nervous systems. All the results reported above encourage further research targeting $CCK_1$ and $CCK_2$ receptors as a possible way to new pharmacological treatments.

## References


Abramov U, Raud S, Koks S, Innos J, Kurrikoff K, et al. 2004. Targeted mutation of CCK(2) receptor gene antagonises behavioural changes induced by social isolation in female, but not in male mice. Behav Brain Res 155: 1-11.

Aquino CJ, Armour DR, Berman JM, Birkemo LS, Carr RAE, et al. 1996. Discovery of 1,5-benzodiazepines with peripheral cholecystokinin (CCK-A) receptor agonist activity. 1. Optimization of the agonist "trigger". J Med Chem 39: 562-569.

Baamonde A, Daugé V, Ruiz-Gayo M, Fulga IG, Turcaud S, et al. 1992. Antidepressant-type effects of endogenous enkephalins protected by systemic RB 101 are mediated by opioid δ and dopamine $D_1$ receptor stimulation. Eur J Pharmacol 216: 157-166.

Baber NS, Dourish CT, Hill DR. 1989. The role of CCK, caerulein, and CCK antagonists in nociception. Pain 39: 307-328.

Barrett RW, Steffey ME, Wolfram CAW. 1989. Type-A CCK receptors in CHP 212 neuroblastoma cells: evidence for association with G protein and activation of phosphoinositide hydrolysis. Mol Pharmacol 35: 394-400.

Becker C, Thiebot MH, Touitou Y, Hamon M, Cesselin F, et al. 2001. Enhanced cortical extracellular levels of cholecystokinin-like material in a model of anticipation of social defeat in the rat. J Neurosci 21: 262-269.

Beinfeld MC. 2003a. Biosynthesis and processing of pro CCK: recent progress and future challenges. Life Sci 72: 747-757.

Beinfeld MC. 2003b. What we know and what we need to know about the role of endogenous CCK in psychostimulant sensitization. Life Sci 73: 643-654.

Beinfeld MC, Garver DL. 1991. Concentration of cholecystokinin in cerebrospinal fluid is decreased in psychosis: relationship to symptoms and drug response. Prog Neuropsychopharmacol Biol Psychiatry 15: 601-609.

Bellier B, Crete D, Million ME, Beslot F, Bado A, et al. 2004. New CCK(2) agonists confirming the heterogeneity of CCK (2) receptors: characterisation of BBL454. Naunyn Schmiedebergs Arch Pharmacol 370: 404-413.

Ben Natan L, Chaillet P, Lecomte JM, Marçais H, Uchida G, et al. 1984. Involvement of endogenous enkephalins in the mouse "behavioral despair" test. Eur J Pharmacol 97: 301-304.

Benoliel JJ, Bourgoin S, Mauborgne A, Legrand JC, Hamon M, et al. 1991. Differential inhibitory/stimulatory modulation of spinal CCK release by mu and delta opioid agonists, and selective blockade of mu-dependent inhibition by kappa receptor stimulation. Neurosci Lett 124: 204-207.

Benoliel JJ, Mauborgne A, Bourgoin S, Legrand JC, Hamon M, et al. 1992. Opioid control of the in vitro release of CCK-like material from the rat substantia nigra. J Neurochem 58: 916-922.

Bernad N, Burgaud BG, Horwell DC, Lewthwaite RA, Martinez J, et al. 2000. The design and synthesis of the high efficacy, non-peptide CCK1 receptor agonist PD170292. Bioorg Med Chem Lett 10: 1245-1248.

Bi S, Moran TH. 2002. Actions of CCK in the controls of food intake and body weight: lessons from the CCK-A receptor deficient OLETF rat. Neuropeptides 36: 171-181.

Bignon E, Bachy A, Boigegrain R, Brodin R, Cottineau M, et al. 1999. SR146131: a new potent, orally active, and selective nonpeptide cholecystokinin subtype 1 receptor agonist. I. In vitro studies. J Pharmacol Exp Ther 289: 742-751.

Blommaert AG, Weng JH, Dorville A, McCort I, Ducos B, et al. 1993. Cholecystokinin peptidomimetics as selective CCK-B antagonists: design, synthesis, and in vitro and in vivo biochemical properties. J Med Chem 36: 2868-2877.

Blommaert AGS, Dhôtel H, Ducos B, Durieux C, Goudreau N, Bado A, Garbay C, Roques BP. 1997. Structure-based design of new constrained cyclic agonists of the cholecystokinin CCK-B receptor. J Med Chem 40: 647-658.

Bock MG, DiPardo RM, Evans BE, Rittle KE, Whitter WL, et al. 1989. Benzodiazepine gastrin and brain cholecystokinin receptor ligands: L-365,260. J Med Chem 32: 13-16.

Boden PR, Higginbottom M, Hill DR, Horwell DC, Hughes J, et al. 1993. Cholecystokinin dipeptoid antagonists: design, synthesis, and anxiolytic profile of some novel CCK-A and CCK-B selective and "mixed" CCK-A/CCK-B antagonists. J Med Chem 36: 552-565.

Bourin M, Malinge M, Vasar E, Bradwejn J. 1996. Two faces of cholecystokinin: anxiety and schizophrenia. Fundam Clin Pharmacol 10: 116-126.

Bradwejn J, de Montigny C. 1984. Benzodiazepines antagonize cholecystokinin-induced activation of rat hippocampal neurons. Nature 312: 363-364.

Bradwejn J, de Montigny C. 1985. Effects of PK 8165, a partial benzodiazepine receptor agonist, on cholecystokinin-induced activation of hippocampal pyramidal neurons: a microiontophoretic study in the rat. Eur J Pharmacol 112: 415-418.

Bradwejn J, Koszycki D. 2001. Cholecystokinin and panic disorder: past and future clinical research strategies. Scand J Clin Lab Invest Suppl 234: 19-27.

Bradwejn J, Koszycki D, Payeur R, Bourin M, Borthwick H. 1992. Replication of action of cholecystokinin tetrapeptide in panic disorder: clinical and behavioral findings. Am J Psychiatry 149: 962-964.

Bradwejn J, Koszycki D, Shriqui C. 1991. Enhanced sensitivity to cholecystokinin tetrapeptide in panic disorder. Arch Gen Psychiatry 48: 603-610.

Bush DE, DeSousa NJ, Vaccarino FJ. 1999. Self-administration of intravenous amphetamine: effect of nucleus accumbens CCKB receptor activation on fixed-ratio responding. Psychopharmacology (Berl) 147: 331-334.

Cain BM, Connolly K, Blum A, Vishnuvardham D, Marchand JE, et al. 2003. Distribution and colocalization of cholecystokinin with the prohormone convertase enzymes PC1, PC2, and PC5 in rat brain. J Comp Neurol 467: 307-325.

Cain BM, Connolly K, Blum AC, Vishnuvardhan D, Marchand JE, et al. 2004. Genetic inactivation of prohormone convertase (PC1) causes a reduction in cholecystokinin (CCK) levels in the hippocampus, amygdala, pons and medulla in mouse brain that correlates with the degree of colocalization of PC1 and CCK mRNA in these structures in rat brain. J Neurochem 89: 307-313.

Carruthers B, Dawbarn D, De Quidt M, Emson PC, Hunter J, et al. 1984. Changes in neuropeptide content of amygdala in schizophrenia. Br J Pharmacol 81(Suppl): 190P.

Chang RSL, Lotti VJ, Monaghan RL, Birnbaum J, Stapley EO, et al. 1985. A potent nonpeptide cholecystokinin antagonist selective for peripheral tissues isolated from *Aspergillus alliaceus*. Science 230: 177-179.

Charpentier B, Dor A, Roy P, England P, Pham H, et al. 1989. Synthesis and binding affinities of cyclic and related linear analogues of $CCK_8$ selective for central receptors. J Med Chem 31: 1184-1190.

Charpentier B, Durieux C, Pélaprat D, Dor A, Reibaud M, et al. 1988b. Enzyme-resistant CCK analogs with high affinities for central receptors. Peptides 9: 835-841.

Charpentier B, Pélaprat D, Durieux C, Dor A, Reibaud M, et al. 1988a. Cyclic cholecystokinin analogues with high selectivity for central receptors. Proc Natl Acad Sci USA 85: 1968-1972.

Charrier D, Dangoumau L, Puech AJ, Hamon M, Thiebot MH. 1995. Failure of CCK receptor ligands to modify anxiety-related behavioural suppression in an operant conflict paradigm in rats. Psychopharmacology (Berl) 121: 127-134.

Chen DY, Deutsch JA, Gonzalez MF, Gu Y. 1993. The induction and suppression of c-fos expression in the rat brain by cholecystokinin and its antagonist L-364,718. Neurosci Lett 149: 91-94.

Corringer PJ, Weng JH, Ducos B, Durieux C, Boudeau P, et al. 1993. CCK-B agonist or antagonist activities of structurally hindered and peptidase-resistant Boc-$CCK_4$ derivatives. J Med Chem 36: 166-172.

Corwin RL, Gibbs J, Smith GP. 1991. Increased food intake after type A but not type B cholecystokinin receptor blockade. Physiol Behav 50: 255-258.

Coudoré-Civiale MA, Courteix C, Fialip J, Boucher M, Eschalier A. 2000. Spinal effect of the cholecystokinin-B receptor antagonist CI-988 on hyperalgesia, allodynia and morphine-induced analgesia in diabetic and mononeuropathic rats. Pain 88: 15-22.

Coudoré-Civiale MA, Meen M, Fournié-Zaluski MC, Boucher M, Roques BP, et al. 2001. Enhancement of the effects of a complete inhibitor of enkephalin-catabolizing enzymes, RB 101, by a cholecystokinin-B receptor antagonist in diabetic rats. Br J Pharmacol 133: 179-185.

Crawley JN. 1985. Comparative distribution of cholecystokinin and other neuropeptides. Why is this peptide different from all other peptides? Ann NY Acad Sci 448: 1-8.

Crawley JN, Corwin RL. 1994. Biological actions of cholecystokinin. Peptides 15: 731-755.

Crespi F. 1998. The role of cholecystokinin (CCK), CCK-A or CCK-B receptor antagonists in the spontaneous preference for drugs of abuse (alcohol or cocaine) in naive rats. Methods Find Exp Clin Pharmacol 20: 679-697.

Dabrowski A, Grady T, Logsdon CD, Williams JA. 1996. Jun kinases are rapidly activated by cholecystokinin in rat pancreas both in vitro and in vivo. J Biol Chem 271: 5686-5690.

Daugé V, Roques BP. 1995. Opioid and CCK systems in anxiety and reward. Cholecystokinin and anxiety: from neuron to behavior. Bradwejn J, Vasar E, editors. Austin: RG Landes Company; pp. 151-171.

Daugé V, Sebret A, Beslot F, Matsui T, Roques BP. 2001. Behavioral profile of CCK2 receptor-deficient mice. Neuropsychopharmacology 25: 690-698.

Dawson GR, Rupniak NM, Iversen SD, Curnow R, Tye S, et al. 1995. Lack of effect of CCKB receptor antagonists in ethological and conditioned animal screens for anxiolytic drugs. Psychopharmacology (Berl) 121: 109-117.

Day HE, McKnight AT, Poat JA, Hughes J. 1994. Evidence that cholecystokinin induces immediate early gene expression in the brainstem, hypothalamus and amygdala of the rat by a CCKA receptor mechanism. Neuropharmacology 33: 719-727.

de Leeuw AS, den Boer JA, Slaap BR, Westenberg HG. 1996. Pentagastrin has panic-inducing properties in obsessive compulsive disorder. Psychopharmacology 126:339-344.

De Montigny C. 1989. Cholecystokinin tetrapeptide induces panic-like attacks in healthy volunteers. Arch Gen Psychiatry 46: 511-517.

De Weerth A, Pisegna JR, Huppi K, Wank SA. 1993. Molecular cloning, functional expression and chromosomal localization of the human cholecystokinin type A receptor. Biochem Biophys Res Commun 194: 811-818.

De Wied D, Sigling HO. 2002. Neuropeptides involved in the pathophysiology of schizophrenia and major depression. Neurotox Res 4: 453-468.

Derrien M, Daugé V, Blommaert A, Roques BP. 1994b. The selective CCK-B agonist, BC 264, impairs socially

reinforced memory in the three-panel runway test in rats. Behav Brain Res 65: 139-146.

Derrien M, Durieux M, Roques BP. 1994a. Antidepressant-like effects of CCK-B antagonists in mice: antagonism by naltrindole. Br J Pharmacol 111: 956-960.

Derrien M, Noble F, Maldonado R, Roques BP. 1993. Cholecystokinin-A but not cholecystokinin-B receptor stimulation induces endogenous opioid-dependent antinociceptive effects in the hot plate test in mice. Neurosci Lett 160: 193-196.

Deschenes RJ, Lorenz LJ, Haun RS, Roos BA, Collier KJ, et al. 1984. Cloning and sequence analysis of cDNA encoding rat preprocholecystokinin. Proc Natl Acad Sci USA 81: 726-730.

DeSousa NJ, Wunderlich GR, De Cabo C, Vaccarino FJ. 1999. The expression of behavioral sensitization to amphetamine: role of CCK(A) receptors. Pharmacol Biochem Behav 62: 31-37.

Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ. 1991. Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem 60: 653-680.

Dourish CT, O'Neill MF, Couglan J, Kitchener SJ, Hawley D, Iversen SD. 1990. The selective CCK-B antagonist L-365,260 enhances morphine analgesia and prevents morphine tolerance in rat. Eur J Pharmacol 175: 35-44.

Dunlop J, Zhang Y, Evans N. 1997. Full and partial agonist activity of C-terminal cholecystokinin peptides at the cloned human CCK-A receptor expressed in Chinese hamster ovary cells. Peptides 18: 865-868.

Durieux C, Coppey M, Zajac JM, Roques BP. 1986. Occurrence of two cholecystokinin binding sites in guinea pig brain cortex. Biochem Biophys Res Commun 137: 1167-1173.

Durieux C, Ruiz-Gayo M, Roques BP. 1991. In vivo binding affinities of cholecystokinin agonists and antagonists determined using the selective CCK-B agonist [$^3$H]pBC 264. Eur J Pharmacol 209: 185-193.

Emson PC, Lee CM, Rehfeld JF. 1980. Cholecystokinin octapeptide: vesicular localization and calcium dependent release from rat brain in vitro. Life Sci 26: 2157-2163.

Epping-Jordan MP, Markou A, Koob GF. 1998. The dopamine D-1 receptor antagonist SCH 23390 injected into the dorsolateral bed nucleus of the stria terminalis decreased cocaine reinforcement in the rat. Brain Res 784: 105-115.

Erel U, Arborelius L, Brodin E. 2004. Increased cholecystokinin release in the rat anterior cingulate cortex during carrageenan-induced arthritis. Brain Res 1022: 39-46.

Evans BE, Bock MG, Rittle KE, de Pardo RM, Whitter WL, et al. 1986. Design of potent, orally effective, nonpeptidal antagonists of the peptide hormone cholecystokinin. Proc Natl Acad Sci USA 83: 4922-4948.

Faris PL, Komisaruk BR, Watkins LR, Mayer DJ. 1983. Evidence for the neuropeptide cholecystokinin as an antagonist of opiate analgesia. Science 219: 310-312.

Faris PL, McLaughlin CL, Baile CA, Olney JW, Komisaruk BR. 1984. Morphine analgesia potentiated but tolerance not affected by active immunization against cholecystokinin. Science 226: 1215-1217.

Farmery SM, Owen F, Poulter M, Crow TJ. 1985. Reduced high affinity cholecystokinin binding in hippocampus and frontal cortex of schizophrenic patients. Life Sci 36: 473-477.

Ferrier IN, Roberts GW, Crow TJ, Johnstone EC, Owens DG, et al. 1983. Reduced cholecystokinin-like and somatostatin-like immunoreactivity in limbic lobe is associated with negative symptoms in schizophrenia. Life Sci 33: 475-482.

Fournié-Zaluski MC, Belleney J, Lux B, Durieux C, Gérard G, et al. 1986. Conformational analysis of neuronal cholecystokinin $CCK_{26–33}$ and related fragments by $^1$H NMR spectroscopy, fluorescence transfer measurements and calculations. Biochemistry 25: 3778-3787.

Fournié-Zaluski MC, Coric P, Turcaud S, Lucas E, Noble F, et al. 1992. Mixed-inhibitor-prodrug as a new approach towards systemically active inhibitors of enkephalin degrading enzymes. J Med Chem 35: 2474-2481.

Frankland PW, Josselyn SA, Bradwejn J, Vaccarino FJ, Yeomans JS. 1997. Activation of amygdala cholecystokininB receptors potentiates the acoustic startle response in the rat. J Neurosci 17: 1838-1847.

Fraser KA, Davison JS. 1993. Meal-induced c-fos expression in brain stem is not dependent on cholecystokinin release. Am J Physiol Regul Integr Comp Physiol 265: R235-R239.

Fuxe K, Andersson K, Locatelli V, Agnati LF, Hokfelt T, et al. 1980. Cholecystokinin peptides produce marked reduction of dopamine turnover in discrete areas in the rat brain following intraventricular injection. Eur J Pharmacol 67: 329-331.

Galas MC, Bernard N, Martinez J. 1992. Pharmacological studies on CCK-B receptors in guinea pig synaptoneurosomes. Eur J Pharmacol 226: 35-41.

Gall C, Lauterborn J, Burks D, Seroogy K. 1987. Co-localization of enkephalins and cholecystokinin in discrete areas of rat brain. Brain Res 403: 403-408.

Gaudreau P, St. Pierre S, Pert CB, Quirion R. 1985. Cholecystokinin receptors in mammalian brain: a comparative characterization and visualization. Ann NY Acad Sci 448: 198-219.

Geracioti TD, Nicholson WE, Orth DN, Ekhator NN, Loosen PT. 1993. Cholecystokinin in human cerebrospinal fluid: concentrations, dynamics, molecular forms and relationship to fasting and feeding in health, depression and alcoholism. Brain Res 629: 260-268.

Gerhardt P, Voits M, Fink H, Huston JP. 1994. Evidence for mnemotropic action of cholecystokinin fragments Boc-CCK-4 and CCK-8S. Peptides 15: 689-697.

Ghijsen WE, Leenders AG, Wiegant VM. 2001. Regulation of cholecystokinin release from central nerve terminals. Peptides 22: 1213-1221.

Gibert-Rahola J, Tejedor P, Chover AJ, Payana M, Rodriguez MM, et al. 1990. RB 38B, a selective endopeptidase inhibitor, induced several of escape deficits caused by inescapable shock pretreatment in rats. Eur J Pharmacol 183: 2317-2325.

Goltermann NR, Stengaard-Pedersen K, Rehfeld JF, Christensen NJ. 1981. Newly synthesized cholecystokinin in subcellular fractions of the rat brain. J Neurochem 36: 959-965.

Goudreau N, Weng JH, Roques BP. 1994. Conformational analysis of CCK-B agonists using $^{1}$H-NMR and restrained molecular dynamics: comparison of biologically active Boc-Trp-(NMe)Nle-Asp-Phe-$NH_2$ and inactive Boc-Trp-(NMe)Phe-Asp-Phe-$NH_2$. Biopolymers 34: 155-159.

Gully D, Fréhel D, Marcy C, Spinazze A, Lespy L, et al. 1993. Peripheral biological activity of SR 27897: a new potent non-peptide antagonist of CCK-A receptors. Eur J Pharmacol 232: 13-19.

Gulpinar MA, Yegen BC. 2004. The physiology of learning and memory: role of peptides and stress. Curr Protein Pept Sci 5: 457-473.

Gustafsson H, Stiller CO, Brodin E. 2000. Peripheral axotomy increases cholecystokinin release in the rat anterior cingulate cortex. Neuroreport 11: 3345-3348.

Hadjiivanova C, Belcheva S, Belcheva I. 2003. Cholecystokinin and learning and memory processes. Acta Physiol Pharmacol Bulg 27: 83-88.

Harhammer R, Schafer U, Henklein P, Ott T, Repke H. 1991. CCK-8-related C-terminal tetrapeptides: affinities for central CCKB and peripheral CCKA receptors. Eur J Pharmacol 209: 263-266.

Harro J, Marcusson J, Oreland L. 1992. Alterations in brain cholecystokinin receptors in suicide victims. Eur Neuropsychopharmacol 2: 57-63.

Harro J, Oreland L. 1993. Cholecystokinin receptors and memory: a radial maze study. Pharmacol Biochem Behav 44: 509-517.

Harro J, Vasar E, Bradwejn J. 1993. Cholecystokinin in animal and human research on anxiety. Trends Pharmacol Sci 14: 244-249.

Hattori E, Yamada K, Ebihara M, Toyota T, Nankai M, et al. 2002. Association study of the short tandem repeat in the 5′ upstream region of the cholecystokinin gene with mood disorders in the Japanese population. Am J Med Genet 114: 523-526.

Hendrie CA, Neill JC, Shepherd JK, Dourish CT. 1993. The effects of CCKA and CCKB antagonists on activity in the black/white exploration model of anxiety in mice. Physiol Behav 54: 689-693.

Hendry SH, Jones EG, DeFelipe J, Schmechel D, Brandon C, et al. 1984. Neuropeptide-containing neurons of the cerebral cortex are also GABAergic. Proc Natl Acad Sci USA 81: 6526-6530.

Henke BR, Aquino CJ, Birkemo LS, Croom DK, Dougherty RW, et al. 1997. Optimization of 3-(1*H*-indazol-3-ylmethyl)-1,5-benzodiazepines as potent, orally active CCK-A agonists. J Med Chem 40: 2706-2725.

Hernando F, Fuentes JA, Fournié-Zaluski MC, Roques BP, Ruiz-Gayo M. 1996. Antidepressant-like effects of CCK-B receptor antagonists: involvement of the opioid system. Eur J Pharmacol 318: 221-229.

Hernando F, Fuentes JA, Roques BP, Ruiz-Gayo M. 1994. The CCK-B receptor antagonist, L-365,260, elicits antidepressant-type effects in the forced-swim test in mice. Eur J Pharmacol 261: 257-263.

Herranz R. 2003. Cholecystokinin antagonists: pharmacological and therapeutic potential. Med Res Rev 23: 559-605.

Hokfelt T, Rehfeld JF, Skirboll L, Ivemark B, Goldstein M, et al. 1980. Evidence for coexistence of dopamine and CCK in meso-limbic neurones. Nature 285: 476-478.

Honda T, Wada E, Battey JF, Wank SA. 1993. Differential gene expression of $CCK_A$ and $CCK_B$ receptors in the rat brain. Mol Cell Neurosci 4: 143-154.

Horwell DC. 1991. Development of CCK-B antagonists. Neuropeptides 19 (Suppl): 57-64.

Hosing VG, Schirmacher A, Kuhlenbaumer G, Freitag C, Sand P, et al. 2004. Cholecystokinin-A and cholecystokinin-B-receptor gene polymorphisms in panic disorder. J Neural Transm Suppl 68: 147-156.

Howbert JJ, Lobb KL, Brown RF, Reel JK, Neel DA, et al. 1992. A novel series of non-peptide CCK and gastrin antagonists: medicinal chemistry and electrophysiological demonstration of antagonism. Multiple cholecystokinin receptors progress toward CNS therapeutic targets. Dourish CT, Cooper SJ, editors. London: Oxford University Press; pp. 28-37.

Hull RAD, Shankley NP, Harper EA, Gerskowitch VP, Black JW. 1993. 2-Naphthalenesulphonyl L-aspartyl-(2-phenethyl)amide (2-NAP)—a selective cholecystokinin CCK-A receptor antagonist. Br J Pharmacol 108: 734-740.

Innis RB, Snyder SH. 1980. Distinct cholecystokinin receptors in brain and pancreas. Proc Natl Acad Sci USA 77: 6917-6921.

Ise K, Akiyoshi J, Horinouchi Y, Tsutsumi T, Isogawa K, et al. 2003. Association between the CCK-A receptor gene and panic disorder. Am J Med Genet 118B: 29-31.

Ito H, Sogabe H, Nakarai T, Sato Y, Tomoi M, et al. 1994. Pharmacological profile of FK-480, a novel cholecystokinin

type-A receptor antagonist: comparison to loxiglumide. J Pharmacol Exp Ther 268: 571-575.

Ivy AC, Oldberg E. 1928. A hormone mechanism for gallbladder contraction and evacuation. Am J Physiol 86: 599-613.

Jagerschmidt A, Popovici T, O'Donohue M, Roques BP. 1994. Identification and characterization of various cholecystokinin B receptor mRNA forms in rat brain tissue and partial determination of the cholecystokinin B receptor gene structure. J Neurochem 63: 1199-1206.

Jensen RT, Huang SC, von Schrenck T, Wank SA, Gardner JD. 1990. Cholecystokinin receptor antagonists: ability to distinguish various classes of cholecystokinin receptors. Gastrointestinal endocrinology: receptors and post-receptor mechanisms. Thompson JT, Townsend CM, Greely GA, Rayford PL, Wooper CW, et al. editors. New York: Academic; pp. 95-113.

Jensen RT, Qian JM, Lin JT, Mantey SA, Pisegna JR, et al. 1994. Distinguishing multiple CCK receptor subtypes: studies with guinea pig chief cells and transfected human CCK receptors. Ann NY Acad Sci 713: 88-106.

Jorpes JE, Mutt V. 1966. Cholecystokinin and pancreozymin, one single hormone? Acta Physiol Scand 66: 196.

Kariya K, Tanaka J, Nomura M. 1994. Systemic administration of CCK-8S, but not CCK-4, enhances dopamine turnover in the posterior nucleus accumbens: a microdialysis study in freely moving rats. Brain Res 657: 1-6.

Kennedy JL, Bradwejn J, Koszycki D, King N, Crowe R, et al. 1999. Investigation of cholecystokinin system genes in panic disorder. Mol Psychiatry 4: 284-285.

Khaznadar T, David C, Crete D, Fournié-Zaluski MC, Roques BP, et al. 1997. Aminopeptidase A involved in the inactivation of endogenous CCK8: a microdialysis study. J Neuropsychopharmacol 11: A50.

Knapp RJ, Vaughn LK, Fang SN, Bogert CL, Yamamura MS, et al. 1990. A new, highly selective CCK-B receptor radioligand ([$^3$H][N-methyl-Nle$^{28,31}$]$CCK_{26–33}$): evidence for CCK-B receptor heterogeneity. J Pharmacol Exp Ther 255: 1278-1286.

Kobayashi S, Ohta M, Miyasaka K, Funakoshi A. 1996. Decrease in exploratory behavior in naturally occurring cholecystokinin (CCK)-A receptor gene knockout rats. Neurosci Lett 214: 61-64.

Koks S, Abramov U, Veraksits A, Bourin M, Matsui T, et al. 2003. CCK2 receptor-deficient mice have increased sensitivity of dopamine D2 receptors. Neuropeptides 37: 25-29.

Koks S, Volke V, Veraksits A, Runkorg K, Sillat T, et al. 2001. Cholecystokinin2 receptor-deficient mice display altered function of brain dopaminergic system. Psychopharmacology 158: 198-204.

Kolodziej SA, Nikiforovich GV, Skeean R, Lignon MF, Martinez J, et al. 1995. Ac-[3- and 4-alkylthioproline$^{31}$]-$CCK_4$ analogs: synthesis, and implication for the CCK-B receptor-bound conformation. J Med Chem 38: 137-149.

Kopin AS, Mathes WF, McBride EW, Nguyen M, Al-Haider W, et al. 1999. The cholecystokinin-A receptor mediates inhibition of food intake yet is not essential for the maintenance of body weight. J Clin Invest 103: 383-391.

Lanaud P, Popovici T, Normand E, Lemoine C, Bloch B, et al. 1989. Distribution of CCK mRNA in particular regions (hippocampus, periaqueductal grey and thalamus) of the rat by in situ hybridization. Neurosci Lett 104: 38-42.

Le Melledo JM, Bradwejn J, Koszycki D, Bichet D. 1995. Premenstrual dysphoric disorder and response to cholecystokinin-tetrapeptide. Arch Gen Psychiatry 52: 605-606.

Lemaire M, Piot O, Roques BP, Böhme AG, Blanchard JC. 1992. Evidence for an endogenous cholecystokininergic balance in social memory. Neuroreport 3: 925-932.

Léna I, Simon H, Roques BP, Daugé V. 1999. Opposing effects of two selective CCK-B agonists, on the retrieval phase of a two-trial memory task after systemic injection in the rat. Neuropharmacology 38: 543-553.

Lo WWY, Hughes J. 1988. Differential regulation of cholecystokinin- and muscarinic-receptor-mediated phosphoinositide turnover in flow 900 cells. Biochem J 251: 625-630.

Löfberg C, Agren H, Harro J, Oreland L. 1998. Cholecystokinin in CSF from depressed patients: possible relations to severity of depression and suicidal behaviour. Eur Neuropsychopharmacol 8: 153-157.

Magnuson DSK, Sullivan AF, Simonnet G, Roques BP, Dickenson AH. 1990. Differential interactions of cholecystokinin and FLFQPQRF-$NH_2$ with μ and δ opioid antinociception in the rat spinal cord. Neuropeptides 16: 213-218.

Makovec F, Chiste R, Bani M, Pacini MA, Setnikar I, et al. 1995. New glutaramic acid derivatives with potent competitive specific cholecystokinin-antagonistic activity. Arzneimittelforschung 35: 1048-1051.

Maldonado R, Valverde O, Ducos B, Blommaert AG, Fournié-Zaluski MC, et al. 1995. Inhibition of morphine withdrawal by the association of RB 101, an inhibitor of enkephalin catabolism, and the CCK-B antagonist PD-134,308. Br J Pharmacol 114: 1031-1039.

Marshall FH, Barnes S, Hughes J, Woodruff GN, Hunter JC. 1991. Cholecystokinin modulates the release of dopamine from the anterior and posterior nucleus accumbens by two different mechanisms. J Neurochem 56: 917-922.

Martin-Martinez M, Bartolomé-Nebreda JM, Gomez-Monterrey I, Gonzalez-Muniz R, Garcia-Lopez MT, et al. 1997. Synthesis and stereochemical structure–activity relationships of 1,3-dioxoperhydropyrido[1,2-*c*]pyrimidine derivatives: potent and selective cholecystokinin-A receptor antagonists. J Med Chem 40: 3402-3407.

Matsushita H, Akiyoshi J, Kai K, Ishii N, Kodama K, et al. 2003. Spatial memory impairment in OLETF rats without cholecystokinin—a receptor. Neuropeptides 37: 271-276.

Menozzi D, Gardner JD, Maton PN. 1989. Properties of receptors for gastrin and CCK on gastric smooth muscle cells. Am J Physiol 257: G73-G79.

Mercer LD, Beart PM. 1997. Histochemistry in rat brain and spinal cord with an antibody directed at the cholecystokinin$_A$ receptor. Neurosci Lett 225: 97-100.

Migaud M, Durieux C, Viereck J, Soroca-Lucas E, Fournié-Zaluski MC, et al. 1996. The in vivo metabolism of cholecystokinin (CCK-8) is essentially ensured by aminopeptidase A. Peptides 17: 601-607.

Migaud M, Roques BP, Durieux C. 1995. Evidence for a high affinity uptake system for cholecystokinin octapeptide (CCK8) in rat cortical synaptosomes. Eur J Neurosci 7: 1074-1079.

Millan MJ. 2003. The neurobiology and control of anxious states. Prog Neurobiol 70: 83-244.

Million ME, Léna I, Da Nascimento S, Noble F, Daugé V, et al. 1997. Development of new potent agonists able to interact with two postulated subsites of the cholecystokinin CCK-B receptor. Lett Pept Sci 4: 407-410.

Miyasaka K, Kobayashi S, Ohta M, Kanai S, Yoshida Y, et al. 2002. Anxiety-related behaviors in cholecystokinin-A, -B, and -AB receptor gene knockout mice in the plus-maze. Neurosci Lett 335: 115-118.

Miyasaka K, Yoshida Y, Matsushita S, Higuchi S, Shirakawa O, et al. 2004. Association of cholecystokinin-A receptor gene polymorphisms and panic disorder in Japanese. Am J Med Genet 127B: 78-80.

Moran TH. 2004. Gut peptides in the control of food intake: 30 years of ideas. Physiol Behav 82: 175-180.

Moran TH, Robinson PH, Goldrich MS, McHugh PR. 1986. Two brain cholecystokinin receptors: implications for behavioral actions. Brain Res 362: 175-179.

Moran TH, Schwartz GJ. 1994. Neurobiology of cholecystokinin. Crit Rev Neurobiol 9: 1-28.

Morino P, Herrera-Marschitz M, Castel MN, Ungerstedt U, Varro A, et al. 1994. Cholecystokinin in cortico-striatal neurons in the rat: immunohistochemical studies at the light and electron microscopical level. Eur J Neurosci 6: 681-692.

Mutt V, Jorpes JE. 1968. Structure of porcine cholecystokinin–pancreozymin. Eur J Biochem 6: 156-162.

Nadzan AM, Garvey DS, Holladay MW, Shiosaki K, Tufano MD, et al. 1991. Design of cholecystokinin analogs with high affinity and selectivity for brain receptors. Peptides, chemistry and biology, Proceedings of the 12th American Peptide Symposium. Smith JA, Rivier JE, editors. Leiden, The Netherlands: ESCOM; pp. 101-102.

Niehoff DL. 1989. Quantitative autoradiographic localization of cholecystokinin receptors in rat and guinea pig brain using $^{125}$I-Bolton-Hunter-CCK$_8$. Peptides 10: 265-274.

Nishida A, Miyata K, Tsutsumi R, Yuki H, Akuzawa S, et al. 1994. Pharmacological profile of (*R*)-1-[2,3-dihydro-1-(2′-methyl-phenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea (YM022), a new potent and selective gastrin/cholecystokinin-B receptor antagonist, in vitro and in vivo. J Pharmacol Exp Ther 269: 725-731.

Noble F, Derrien M, Roques BP. 1993. Modulation of opioid analgesia by CCK at the supraspinal level: evidence of regulatory mechanisms between CCK and enkephalin systems in the control of pain. Br J Pharmacol 109: 1064-1070.

Noble F, Wank S, Crawley J, Bradwejn J, Seroogy K, et al. 1999. International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol Rev 51: 745-781.

O'Dowd B, Hnatowich M, Caron MG, Lefkowitz RJ, Bouvier M. 1988. Site-directed mutagenesis of the cytoplasmatic domains of the human β-2 adrenergic receptor. Localization of regions involved in G-protein-receptor coupling. J Biol Chem 263: 15985-15992.

Okubo T, Harada S, Higuchi S, Matsushita S. 2002. Investigation of quantitative trait loci in the CCKAR gene with susceptibility to alcoholism. Alcohol Clin Exp Res 26: 2S-5S.

Ovchinikov YA, Ablulajew NG, Bogachuck AS. 1988. Two adjacent cysteine residues in the C-terminal cytoplasmatic fragment of bovine rhodopsin are palmitoylated. FEBS Lett 230: 1-5.

Ozcelebi F, Miller LJ. 1995. Phosphopeptide mapping of cholecystokinin receptors on agonist-stimulated native pancreatic acinar cells. J Biol Chem 270: 3435-3441.

Patel S, Smith AJ, Chapman KL, Fletcher AE, Kemp JA, et al. 1994. Biological properties of the benzodiazepine amide derivative L-740,093, a cholecystokinin-B/gastrin receptor antagonist with high affinity in vitro and high potency in vivo. Mol Pharmacol 46: 943-948.

Pendley CE, Fitzpatrick LR, Capolino AJ, Davis MA, Esterline NJ, et al. 1995. RP 73870, a gastrin/cholecystokinin-B receptor antagonist with potent anti-ulcer activity in the rat. J Pharmacol Exp Ther 273: 1015-1022.

Pélaprat D, Broer Y, Studler JM, Peschanski M, Tassin JP, et al. 1987. Autoradiography of CCK receptors in the rat brain using [$^3$H]Boc[Nle$_{28,31}$]CCK$_{27–33}$ and [$^{125}$I]Bolton-Hunter CCK$_8$. Neurochem Int 10: 495-508.

Pohl M, Benoliel JJ, Bourgoin S, Lombard MC, Mauborgne A, et al. 1990. Regional distribution of calcitonin gene-related peptide-, substance P-, cholecystokinin-, Met$^5$-enkephalin-, and dynorphin A (1-8)-like materials in the spinal cord and dorsal root ganglia of adult rats: effects of dorsal rhizotomy and neonatal capsaicin. J Neurochem 55: 1122-1130.

Pommier B, Da Nascimento S, Dumont S, Bellier B, Million E, Garbay C, Roques BP, Noble F. 1999. The CCK-B receptor is coupled to two effector pathways through pertussis toxin sensitive and insensitive G proteins. J Neurochem 73: 281-288.

Pommier B, Marie-Claire C, Da Nascimento S, Wang HL, Roques BP, et al. 2003. Further evidence that the CCK2 receptor is coupled to two transduction pathways using site-directed mutagenesis. J Neurochem 85: 454-461.

Pu SF, Zhuang HX, Lu ZB, Wu XR, Han JS. 1994. Cholecystokinin gene expression in rat amygdaloid neurons. Normal distribution and effect of morphine tolerance. Mol Brain Res 21: 183-189.

Radu D, Ahlin A, Svanborg P, Lindefors N. 2003. Pentagastrin test for anxiety—psychophysiology and personality. Psychopharmacology (Berl) 166: 139-145.

Rattray M, De Belleroche J. 1987. Morphine action on cholecystokinin octapeptide release from rat periaqueductal grey slices: sensitization by naloxone. Neuropeptides 10: 189-200.

Rehfeld JF, Bungaard JR, Friis-Hansen L, Goetze JP. 2003. On the tissue-specific processing of procholecystokinin in the brain and gut—a short review. J Physiol Pharmacol 54 (Suppl. 4): 73-79.

Rehfeld JF, Holst JJ, Jensen SL. 1982. The molecular nature of vascularly released cholecystokinin from the isolated perfused porcine duodenum. Regul Pept 3: 15-28.

Rehfeld JF, Lindberg I, Friis-Hansen L. 2002. Increased synthesis but decreased processing of neuronal proCCK in prohormone convertase 2 and 7B2 knockout animals. J Neurochem 83: 1329-1337.

Rehfeld JH, Nielsen FC. 1995. Molecular forms and regional distribution of cholecystokinin in the central nervous system. Cholecystokinin and anxiety. Bradwejn J, Vasar E, editors. Austin: RG Landes Company; pp. 33-56.

Reidelberger RD, Varga G, Solomon TE. 1991. Effects of selective cholecystokinin antagonists L364,718 and L365,260 on food intake in rats. Peptides 12: 1215-1221.

Revel L, Ferrari F, Makovec F, Rovati LC, Impicciatore M. 1992. Characterization of antigastrin activity in vivo of CR 2194, a new R-4-benzamido-5-oxo-pentanoic acid derivative. Eur J Pharmacol 216: 217-224.

Rodriguez RE, Sacristan MP. 1989. In vivo release of CCK-8 from the dorsal horn of the rat: inhibition by DAGOL. FEBS Lett 250: 215-217.

Roques BP. 2000. Novel approaches to targeting neuropeptide systems. Trends Pharmacol Sci 21: 475-483.

Roques BP, Noble F. 1995. NIDA Research Monograph, Dual inhibitors of enkephalin-degrading enzymes (neutral endopeptidase 24.11 and aminopeptidase N) as potential new medications in the management of pain and opioid addiction. Discovery of novel opioid medications, Rapaka RS, Sorer H, editors. pp. 104–145.

Roques BP, Noble F. 1996. Association of enkephalin catabolism inhibitors and CCK-B antagonists: a potential use in the management of pain and opioid addiction. Neurochem Res 21: 1395-1409.

Roques BP, Noble F, Daugé V, Fournié-Zaluski MC, Beaumont A. 1993. Neutral endopeptidase 24.11: structure, inhibition, and experimental and clinical pharmacology. Pharmacol Rev 45: 87-146.

Rose C, Vargas F, Facchinetti P, Bourgeat P, Bambal RB, et al. 1996. Characterization and inhibition of a cholecystokinin-inactivating serine peptidase. Nature (Lond) 380: 403-409.

Ruiz-Gayo M, Daugé V, Menant I, Bégué D, Gacel G, et al. 1985. Synthesis and biological activity of Boc(Nle28, Nle31)CCK27-33 a highly potent CCK8 analogue. Peptides 6: 415-420.

Ruiz-Gayo M, Durieux C, Fournié-Zaluski MC, Roques BP. 1992. Stimulation of δ opioid receptors reduces the in vivo binding of the CCK-B selective agonist [$^3$H] pBC264: evidence for a physiological regulation of CCKergic systems by endogenous enkephalins. J Neurochem 59: 1805-1811.

Saito AH, Sankaran H, Goldfine ID, Williams JA. 1980. Cholecystokinin receptors in the brain: characterization and distribution. Science 208: 1155-1156.

Sanjuan J, Toirac I, Gonzalez JC, Leal C, Molto MD, et al. 2004. A possible association between the CCK-AR gene and persistent auditory hallucinations in schizophrenia. Eur Psychiatry 19: 349-353.

Sankaran H, Goldfine ID, Deveney CW, Wong KY, Williams JA. 1980. Binding of cholecystokinin to high affinity receptors on isolated rat pancreatic acini. J Biol Chem 255: 1849-1853.

Schalling M, Friberg K, Seroogy K, Riederer P, Bird E, et al. 1990. Analysis of expression of cholecystokinin in dopamine cells in the ventral mesencephalon of several species and in humans with schizophrenia. Proc Natl Acad Sci USA 87: 8427-8431.

Sebret A, Léna I, Crété D, Matsui T, Roques BP, et al. 1999. Rat hippocampal neurons are critically involved in physiological improvement of memory processes induced by cholecystokinin-B receptor stimulation. J Neurosci 19: 7230-7237.

Seidah NG, Chretien M. 1999. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res 848: 45-62.

Sherrill RG, Berman JM, Birkemo L, Croom DK, Dezube M, et al. 2001. 1,4-Benzodiazepine peripheral cholecystokinin (CCK-A) receptor agonists. Bioorg Med Chem Lett 11: 1145-1148.

Shiosaki K, Graig R, Lin CW, Barrett R, Miller T, et al. 1990. Toward development of peptidomimetics: diketopiperazine templates for the Trp-Met segment of $CCK_4$. Peptides:

chemistry, structure and biology. Proceedings of the 11th American Peptide Symposium. Rivier JE, Marshall GR, editors. Leiden: ESCOM; pp. 978-980.

Shlik J, Aluoja A, Vasar V, Vasar E, Podar T, Bradwejn J. 1997. Effects of citalopram treatment of behavioural, cardiovascular, and neuroendocrine response to cholecystokinin tetrapeptide challenge in patients with panic disorder. J Psychiatry Neurosci 22: 332-340.

Shlik J, Koszycki D, Bradwejn J. 1998. Decrease in short-term memory function induced by CCK-4 in healthy volunteers. Peptides 19: 969-975.

Silvente-Poirot S, Escrieut C, Wank SA. 1998. Role of the extracellular domains of the cholecystokinin receptor in agonist binding. Mol Pharmacol 54: 364-371.

Simmons RD, Blosser JC, Rosamond JR. 1994. FPL 14294: a novel CCK-8 agonist with potent intranasal anorectic activity in the rat. Pharmacol Biochem Behav 47: 701-708.

Singh L, Field MJ, Hughes J, Menzies R, Oles RJ, et al. 1991. The behavioural properties of CI-988, a selective cholecystokininB receptor antagonist. Br J Pharmacol 104: 239-245.

Singh P, Owlia A, Espeijo R, Dai B. 1995. Novel gastrin receptors mediate mitogenic effects of gastrin processing intermediates of gastrin on Swiss 3T3 fibroblasts. J Biol Chem 270: 8429-8438.

Smadja C, Maldonado R, Turcaud S, Fournié-Zaluski MC, Roques BP. 1995. Opposite role of CCK-A and CCK-B receptors in the modulation of endogenous enkephalins antidepressant-like effects. Psychopharmacology 128: 400-408.

Smadja C, Ruiz F, Coric P, Fournie-Zaluski MC, Roques BP, et al. 1997. CCK-B receptors in the limbic system modulate the antidepressant-like effects induced by endogenous enkephalins. Psychopharmacology (Berl) 132: 227-236.

Smith GP, Gibbs J. 1992. The development and proof of the CCK hypothesis of satiety. Multiple cholecystokinin receptors in the CNS. Dourish CT, Cooper SJ, Iversen SD, Iversen LL, editors. Oxford: Oxford University Press; pp. 166-182.

Tachikawa H, Harada S, Kawanishi Y, Okubo T, Shiraishi H. 1999. Novel polymorphism in the promoter and coding regions of the human cholecystokinin B receptor gene: an association analysis with schizophrenia. Am J Med Genet 88: 700-704.

Tachikawa H, Harada S, Kawanishi Y, Okubo T, Shiraishi H. 2000. Novel polymorphisms of the human cholecystokinin A receptor gene: an association analysis with schizophrenia. Am J Med Genet 96: 141-145.

Taghzouti K, Léna I, Dellu F, Roques BP, Daugé V, et al. 1999. Cognitive enhancing effects in young and old rats of pBC 264, a selective CCK-B receptor agonist. Psychopharmacology 143: 141-149.

Takinami Y, Yuki H, Nishida A, Akuzawa S, Uchida A, et al. 1997. YF476 is a new potent and selective gastrin/cholecystokinin-B receptor antagonist in vitro and in vivo. Aliment Pharmacol Ther 11: 113-120.

Talkad VD, Fortune KP, Pollo DA, Shah GN, Wank SA, et al. 1994. Direct demonstration of three different states of the pancreatic cholecystokinin receptor. Proc Natl Acad Sci USA 91: 1868-1872.

Taniguchi T, Matsui T, Ito M, Murayama T, Tsukamoto T, et al. 1994. Cholecystokinin-B/gastrin receptor signaling pathway involves tyrosine phosphorylations of $p125^{FAK}$ and $p42^{MAP}$. Oncogene 9: 861-867.

Tejedor-Real R, Mico JA, Maldonado R, Roques BP, Gibert-Rahola J. 1993. Effect of a mixed (RB 38A) and selective (RB 38B) inhibitors of enkephalin-degrading enzymes on a model of depression in the rat. Biol Psychiatry 34: 100-107.

Tejedor-Real R, Mico TP, Maldonado R, Roques BP, Gibert-Rahola J. 1995. Implication of endogenous opioid system in the learned helplessness model of depression. Pharmacol Biochem Behav 52: 145-152.

Tortorici V, Nogueira L, Salas R, Vanegas H. 2003. Involvement of local cholecystokinin in the tolerance induced by morphine microinjections into the periaqueductal gray of rats. Pain 102: 9-16.

Trivedi BK, Padia JK, Holmes A, Rose S, Wright DS, et al. 1998. Second generation "peptoid" CCK-B receptor antagonists: identification and development of *N*-(adamantyloxycarbonyl)-a-methyl-(*R*)-tryptophan derivative (CI-1015) with an improved pharmacokinetic profile. J Med Chem 41: 38-45.

Valverde O, Blommaert AGS, Fournié-Zaluski MC, Roques BP, Maldonado R. 1995. Weak tolerance to the antinociceptive effect induced by the association of a peptidase inhibitor and a CCK-B antagonist. Eur J Pharmacol 286: 79-93.

Valverde O, Maldonado R, Fournié-Zaluski MC, Roques BP. 1994. Cholecystokinin B antagonists strongly potentiate antinociception mediated by endogenous enkephalins. J Pharmacol Exp Ther 270: 77-88.

van der Kooy D, Hunt SP, Steinbusch HW, Verhofstad AA. 1981. Separate populations of cholecystokinin and 5-hydroxytryptamine-containing neuronal cells in the rat dorsal raphe, and their contribution to the ascending raphe projections. Neurosci Lett 26: 25-30.

van Vliet IM, Westenberg HG, Slaap BR, den Boer JA, Ho Pian KL. 1997. Anxiogenic effects of pentagastrin in patients with social phobia and healthy controls. Biol Psychiatry 42: 76-78.

Vanderhaeghen JJ, Signeau JC, Gepts W. 1975. New peptide in the vertebrate CNS reacting with antigastrin antibodies. Nature (Lond) 257: 604-605.

Vanderschuren LJ, Kalivas PW. 2000. Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. Psychopharmacology (Berl) 151: 99-120.

Verhage M, McMahon HT, Ghijsen WE, Boomsma F, Scholten G, Wiegant VM, Nicholls DG. 1991. Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. Neuron 6: 517-524.

Voigt MM, Wang RY, Westfall TC. 1985. The effects of cholecystokinin on the in vivo release of newly synthesized [3H] dopamine from the nucleus accumbens of the rat. J Neurosci 5: 2744-2749.

Wank SA. 1995. Cholecystokinin receptors. Am J Physiol 269: G628-G646.

Wank SA, Harkins RT, Jensen RT, Shapira H, de Weerth A, et al. 1992. Purification, molecular cloning and functional expression of the cholecystokinin receptor from rat pancreas. Proc Natl Acad Sci USA 89: 3125-3129.

Watkins LR, Kinscheck IB, Mayer DJ. 1984. Potentiation of opiate analgesia and apparent reversal of morphine tolerance by proglumide. Science 224: 395-396.

Wei J, Hemmings GP. 1999. The CCK-A receptor gene possibly associated with auditory hallucinations in schizophrenia. Eur Psychiatry 14: 67-70.

Wettstein JG, Bueno L, Junien JL. 1994. CCK antagonists: pharmacology and therapeutic interest. Pharmacol Ther 62: 267-282.

Whiteford HA, Stedman TJ, Welham J, Csernansky JG, Pond SM. 1992. Placebo-controlled, double-blind study of the effects of proglumide in the treatment of schizophrenia. J Clin Psychopharmacol 12: 337-340.

Wiesenfeld-Hallin Z, Lucas GA, Alster P, Xu XJ, Hökfelt T. 1999. Cholecystokinin/opioid interactions. Brain Res 848: 78-89.

Willner P. 1990. Animal models of depression: an overview. Pharmacol Ther 45: 425-455.

Wolkowitz OM, Gertz B, Weingartner H, Beccaria L, Thompson K, et al. 1990. Hunger in humans induced by MK-329, a specific peripheral-type cholecystokinin receptor antagonist. Biol Psychiatry 28: 169-173.

Wunderlich GR, DeSousa NJ, Vaccarino FJ. 2000. Cholecystokinin modulates both the development and the expression of behavioral sensitization to amphetamine in the rat. Psychopharmacology (Berl) 151: 283-290.

Wunderlich GR, Raymond R, De Sousa NJ, Nobrega JN, Vaccarino FJ. 2002. Decreased CCK(B) receptor binding in rat amygdala in animals demonstrating greater anxiety-like behavior. Psychopharmacology (Berl) 164: 193-199.

Wunderlich GR, Rotzinger S, Bush DE, DeSousa NJ, Vaccarino FJ. 2004. Cholecystokinin modulation of locomotor behavior in rats is sensitized by chronic amphetamine and chronic restraint stress exposure. Brain Res 1001: 95-107.

Yule DI, Tseng MJ, Williams JA, Logsdon CD. 1993. A cloned CCK-A receptor transduces multiple signals in response to full and partial agonists. Am J Physiol 265: G999-G1004.

Zarbin MA, Innis RB, Wamsley JK, Snyder SH, Kuhar MJ. 1983. Autoradiographic localization of cholecystokinin receptors in rodent brain. J Neurosci 3: 877-906.

Zhang LJ, Lu XY, Han JS. 1992. Influences of cholecystokinin octapeptide on phosphoinositide turnover in neonatal-rat brain cells. Biochem J 285: 847-850.

Zhou Y, Sun YH, Zhang ZW, Han JS. 1992. Accelerated expression of cholecystokinin gene in the brain of rats rendered tolerant to morphine. Neuroreport 3: 1121-1123.

Zhu PC, Thureson-Klein A, Klein RL. 1986. Exocytosis from large dense cored vesicles outside the active synaptic zones of terminals within the trigeminal subnucleus caudalis: a possible mechanism for neuropeptide release. Neuroscience 19: 43-54.

Zittel TT, Glatzle J, Kreis M, Starlinger M, Eichner M, et al. 1999. C-Fos protein expression in the solitary tract correlates with cholecystokinin dose injected and food intake. Brain Res 848: 1-11.

# 25 Oxytocin and Vasopressin: Genetics and Behavioral Implications

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**Abstract:** Oxytocin (OT) and vasopressin (VP) can profoundly affect animal physiology and behavior. Over the past 20 years, the genes that encode OT and VP, as well as their respective receptors, have been identified and intensively studied leading to a greater understanding of the hormones' functions. The use of transgenic animals, including knockout mice, and viral vectors have opened new vistas of research on the behavioral roles of OT and VP. In this chapter, we briefly review the history and the evolutionary origins of OT and VP, as well as their structures, regulation, and neuroanatomy. Finally, we highlight recently explored roles for OT and VP in physiology and behavior.

**List of Abbreviations:** ACTH, adrenocorticotropic hormone; AH, anterior hypothalamus; AP-2, activator protein-2; ATF-2, activating transcription factor-2; AVT, arginine vasotocin; BNST, bed nucleus of the stria terminalis; CeM, central amygdala; CNS, central nervous system; CRF, corticotropin releasing factor; DAG, diacylglycerol; ERE, estrogen response element; GRE, glucocorticoid response element; ICV, intracerebroventricularly; IGR, intergenic region; $IP_3$, 1,4,5 inositol triphosphate; LS, lateral septum; MeA, medial amygdala; MPOA, medial preoptic area; MPOA-AH, medial preoptic area-anterior hypothalamus; OT, oxytocin; OTKO, oxytocin knockout; OTR, oxytocin receptor; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; V1aR, vasopressin 1a receptor; V1aRKO, vasopressin 1a receptor knockout; V1bR, vasopressin 1b receptor; V1bRKO, vasopressin 1b receptor knockout; VACM-1, vasopressin-activated calcium-mobilizing receptor; VLH, ventrolateral hypothalamus; VMH, ventromedial hypothalamus; VP, vasopressin; VP-ir, vasopressin immunoreactivity

## 1 Overview

This is an exciting time in the field of oxytocin (OT) and vasopressin (VP) research. The important roles for OT and VP in the brain and in behavior are just now becoming understood. The use of modern molecular biological techniques as well as behavioral studies have implicated OT and VP in the regulation of a variety of behaviors. The use of viral vectors and transgenic animals, including knockout mice, has provided valuable insights into the complex roles these two hormones play in the regulation of behavior. This chapter will briefly review the history and the evolutionary origins of OT and VP, as well as their receptors, structures, regulation, and neuroanatomy. Finally, the chapter highlights recently explored roles for OT and VP in physiology and behavior.

## 2 History

The neurohypophysial hormones OT and VP were originally detected by Oliver and Schäfer in 1895 who demonstrated that extracts of the pituitary altered blood pressure (Oliver and Schäfer, 1895). In the decades that followed, other actions of posterior pituitary extracts were determined: in 1906, the uterine-contracting properties (Dale, 1906); in 1910, the milk-ejection properties (Ott and Scott, 1910); and in 1913, the antidiuretic properties (Farini, 1913; Vongraven, 1913). However, it was not until 1952 that du Vigneaud and colleagues isolated two distinct peptides to which specific activities could be ascribed (du Vigneaud, 1952). Following this finding, the amino acid sequences and structures of OT (Tuppy, 1953; du Vigneaud et al., 1953b) and VP (Turner et al., 1951; Archer and du Vigneaud, 1953; du Vigneaud et al., 1953a) were elucidated, followed shortly by their syntheses (du Vigneaud et al., 1954a, b). In 1955, du Vigneaud won the Nobel Prize in Chemistry due, in part, to his early descriptions and syntheses of OT and VP.

Since the 1950s, research examining the roles of OT and VP in the brain and periphery has intensified. The development of specific agonists and antagonists for OT and VP receptors has allowed for a better elucidation of the specific contributions to physiology and behavior that each peptide makes (Manning and Sawyer, 1991; Barberis et al., 1999; Serradeil-Le Gal et al., 2002). Pharmacological studies, as well as transgenic animal studies, have implicated OT and VP in the regulation of social behaviors across species. Enough scientific progress has been made so that we can now begin to integrate molecular biology and

behavior to gain a better understanding of OT and VP from the regulatory level of transcription to that of behavior.

## 3 Structure and Expression of Oxytocin and Vasopressin

### 3.1 Conservation Across Phyla

OT and VP are ancient neuropeptides that are members of a peptide family that is highly conserved across phyla (*Table 25-1*) and arose through the duplication of an ancestral vasotocin gene (Acher and Chauvet, 1995; Acher et al., 1995). OT and VP are nonapeptides with the same ring structure formed by a disulfide bridge (Hruby et al., 1990). Only the third and eighth amino acid residues differ between

**Table 25-1**
**Vasopressin/oxytocin superfamily**

| | | |
|---|---|---|
| **Vertebrate vasopressin family** | | |
| **Cys**-Tyr-Phe-Gln-**Asn**-**Cys**-**Pro**-Arg-**Gly**-$NH_2$ | Vasopressin (ADH) | Mammals[a] |
| **Cys**-Tyr-Phe-Gln-**Asn**-**Cys**-**Pro**-Lys-**Gly**-$NH_2$ | Lysipressin | Pigs, hippopotamuses, warthogs, some marsupials |
| **Cys**-Phe-Phe-Gln-**Asn**-**Cys**-**Pro**-Arg-**Gly**-$NH_2$ | Phenypressin | Some marsupials |
| **Cys**-Tyr-Ile-Gln-**Asn**-**Cys**-**Pro**-Arg-**Gly**-$NH_2$ | Vasotocin[b] | Nonmammals |
| **Vertebrate Oxytocin Family** | | |
| **Cys**-Tyr-Ile-Gln-**Asn**-**Cys**-**Pro**-Leu-**Gly**-$NH_2$ | Oxytocin | Mammals[c], ratfish |
| **Cys**-Tyr-Ile-Gln-**Asn**-**Cys**-**Pro**-Ile-**Gly**-$NH_2$ | Mesotocin | Marsupials, birds, reptiles, amphibians, lungfishes |
| **Cys**-Tyr-Ile-Ser-**Asn**-**Cys**-**Pro**-Ile-**Gly**-$NH_2$ | Isotocin | Bony fishes |
| **Cys**-Tyr-Ile-N/Q-**Asn**-**Cys**-**Pro**-L/V-**Gly**-$NH_2$ | Various tocins | Sharks |
| **Invertebrate VP/OT Superfamily** | | |
| **Cys**-Leu-Ile-Thr-**Asn**-**Cys**-**Pro**-Arg-**Gly**-$NH_2$ | Diuretic hormone | Locust |
| **Cys**-Phe-Val-Arg-**Asn**-**Cys**-**Pro**-Thr-**Gly**-$NH_2$ | Annetocin | Earthworm |
| **Cys**-Phe-Ile-Arg-**Asn**-**Cys**-**Pro**-Lys-**Gly**-$NH_2$ | Lys-Connopressin | Geography and imperial cones, pond snail, sea hare, leech |
| **Cys**-Ile-Ile-Arg-**Asn**-**Cys**-**Pro**-Arg-**Gly**-$NH_2$ | Arg-Connopressin | Striped cone |
| **Cys**-Tyr-Phe-Arg-**Asn**-**Cys**-**Pro**-Ile-**Gly**-$NH_2$ | Cephalotocin | Octopus |
| **Cys**-Phe-Trp-Thr-Ser-**Cys**-**Pro**-Ile-**Gly**-$NH_2$ | Octopressin | Octopus |

[a]Vasopressin is not found in some marsupials, pigs, and some other mammals

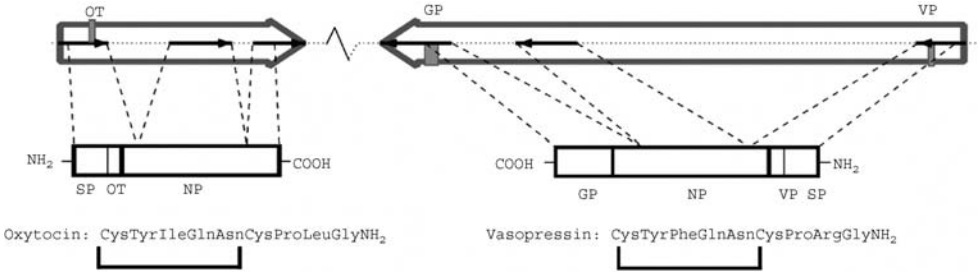
[b]Vasotocin is the progenitor of the vertebrate neurohypophysial hormones. Only vasotocin is found in hagfish and lampreys

[c]Oxytocin is also found in some marsupials (Agnatha appeared 500 million years ago)

the two peptides (Table 25-1, Figure 25-1). Even in the most primitive of organisms, such as the freshwater hydra, an OT/VP-like compound is found (Grimmelikhuijzen, 1984). In general, nonmammalian tetrapods use mesotocin and vasotocin instead of the mammalian OT and VP, respectively. Bony fish have isotocin and vasotocin which correspond to the mammalian OT and VP (Acher, 1990). Exceptions to these rules are shown in Table 25-1 (e.g., the primitive ratfish has OT and some mammals have mesotocin).

**Figure 25-1**

**Schematic representation of the oxytocin and vasopressin genes (*top*, *large arrows*), mRNAs (*middle boxes*), and the neuropeptides themselves (*bottom*). The arrows point from the 5′- to the 3′- ends of the genes. $NH_2$ and COOH indicate the amino and carboxy termini, respectively. Abbreviations: GP, glycopeptide; SP, signalpeptide; NP, neurophysin**




## 3.2 Chromosomal Arrangement

The OT and VP genes are similar in structure and are oriented in opposing transcriptional directions on the same chromosome (Hara et al., 1990) (Figure 25-1). These genes are found on chromosome 2 in mice, chromosome 3 in rats, and chromosome 20 in humans (Dutil et al., 2001) (Table 25-2). The transcriptional units are separated by a region of DNA known as the intergenic region (IGR). The IGR shows variability across species, being 10–11 kbp in length in rat and human (Mohr et al., 1998; Gainer et al., 2001) and approximately 3.6 kbp in length in mouse (Hara et al., 1990). The IGR is particularly interesting because portions of it appear to be necessary for normal OT and VP gene expression within the hypothalamus (Fields et al., 2003; Young III and Gainer, 2003). The OT and VP genes are composed of three exons: the first exon encodes the signal peptide, the nonapeptide, and the first nine amino acid residues of the neurophysin protein; the second exon encodes the central portion of the neurophysin; and the third exon encodes the C-terminal part of the neurophysin as well as the glycopeptide of the VP preprohormone. The greatest amount of variability in sequence across species is found in exon 3 while the greatest conservation is found in exon 2 (Ivell and Richter, 1984; Ruppert et al., 1984; Sausville et al., 1985; Hara et al., 1990).

## 3.3 Coding and Synthesis

As noted above, both OT and VP are synthesized as part of a precursor preprohormone. Each preprohormone is cleaved resulting in the release of a nonapeptide, a neurophysin, and, in the case of VP, a glycopeptide (also known as copeptin), as it is transported along the axon (Brownstein et al., 1980). The bulk of the processing of the preprohormones occurs in the acidic environment within large, dense core vesicles (approximately 160–200 nm in diameter), and includes proteolysis by prohormone convertases and carboxypeptidase H/E and amidation of the carboxy-terminal glycine to yield the final OT and VP nonapeptides (see Burbach et al., 2001; Acher et al., 2002; von Eggelkraut-Gottanka and

**Table 25-2**
**Gene information about vasopressin, oxytocin, and their receptors**

| Gene | Species | Symbol[a] | Chromosome | Known exons | Amino acids[b] | LocusID[c] |
|---|---|---|---|---|---|---|
| Vasopressin | Human | AVP | 20 (p13) | 3 | 164 | 551 |
| | Rat | Avp | 3 (q41-q42) | 3 | 164 | 24211 |
| | Mouse | Avp | 2 (73.2cM) | 3 | 168 | 11998 |
| Oxytocin | Human | OXT | 20 (p13) | 3 | 125 | 5020 |
| | Rat | Oxt | 3 (q36) | 3 | 125 | 25504 |
| | Mouse | Oxt | 2 (73.5cM) | 3 | 125 | 18429 |
| Vasopressin 1a receptor | Human | AVPR1A | 12 (q14-q15) | 2 | 418 | 552 |
| | Rat | Avpr1a | 7 (q21) | 2 | 424 | 25107 |
| | Mouse | Avpr1a | 10 D3 (122.1cM) | 2 | 423 | 54140 |
| Vasopressin 1b receptor | Human | AVPR1B | 1 (q32) | 3 | 424 | 553 |
| | Rat | Avpr1b | 13 (q13) | 3 | 425 | 29462 |
| | Mouse | Avpr1b | 1 E4 (131.5cM) | 3 | 421 | 26361 |
| Vasopressin 2 receptor | Human | AVPR2 | X (q28) | 3 | 371 | 554 |
| | Rat | Avpr2 | X (q37) | 3 | 371 | 25108 |
| | Mouse | Avpr2 | X (29.52 cM) | 3 | 371 | 12000 |
| Oxytocin receptor | Human | OXTR | 3 (p25) | 4 | 389 | 5021 |
| | Rat | Oxtr | 4 (q42) | 4 | 388 | 25342 |
| | Mouse | Oxtr | 6 E3 (113cM) | 4 | 388 | 18430 |
| Dual AII/VP receptor[d] | Human | NALP6 | 11 (p15) | 8 | 513 | 171389 |
| | Rat | Nalp6 | 1 (q41) | 7 | 483 | 171390 |
| | Mouse | Nalp6 | 7 F4 | 7 | 490 | 101613 |
| VACM-1 receptor[e] | Human | CUL5 | 11 (q22-q23) | 19 | 780 | 8065 |
| | Rat | Cul5 | 8 (q24) | 18 | 780 | 64624 |
| | Mouse | Cul5 | 9 C (53.8cM) | 19 | 780 | 75717 |

[a]Offical gene symbol
[b]Number of amino acids in the preprohormone for vasopressin and oxytocin
[c]Available at http://www.ncbi.nlm.nih.gov/LocusLink/index.html
[d]Dual angiotensin II/vasopressin receptor
[e]Vasopressin-activated calcium-mobilizing receptor protein (VACM-1; Cullin-5)

Beck-Sickinger, 2004 and references within). While the neurophysins themselves do not seem to be biologically active, it has been suggested that they may protect either OT or VP from enzymatic damage or are important for normal packaging (de Bree, 2000) and processing (Legros and Geenen, 1996) in neurosecretory vesicles. Recently, it has been suggested that the function of the glycopeptide is to help in the folding of the VP precursor which, in the absence of the glycopeptide, is less stable than the OT counterpart (Barat et al., 2004).

By far, most OT and VP are synthesized within the magnocellular neurons of the hypothalamic supraoptic nuclei (SON) and paraventricular nuclei (PVN), and are transported along their axons to the

posterior pituitary where they are stored and ultimately released into the blood stream. Once released, VP regulates salt and water homeostasis and OT regulates parturition and lactation. Within the brain, OT and VP that are not routed to the pituitary are synthesized by and transported from smaller, parvocellular neurons located in the PVN and elsewhere. However, even the magnocellular neurons of the SON and PVN can release OT and VP from their dendrites to produce important local effects (for reviews and further references, see: Ludwig, 1998; Hirasawa et al., 2004; Landgraf and Neumann, 2004; Morris and Ludwig, 2004).

The relative numbers and the distributions of OT and VP neurons within the SON and PVN vary between species. In humans, for example, VP neurons vastly outnumber OT neurons in the SON, but are nearly equal in the PVN (see Sukhov et al., 1993 and references within). In rats, however, the numbers of these neurons are approximately equal in the two nuclei but distributed in more clearly delineated and generally separate areas (Hou-Yu et al., 1986). In an osmotically normal state, about 2–3% of magnocellular neurons contain both OT and VP, but the percentage of colocalization dramatically increases during a hyperosmolar state or lactation (Kiyama and Emson, 1990; Mezey and Kiss, 1991; Glasgow et al., 1999; Telleria-Diaz et al., 2001). The increased colocalization of VP and OT is probably to further water retention, but this is speculative. Even in those cells considered exclusively vasopressinergic or oxytocinergic, there appears to be expression of the "absent" gene at about 0.5% of the major ones (Xi et al., 1999).

There are wide distributions of OT and VP fibers within the CNS. Fibers can be found from the olfactory bulbs to the intermediate lateral column of the spinal cord (Buijs et al., 1983; De Vries et al., 1985, 1986; Buijs, 1987). In general, VP tends to be found in higher concentrations in the more rostral regions of the brain and OT in the more caudal regions (Sofroniew, 1985a; Gainer and Wray, 1994). Most of the VP and OT found in these subcortical regions are likely involved in the regulation of social and reproductive behaviors, which will be discussed later in this chapter.

## 3.4 Vasopressin

### 3.4.1 CNS Distribution

As mentioned before, the majority of VP within the CNS is expressed within the magnocellular neurons of the SON and PVN, from where it is transported to the posterior pituitary. The evidence for any extrapituitary projections from the SON is scant (Mason et al., 1984; Alonso et al., 1986). In contrast, parvocellular neurons of the PVN provide robust projections, especially to the brainstem and spinal cord. Areas innervated by VP fibers include the hippocampus and subiculum, diagonal band of Broca, locus coeruleus, solitary tract nucleus, dorsal motor nucleus of the vagus, medullary adrenergic groups, and spinal cord (Buijs, 1978; Sawchenko and Swanson, 1982; De Vries and Buijs, 1983; Millan et al., 1984).

VP is also expressed within parvocellular neurons of the suprachiasmatic nucleus (SCN), bed nucleus of the stria terminalis (BNST), and medial amygdala (MeA) (Sofroniew, 1983). Within the BNST and MeA, VP-expressing cells are sex-steroid-dependent (see below) with the males having more VP immunoreactive cells than females in some species (Caffè and Van Leeuwen, 1983; Van Leeuwen et al., 1985; De Vries et al., 1986; Buijs, 1987), but not all. For example, there appears to be no sex difference in VP-immunoreactivity (VP-ir) within the BNST and MeA of Syrian hamsters; instead, galanin may have replaced VP as the gender-dependent peptide (Miller et al., 1999). Nevertheless, there are sexual dimorphisms in brain AVT in bullfrogs and newts, suggesting that across phyla VP and VP-like compounds are sensitive to gonadal steroids (Boyd and Moore, 1992). The BNST and MeA send VP fibers to the olfactory tubercle, nucleus of the diagonal band, ventral pallidum, lateral septum, ventral hippocampus, paraventricular thalamic nuclei, zona incerta, lateral habenula, ventral tegmental area, substantia nigra, periventricular gray, median and dorsal raphe nuclei, and the locus coeruleus (De Vries et al., 1985). Neurons immunoreactive for VP have also been described within the medial and lateral septum, vertical limb of the nucleus of diagonal band of Broca, and locus coeruleus (Sofroniew, 1985b), but only those in the diagonal band have been confirmed by hybridization histochemistry (Urban et al., 1990; Planas et al., 1995; Hallbeck et al., 1999). The patterns of

VP immunostaining in four different vole species are similar to each other and to other rodents and also show similar gender dimorphisms regardless of their social behavior (see below and Wang et al., 1996). A recent hybridization histochemical study in the rat has found VP expression within several new areas, including pyramidal cells of the hippocampus, parabrachial nucleus, and a portion of the mesencephalic reticular nucleus (Hallbeck et al., 1999).

### 3.4.2 Regulation

In the 5′-flanking region of both the OT and VP genes, there are important regulatory elements. In most eukaryotic cells, TATA and CAAT boxes are found close to the start site of transcription and regulate basal transcription. However, the promoters for OT and VP do not contain CAAT boxes but instead have "atypical" TATA boxes (Gainer and Wray, 1994). This atypical TATA box in the VP gene (CATA) may be involved in cell-specific and physiological regulation (Ho and Murphy, 2002). As described below, OT and VP are expressed in cells in different locations within the brain (and periphery, at least for OT). How expression is specifically targeted to those areas is still largely a mystery. The majority of studies examining this issue have used transgenic animals, and certain minimal requirements for expression in some areas are being ascertained. The interested reader is referred to a recent review (Young III and Gainer, 2003). The pathways from the cell surface receptors to the *cis*-acting elements have been difficult to elucidate due to the lack of pure neuronal culture systems that express OT or VP. Nonetheless, progress is being made and a brief survey of regulatory components is presented (see Itoi et al., 2004 for a review). Many of the observations are phenomenological and await direct connection between physiological state or perturbation and VP regulation.

Perhaps the best understood regulator of VP is corticosterone. This glucocorticoid suppresses expression of VP (as well as corticotropin-releasing factor, CRF) in the parvocellular neurons of the PVN (Tramu et al., 1983; Kiss et al., 1984; Sawchenko et al., 1984; Schipper et al., 1984). A sequence resembling the consensus glucocorticoid response element (GRE) has been identified, which spans from −622 to −608 in the rat VP promoter and is proposed to act as a negative response element (Mohr and Richter, 1990). Although negative regulation by a glucocorticoid has been shown in a heterologous cell system (Iwasaki et al., 1997), a specific GRE has not been demonstrated. However, in more neuronal cell cultures, removal of sequence 5′ of −588 of the rat promoter does lead to increased reporter expression (Kim et al., 2001).

The VP gene appears to be regulated by other nuclear hormone receptors, in addition to the glucocorticoid receptor, in a region-specific manner. De Vries and colleagues noted that the density of VP fibers in the lateral septum (LS) is elevated in males compared with females and that testosterone administered to females and castrated males increases the density of VP fibers within the LS (De Vries et al., 1983). They also observed that gonadectomy in males or females reduces VP in certain regions of the brain that receive innervation from the BNST and MeA, but no reduction was observed in regions that receive innervation from the magnocellular neurons of the PVN and SON (De Vries et al., 1984, 1985). VP mRNA levels in the BNST and MeA are also testosterone dependent (Miller et al., 1989, 1992; Wang and De Vries, 1995). Their studies, as well as more recent ones by others using knockout mice, also indicate that testosterone can act through both the androgen receptor and the estrogen receptor but is a stronger regulator through the estrogen receptor (Brot et al., 1993; De Vries et al., 1994; Wang and De Vries, 1995; Plumari et al., 2002; Han and De Vries, 2003; Scordalakes and Rissman, 2004). Gonadectomy has no effect on VP-ir in Syrian hamsters (Albers et al., 1991c), but it does affect VP-ir in Siberian hamsters (Dubois-Dauphin et al., 1994). Therefore, it is not clear how widely this gonadal steroid dependency is conserved across mammals. Also, in chronically hyperosmolar rats there is evidence that gonadal steroids may modulate OT and VP expression (Crowley and Amico, 1993; O'Keefe et al., 1995). Androgen receptors are found in nonmagnocellular responsive neurons (Zhou et al., 1994). Estrogen receptor beta is expressed in VP magnocellular neurons (Alves et al., 1998). An estrogen response element (ERE) is found over 4kb downstream of the transcriptional start site of the rat VP gene (Shapiro et al., 2000), but whether this is the site where androgens and/or its metabolites exert their effects is unknown.

Hypovolemia or hyperosmolality are strong stimuli for the expression of VP (and OT) in magnocellular neurons of the PVN and SON, but the intracellular pathways are unclear. Hyperosmolality activates the cAMP pathway (Young III et al., 1987). Elevation of cAMP in cultured hypothalamic neurons stimulates VP expression (Sladek and Gallagher, 1993). In the absence of synaptic transmission, elevation of cAMP increases VP expression in parvocellular PVN neurons (Arima et al., 2001), and this increase can be inhibited by glucocorticoids (Kuwahara et al., 2003). It is possible that this osmotic response is mediated through two different cAMP-responsive elements (−227 to −220 and −123 to −116) (Iwasaki et al., 1997). Similarly, animals in a hyperosmotic condition have increased expression of the immediate early genes c-fos and c-jun in these nuclei (Carter and Murphy, 1990), as well as increases in the alpha isoform of activator protein-2 (AP-2) and activating transcription factor-2 (ATF-2) (Meeker and Fernandes, 2002). In the VP promoter, putative regulatory elements for cAMP and AP-2 have been identified (Mohr and Richter, 1990). Cell extracts from a nonneuronal cell line were used to show that the proximal VP promoter contains an AP-1, five E-Box, and two GC-rich transcription binding sites (Grace et al., 1999).

The SCN, site of the mammalian circadian pacemaker (see below), has also been extensively investigated. VP expression within the SCN undergoes a circadian rhythm with peak mRNA levels during the day (light-phase) in rats (Uhl and Reppert, 1986; Burbach et al., 1988; Young III et al., 1993). The SCN receives excitatory input from the retina and both excitatory and inhibitory inputs from elsewhere in the CNS, in addition to input from intrinsic neurons (Albers et al., 1991a, b; Moore, 1992). How these inputs are transduced to affect VP expression is unclear. Numerous studies have shown that regulation of immediate early genes undergo circadian rhythms (e.g., Schwartz et al., 2000), and tremendous progress has been made recently in understanding the underlying molecular biology of the circadian clock (Hastings and Herzog, 2004). For example, the E-Box element, found in many genes including VP, is recognized by transcription factors containing the basic helix–loop–helix motif and is important in the generation of rhythmic expression by BMAL1 and CLOCK proteins (Jin et al., 1999; Hastings and Herzog, 2004). Muñoz et al. defined some of the E-Box flanking sequence involved in VP circadian transcription through BMAL1 and CLOCK (Muñoz et al., 2002). Arima et al. (2002) provide evidence that the circadian rhythm of VP expression requires neural activity and a MAP kinase pathway.

Two other mechanisms appear to be involved in regulating VP expression. The first is the regulation of the poly(A) tail of the mRNA. Increases in plasma osmolality increase the length of the poly(A) tail in rats and that may serve to prolong the half-life of the VP message (Carrazana et al., 1988; Zingg et al., 1988). Interestingly, the increase in mRNA levels and the increase in mRNA poly(A) tail length induced by hyperosmolality are regulated separately. Administration of *p*-chlorophenylalanine blocks the increase in mRNA levels but does not prevent the increase in tail length (Carter and Murphy, 1989). Tail length can also be shortened by starvation (Chooi et al., 1992). The second mechanism for regulating VP expression is the distribution of the VP mRNA within the cell and its processes. Because VP mRNA is found in both axons and dendrites, sorting to different neuronal processes may be important in regulating where VP is synthesized and released, although studies examining this idea are in their infancy (Trembleau et al., 1996; Mohr and Richter, 2004). It is worth noting, as well, that many small cell lung cancers express VP, accounting for some of its pathology. These cells have been used to identify potential regulatory sites within the VP gene (North, 2000; Coulson, 2002).

## 3.5 Oxytocin

### 3.5.1 CNS Distribution

OT synthesized in the magnocellular neurons of the PVN and SON project to the posterior pituitary. Parvocellular (or, at least smaller than the magnocellular) neurons in the PVN project to similar areas in the brainstem and spinal cord as the VP neurons described previously. Parvocellular OT neurons outside the PVN have been described in mice (Castel and Morris, 1988; Jirikowski et al., 1990) and various vole species (Wang et al., 1996). However, in the rat, it appears that the PVN is responsible for most, if not all, brain OT

projections (De Vries and Buijs, 1983; Rinaman, 1998; although see Jirikowski et al., 1988). OT is not expressed in the SCN, and there are very few or no OT neurons in the BNST and MeA.

### 3.5.2 Regulation

Most of the work examining the promoter region of the OT gene has focused on nuclear hormone receptors and orphan receptors. Estrogen binding is observed in 10–40% of the magnocellular OT neurons of the rat PVN and SON and only occasionally in mouse OT neurons elsewhere (Jirikowski et al., 1990). OT-immunoreactive neurons are observed after short-term estradiol treatment in the rat septohippocampal nucleus, lateral subcommissural area, medial preoptic area (MPOA), perifornical regions, zona incerta, and ansa lenticularis, along with more fibers in the LS, preoptic area, striatum, and amygdala (Jirikowski et al., 1988). The human and rat promoters have proven EREs (Burbach et al., 1990; Richard and Zingg, 1990; Mohr and Schmitz, 1991), but the bovine promoter apparently does not (Adan et al., 1991). Subsequently, the most proximal ERE in the rat promoter was shown to be a composite hormone response element that responds to a variety of nuclear hormone receptors and orphan receptors (Adan et al., 1993).

There are ample studies demonstrating that in cell culture both estrogen receptor alpha and beta, thyroid hormone receptors, retinoic acid receptors, and some orphan receptors can stimulate the transcription of OT (Richard and Zingg, 1990; Adan et al., 1992; Burbach et al., 1992, 1993; Vasudevan et al., 2001). However, one study provides strong evidence that retinoic acid actually represses activity from this element (Lipkin et al., 1992), but the reason for this discrepancy is unknown. Interesting recent studies suggest that steroid hormone receptors may not, in fact, act directly upon the promoter to affect transcription, but instead influence other transacting factors, either directly or through kinases (Stedronsky et al., 2002).

In vivo studies support the idea that both OT and VP are regulated by estrogen and thyroid hormones in the hypothalamus (Burbach and Adan, 1993; Dellovade et al., 1999; Ciosek, 2002; Nomura et al., 2002; Patisaul et al., 2003); although this may not always be through direct effects within the synthesizing neurons (Sladek and Somponpun, 2004). Progesterone may also directly activate expression of OT in the PVN (Thomas et al., 1999).

As with VP, hyperosmolality increases OT mRNA levels independent of an increase in poly(A) tail length (Carter and Murphy, 1989), but this effect is dependent upon gonadal steroids, since gonadectomy blocks this increase (Crowley and Amico, 1993). Increases in OT mRNA are also accompanied by increases in c-fos protein (Giovannelli et al., 1992). Interestingly, OT transcript poly(A) tail length also increases during pregnancy and lactation (Zingg and Lefebvre, 1989).

## 4 Vasopressin and Oxytocin Receptors

### 4.1 The Vasopressin Receptors

There are two principle classes of VP receptors: V1 and V2 receptors (V1R and V2R, respectively), both of which are seven transmembrane G-protein-coupled receptors. Activation of the V2R increases cAMP that mediates the classical antidiuretic effects of vasopressin. The V1Rs are coupled to $G_{\alpha q/11}$ GTP binding proteins, which along with $G_{\beta\lambda}$, activate phopholipase C (PLC) activity (Michell et al., 1979; Jard et al., 1987). PLC then generates 1,4,5-inositol triphosphate ($IP_3$) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate ($PIP_2$). $IP_3$ facilitates the release of intracellular $Ca^{2+}$ stores while DAG activates protein kinase C to modulate cellular activity. There are two subtypes of the V1R: V1aR and V1bR. In the periphery, V1aR mediates the effects of VP on vasoconstriction and can be found in liver, kidney, platelets, and smooth muscle (Ostrowski et al., 1992; Watters et al., 1998). Centrally, V1aR is found in a variety of brain nuclei (Ostrowski et al., 1992, 1994; Szot et al., 1994), where it has been implicated in the regulation of several social behaviors, including social recognition, affiliative behavior, aggressive behavior, and scent marking behavior (Ferris et al., 1984, 1997; Albers and Ferris, 1985; Albers et al., 1986; Winslow et al., 1993).

The V1bR (sometimes called V3R) was originally described in the pituitary (Antoni, 1984; Jard et al., 1986; Arsenijevic et al., 1994) and finally cloned (Lolait et al., 1995). Subsequently, V1bR was also found in the brain as well as in several peripheral tissues (Arsenijevic et al., 1994; Lolait et al., 1995). More recently, V1bR has been linked to stress adaptation (Volpi et al., 2004a) and aggressive behavior in mice (Wersinger et al., 2002). There is no conclusive evidence for expression of the V2R within the CNS. Transcription of the ARHGAP4 gene within the CNS overlaps the transcription of V2R, and reverse transcriptase-PCR analysis must must be carried out under specific conditions to avoid this "contaminant" (Foletta et al., 2002). Readers interested in the actions of VP at the renal V2R and V1aR are referred to some excellent reviews (Bankir, 2001; Inoue et al., 2001).

Recent work has indicated that there are other proteins in the brain that bind VP (◉ *Table 25-2*). The vasopressin-activated calcium-mobilizing receptor protein (i.e. VACM-1or Cullin-5) and the dual angiotensin II/vasopressin receptors have widespread neuronal distributions (Hurbin et al., 2000; Ceremuga et al., 2003a). The former receptor is coupled to the phosphoinositol pathway (Burnatowska-Hledin et al., 1995) and the latter to the adenylate cyclase system (Ruiz-Opazo et al., 1995). The roles, of these receptors in the brain, including behavioral roles, are unknown, however, levels of Cullin-5 increase in the cerebral cortex and, to a lesser extent, in the hypothalamus following water deprivation (Ceremuga et al., 2003b). (◉ *Table 25-2*) summarizes genetic information about VP, OT, and their receptors.

### 4.1.1 CNS Distribution

The distribution of V1aR expression within the CNS has been primarily studied using receptor autoradiography and hybridization histochemistry. Receptor autoradiography identifies the locations of binding to the receptor protein, whereas hybridization histochemistry identifies the cells that transcribe the receptor gene. The former technique was greatly facilitated through the use of specific and potent $^{125}$I-labeled V1aR antagonists (Johnson et al., 1993; Kremarik et al., 1993). Prominent V1aR binding is present in the rat LS, neocortical layer IV, hippocampal formation, amygdalostriatal area, BNST, various hypothalamic areas (including SCN), ventral tegmental area, substantia nigra, superior colliculus, dorsal raphe, nucleus of the solitary tract, and inferior olive (Johnson et al., 1993). V1aR binding is moderate throughout the spinal cord, but with higher binding in the dorsolateral motoneurons in general and all motoneurons in the lumbar 5/6 levels where innervation to the perineal muscles originates (Tribollet et al., 1997).

Neurons containing V1aR transcripts are found extensively throughout the rat CNS, being especially prominent, for example, in the olfactory bulb, hippocampal formation, LS, SCN, PVN, anterior hypothalamic area, arcuate nucleus, lateral habenula, ventral tegmental area, substantia nigra (pars compacta), superior colliculus, raphe nuclei, locus coeruleus, inferior olive, area postrema, and nucleus of the solitary tract (Ostrowski et al., 1994; Szot et al., 1994). Transcripts are also detected in the choroid plexus and endothelial cells. The distributions of VP (and OT) binding have been examined in a number of rodent species, and they are remarkably similar. Differences in binding in selected areas may mediate important adaptations or behavioral traits, and these differences will be mentioned below when correlations are possible.

The V1bR was originally described in the anterior pituitary where it facilitates the release of adrenocorticotropic hormone (ACTH) from the corticotropes (Jard et al., 1987; Antoni, 1993). V1bR in the pituitary helps mediate the effects of VP on the hypothalamic–pituitary–adrenal axis, which is the regulator of the stress response in mammals (Volpi et al., 2004a). V1bR mRNA is also found in a variety of peripheral tissues including kidney, thymus, heart, lung, spleen, uterus, and breast (Lolait et al., 1995), although its role in these tissues remains unclear. Only recently have V1bR transcripts as well as V1bR immunoreactive cell bodies been found in the rat brain, including in the olfactory bulb, piriform cortical layer II, septum, cerebral cortex, hippocampus, PVN, SCN, cerebellum, and red nucleus. (Lolait et al., 1995; Saito et al., 1995; Vaccari et al., 1998; Hernando et al., 2001; Stemmelin et al., 2005). It should be noted, however, that V1bR distribution has not been mapped by receptor autoradiography due to the lack of specific radiolabeled ligands. Fortunately, the development of a V1bR knockout (V1bRKO) mouse has offered critical insight into the role of V1bR in the mouse brain (below).

### 4.1.2 Regulation

Within the CNS, V1aR transcription and translation are sensitive to gonadal steroids. In the photoperiodic Syrian and Siberian hamsters, exposure to short "winter-like" photoperiods results in dramatic reductions in V1aR binding within brain areas associated with the neural regulation of social behavior (Dubois-Dauphin et al., 1994; Caldwell and Albers, 2003, 2004b). Likewise, gonadectomy and lactation can decrease V1aR mRNA and receptor binding within the hypothalamus (Johnson et al., 1995; Delville et al., 1995; Young et al., 2000). In young rats, estrogen increases V1aR mRNA in the preoptic area of the hypothalamus (Funabashi et al., 2000). Castration leads to reduced binding in the pudendal nuclei of L5/L6 (Tribollet et al., 1997). While it is clear that gonadal hormones can affect V1aR transcription and translation within specific parts of the brain, the mechanisms underlying these changes remain unknown. Presumably, gonadal steroids could directly affect V1aR transcription through response elements, but none have been identified yet. There do, however, appear to be putative GREs within the 5′-flanking region of the V1aR gene. In rats, adrenal steroids can affect V1aR mRNA expression and V1aR binding within the LS and BNST (Watters et al., 1996). There is still much work to do to understand the regulation of V1aR transcription and its complex relationship with steroid hormones.

A recent study found that expression of the V1aR increased after traumatic head injury (Szmydynger-Chodobska et al., 2004). The increase was observed in astrocytes of the damaged frontal cortex, and the receptor was observed to move from the cell body to processes with time. It is interesting to speculate as to whether VP plays a role in cerebral edema that may accompany brain trauma.

Some very provocative work has focused on interspecies and individual variations in the V1aR promoter of voles. Across vole species there are profound differences in social structure and behavior. Prairie voles (*Microtus ochrogaster*) and pine voles (*Microtus pinetorum*) tend to be social and monogamous while montane voles (*Microtus montanus*) and meadow voles (*Microtus pennsylvanicus*) tend to be asocial and polygamous. These social behaviors are mediated, at least in part, by VP action on the V1aR (Winslow et al., 1993; Wang et al., 1994; Cho et al., 1999; Young et al., 1999; Liu et al., 2001). There are striking differences in the distribution and density of V1aR between these species (Young et al., 1997b, 1999). Within the promoter region of the V1aR gene of the prairie and pine voles, there is an approximately 400-bp sequence of repetitive DNA, known as a microsatellite sequence, which is absent from the promoter region of the V1aR gene in the montane and meadow voles. The length of this microsatellite sequence shows a correlation with V1aR expression patterns and ultimately behavior (Hammock and Young, 2002; Hammock et al., 2004). We will come back to the role of this microsatellite polymorphism in the mediation of behavior later in this chapter.

While V1bR has only been described relatively recently, there has been some work examining its regulation in the pituitary. In the rat, pituitary expression of V1bR appears to be positively regulated by corticosteroids and perhaps by VP (Rabadan-Diehl et al., 1997; Rabadan-Diehl and Aguilera, 1998). V1bR expression appears to increase or decrease depending on the stressor (Rabadan-Diehl et al., 1995; Aguilera and Rabadan-Diehl, 2000; Qahwash et al., 2002). In the rat, there are AP-1 and AP-2 sites and a GRE in the 5′-flanking region. In humans there is a half-palindromic sequence for estrogen, a cAMP response element, and a GRE (Aguilera et al., 2003). The V1bR promoter also contains a GAGA box essential for expression (Volpi et al., 2002). The V1bR 5′-untranslated region contains small expressed minicistrons or open reading frames that appear to inhibit V1bR expression posttranscriptionally as well as an internal ribosomal entry site that may be uncovered when increased expression is desired (Nomura et al., 2001; Aguilera et al., 2003). How these regulatory elements all interact to regulate the V1bR gene is still being investigated (Volpi et al., 2004b). Nothing is known about V1bR regulation in the brain proper and will likely be a fruitful field for investigation.

## 4.2 The Oxytocin Receptor

Physiologically and behaviorally, OT regulates reproduction across species, from its peripheral effects on milk ejection and uterine contractions to its central effects on sexual behavior, maternal behavior, and pair

bonding. A single OT receptor (OTR) appears to transduce the actions of OT. This receptor was first isolated and identified by Kimura and colleagues in 1992.

The OTR is also a member of the G-protein-coupled receptor family. Like other members of this family, the OTR contains seven transmembrane domains and is similar in structure to the VP receptors. The OTR is also coupled to $G_{\alpha q/11}$ GTP-binding proteins and $G_{\beta\lambda}$ (Ku et al., 1995; Gimpl and Fahrenholz, 2001; Zingg and Laporte, 2003) to cause the hydrolysis of phosphatidylinositol and act through pathways similar to that of the V1Rs.

### 4.2.1 CNS Distribution

Initially, the location of OTR expression was determined by receptor autoradiography, using a potent and specific $^{125}$I-labeled antagonist (Kremarik et al., 1993; Veinante and Freund-Mercier, 1997). In the rat, OT binding is found in numerous regions, especially in the hippocampal formation (ventral subiculum particularly), LS, central amygdala (CeA), olfactory tubercle, accumbens nucleus shell, dorsal caudate-putamen, BNST, MeA, and ventromedial hypothalamus (VMH). Binding in the spinal cord is light and confined to the superficial dorsal horn (Tribollet et al., 1997).

Hybridization histochemistry reveals OTR transcripts in many areas of the rat CNS, including main and accessory olfactory bulbs, neocortical layers II and III, piriform cortical layer II, hippocampal formation, olfactory tubercle, BNST, medial habenula, VMH, PVN, and SON. Expression is lower in the midbrain, pons, and medulla (Vaccari et al., 1998). Recently, an OTR–*lacZ* reporter mouse has shown additional OTR gene expression in the medial septum, parts of the amygdala and mammillary nuclei, and some brainstem nuclei (Gould and Zingg, 2003).

The distribution of the OTR is highly species specific, as is elegantly illustrated in receptor binding differences between two closely related species of voles, the polygamous montane vole and the monogamous prairie vole (Insel and Shapiro, 1992; Insel et al., 1997). Differences in the distributions of the OTRs have also been shown among mice, rats, voles, hamsters, and guinea pigs (Insel et al., 1993). These differences in OTR distribution across species are thought to confer differing behavioral phenotypes, as discussed below.

### 4.2.2 Regulation

In a variety of species, both transcription and translation of the OTR gene are sensitive to gonadal steroids. In rats, OTR binding and mRNA levels in the brain and myometrium of the uterus are increased with estradiol and testosterone treatment (Tribollet et al., 1990; Stevenson et al., 1994; Larcher et al., 1995; Breton and Zingg, 1997). Curiously, estrogen tremendously increases the expression of the OTR in the kidney (Ostrowski et al., 1995; Breton et al., 1996). Castration and inhibition of estrogen synthesis results in a decrease in OTR binding in the rat brain (Tribollet et al., 1990). OTR within the VMH, an important nucleus for the regulation of sex behavior, has been the focus of intense study. OTR expression within the VMH of both males and females is particularly sensitive to gonadal steroids (de Kloet et al., 1985, 1986; Coirini et al., 1989; Johnson et al., 1991; Bale and Dorsa, 1995; Bale et al., 1995; Quinones-Jenab et al., 1997). Interestingly, the mouse is the only known species in which there is a complete palindromic ERE in the promoter of the OTR gene (Bale and Dorsa, 1997); rats and humans have only half-palindromic EREs (Inoue et al., 1994; Rozen et al., 1995). It does appear likely, however, that estrogen can act on the half-palindromic EREs with low affinity (Sanchez et al., 2002). The OTR gene has several other response elements in its promoter, including an interleukin response element, a cAMP response element, and AP-1, AP-2, AP-3, and AP-4 sites (Rozen et al., 1995; Bale and Dorsa, 1998; Gimpl and Fahrenholz, 2001). While there seems to be ample information on potential modulators of OTR synthesis, there is much work to do to understand their interaction with one another. For a more detailed description of the OTR system, there are several excellent reviews including ones by Gimpl and Fahrenholz (2001), Kimura et al. (2003), and Zingg and Laporte (2003).

## 5 Behavioral and Physiological Effects of Vasopressin and Oxytocin

Across species OT facilitates bonding behaviors between offspring and parents and between males and females, whereas VP appears to be more involved in the regulation of aggression and male parental care. Understanding the involvement of OT and VP in the regulation of sexual and social behaviors has been, and continues to be, the source of exciting research in behavioral neuroendocrinology. Our understanding of how OT and VP systems interact with one another and with other neurotransmitter systems to affect behavior is still in its infancy. However, some recent work has begun to shed some light on their profound and lasting effects on behavior across species. The use of transgenic animal models and viral vectors has moved the field forward and provided valuable insight into the individual roles of OT and VP in the mediation of behavior. This section will survey past and current findings on the roles of OT and VP in the regulation of a variety of behaviors. A summary of this section can be found in ❯ *Table 25-3*.

**Table 25-3**
**Summary of the behavioral effects of OT and VP**

| Behavioral classes | Behaviors | Effects of OT | Effects of VP |
|---|---|---|---|
| Reproductive behaviors | Maternal behavior | ⇑ when injected ICV and into the MPOA | Maternal aggression impaired in V1bRKO mice |
| | Female sexual behavior | ⇑ when injected into the MPOA-AH and VMH | No known effect |
| | Male sexual behavior | ⇑ in erection when injected ICV | No known effect |
| Social behaviors Aggression | Female aggression | ⇑ when injected into the MPOA-AH (hamsters) and CeM (rats). | Maternal aggression impaired in V1bRKO mice |
| | Male aggression | ⇔ conflicting reports | ⇑ when injected into the AH and LS impaired in V1bRKO mice |
| Social memory | Social recognition | ⇔ conflicting reports, but is impaired in OTKO mice | impaired in V1aRKO and V1bRKO mice |
| | Partner preference | ⇑ when injected ICV in females and in males following neonatal exposure | ⇑ when injected ICV in male prairie voles; dependent on V1aR distribution |
| Other Behavioral and Physiological Effects | Scent marking and grooming | ⇑ when injected ICV in males and females (rats) | ⇑ when injected into the MPOA-AH (hamsters) |
| | Anxiety and depression | ⇓ in anxiety when injected ICV in females | Correlation in VP release from the PVN and ⇑ in anxiety (rats); V1aRKO mice show reduced anxiety |
| | Learning and memory | ⇑ in spatial learning in female; acts as an amnestic in some tests | ⇑ in spatial learning when injected into the ventral hippocampus of scopolamine-treated male rats |
| | Fever | No known effect | ⇓ when infused into the LS |

### 5.1 Reproductive Behaviors

#### 5.1.1 Maternal Behavior

The term "maternal behavior" encompasses a spectrum of behaviors describing the care of offspring by a female of a species. In a variety of species, OT is important for the regulation of maternal behaviors. In rats,

OT infused intracerebroventricularly (ICV) or directly into the medial preoptic area (MPOA) can stimulate maternal behavior (Pedersen and Prange, 1979; Pedersen et al., 1982; Fahrbach et al., 1985; Pedersen, 1997). Similarly, in mice OT injected ICV increases maternal behavior (McCarthy, 1990). Lesions of OT-producing neurons in estrogen-primed virgin female rats inhibit maternal behavior (Insel and Harbaugh, 1989), and centrally administered OT antisera or OTR antagonists reduce maternal behaviors (Pedersen et al., 1982; Fahrbach et al., 1985; van Leengoed et al., 1987). In sheep, high levels of OT within the limbic system are important for maternal behavior and OT injected ICV can induce maternal behavior in sexually naïve animals. It may be that in sheep OT is a more potent regulator of maternal behavior because its effects are faster acting than those seen in rodents (Keverne and Kendrick, 1994; Kendrick et al., 1997).

While OT can directly influence maternal behavior, estrogen also interacts with the OT system. Estrogen can affect the transduction of OT signals by altering OTR transcription and translation. As estrogen concentrations change across the estrous cycle and during pregnancy, there are coinciding changes in OTR expression. In rats, as estrogen levels increase following parturition, there is an activation of c-fos and fos-B in OTR expressing cells within the MPOA, BNST, and amygdala (Lin et al., 2003). Exogenous estrogen treatment increases OTR density within the MPOA, resulting in the support of OT mediated maternal behaviors (Patchev et al., 1993; Young et al., 1997a). Estrogen induced OTR in the LS, CeA, PVN, and BNST can affect grooming and licking behavior in rats, both of which are part of the normal repertoire of maternal behaviors (Champagne et al., 2001; Francis et al., 2002). In light of these data, it is obvious that OT is a regulator of maternal behavior in rats. Surprisingly, mice with a disruption of their OT gene (OTKO) display normal maternal behaviors (Winslow and Insel, 2002). Oxytocin knockout (OTKO) mice show normal parturition, licking, and grooming behaviors, but do not lactate. The conflicting results between in vivo pharmacological studies and the OTKO studies may be due to a developmental compensation in OTKO mice. This idea is supported by a recent study that found that within the VMH of OTKO mice, VP can act on the OTR (Ragnauth et al., 2004). If VP is substituting for OT in OTKO mice, it would then explain the presence of normal maternal behavior in OTKO mice.

### 5.1.2 Female Sexual Behavior

OT within the CNS also regulates female sexual receptivity across species. In female Syrian hamsters, OT microinjected into the medial preoptic area-anterior hypothalamic continuum (MPOA-AH) or into the VMH induces sexual responsiveness, as measured by the duration and frequency of sexual receptivity, i.e., lordosis behavior (Whitman and Albers, 1995). In rats, OT injected into the MPOA-AH or medial basal hypothalamus in combination with estrogen or estrogen conjugated to bovine serum albumin (so it cannot pass through the cell membrane and into the cell to act on classical estrogen receptors), increases sexual receptivity (Caldwell et al., 1989; Caldwell and Moe, 1999). Conversely, OTR antagonists injected ICV in rats or into the MPOA-AH of Syrian hamsters reduce sexual receptivity (Benelli et al., 1994; Whitman and Albers, 1995). Also, when infused into the VMH of female rats, antisense oligonucleotides against OTR prevent female sexual receptivity (McCarthy et al., 1994). Aside from its regulatory effects on lordosis, OT injected into the MPOA-AH of Syrian hamsters increases their ultrasonic vocalizations, an important component of sex behavior (Floody et al., 1987). Interestingly, low concentrations of OT infused into the lateral ventricles can actually reduce lordosis, suggesting that the effects of OT on receptivity do not follow a simple dose–response curve (Schulze and Gorzalka, 1992). Again surprisingly, female (and male) sexual behavior is not significantly affected in OTKO mice (Nishimori et al., 1996; DeVries et al., 1997). Whether this is permitted through compensation and/or redundancy of this vital behavior remains unanswered.

### 5.1.3 Male Sexual Behavior

In males, OT neurons originating from the PVN and projecting to extrahypothalamic brain areas and spinal cord are involved in aspects of male sexual behavior, including copulation and erection. Humans show increases in plasma OT at ejaculation (Carmichael et al., 1987; Murphy et al., 1987) and in male rats, OT is

elevated following exposure to females (Hillegaart et al., 1998). At least in rodents, these increases in OT correlate with the intensity of copulation (Hillegaart et al., 1998). OT administered centrally can induce penile erection that castration or hypophysectomy can abolish (Argiolas et al., 1985; Argiolas, 1999). OT antagonists injected ICV reduce mounts and intromissions, and ejaculations are completely abolished (Argiolas et al., 1988). One of the critical sites for the neural regulation of erection is the PVN. Lesions of OT neurons of the PVN prevents erection and injections of OTR antagonists into the lateral ventricles also prevent erection (for review see Argiolas, 1999; Andersson, 2001; Argiolas and Melis, 2004). Even in amphibians such as the rough-skinned newt (*Taricha granulosa*), clasping behaviors are affected by the OT homologue AVT (Moore, 1983), suggesting that OT is important to displays of sexual behavior across species.

## 5.2 Social Behaviors

### 5.2.1 Aggression

In a variety of species, aggressive behavior is important to the development and maintenance of social structures. Defense of territory, protection of young, formation of social hierarchies, and competition for mates are just some of the reasons why animals display aggressive behaviors. In general, males are more aggressive than females, though females often show increased aggression during pregnancy and in the subsequent postpartum period. Recently, interest in understanding the neural underpinnings of violent and aggressive behavior has increased. Aggression is notoriously difficult to study, but the use of specific pharmacological agents and transgenic mouse models have aided in our understanding of the neural regulation of aggression and have shown that OT and VP have important roles in the regulation of aggression across species.

**5.2.1.1 Female Aggression** In many mammalian species, females are only aggressive during pregnancy and following parturition. There are, however, notable exceptions in some mammalian species, such as spotted hyenas (Holekamp and Smale, 2000) and Syrian hamsters (Payne and Swanson, 1970). In female Syrian hamsters, injections of OT into the MPOA-AH can reduce the duration of aggressive behavior directed toward a nonaggressive, female intruder (Harmon et al., 2002a). Even in female voles, OT injected ICV can decrease male-directed aggression (Bales and Carter, 2003b). Thus, it appears that OT can reduce nonmaternal aggressive behaviors.

The role of OT in the regulation of maternal aggressive behavior remains somewhat murky. While OT can facilitate maternal aggression in female Syrian hamsters when microinjected into the amygdala (Ferris et al., 1992), OT antagonists injected into the CeM of rats also increases maternal aggression (Lubin et al., 2003). Reduction of OT using antisense oligonucleotides and lesions of the PVN also reduce maternal aggression in rats (Giovenardi et al., 1998). Most of the studies that have implicated OT in the mediation of maternal aggression have used gestational cocaine treatment. Gestational cocaine treatment reduces OT and OTR in several brain areas (Johns et al., 2004). As a result, there is a subsequent increase in maternal aggression thought to be due in part to these changes in the OT neurocircuitry (Johns et al., 1997; Elliott et al., 2001). While OTKO mice would seem the obvious model in which to examine OT regulated maternal aggression, to our knowledge maternal aggression has not been examined in OTKO mice. However, the results of studies using these mice might be difficult to interpret in light of the recent work suggesting that VP can act directly on the OTR in OTKO mice (Ragnauth et al., 2004).

**5.2.1.2 Male Aggression** VP has been implicated in the modulation of male aggressive behavior across species. In birds, fish, rodents, and primates, VP or nonmammalian VP homologues can affect aggressive behavior (Ferris and Potegal, 1988; Winslow and Insel, 1991b; Goodson and Adkins-Regan, 1999; Semsar et al., 2001). Some neurocircuitry underlying aggressive behavior has been described. In Syrian hamsters, the anterior hypothalamus (AH), which has reciprocal connections with the ventrolateral hypothalamus (VLH), MeA, and BNST, is an important site in the regulation of aggressive behavior (Delville et al., 2000)

and will be discussed in more detail below. In rats and mice, gonadal steroid-dependent VP projections from the BNST and the MeA to the LS (De Vries et al., 1984, 1985; Van Leeuwen et al., 1985; Miller et al., 1992) have been implicated in the modulation of aggressive behavior (Scordalakes and Rissman, 2004). In rats and prairie voles, VP injected into the LS can induce agonistic behavior (Koolhaas et al., 1991; Winslow et al., 1993; Wang et al., 1994). However, in wild-type (WT) rats there is a negative correlation between reduced VP in the LS and aggressive behavior (Everts et al., 1997). While the exact role of VP in the LS is not certain, it may be that the LS regulates the emotional aspects of aggressive behavior (Desmedt et al., 1999; Everts and Koolhaas, 1999).

Aggressive behavior in Syrian hamsters is particularly well-studied (Ferris and Delville, 1994). Microinjections of specific V1aR antagonists into the VLH and the AH can reduce agonistic behavior (Ferris and Potegal, 1988; Delville et al., 1996). Interestingly, only in socially dominant animals do microinjections of VP into the AH facilitate offensive aggressive behavior (Ferris et al., 1997; Caldwell and Albers, 2004a). This seems to be a common theme across species; while VP is an important modulator of aggressive behavior, its effects are specific to the social status of the animal. For instance, only in "dominant" squirrel monkeys does an ICV injection of VP increases aggression (Winslow and Insel, 1991b). Even conditions such as housing, which are known to affect social status (Grelk et al., 1974), alter VP neurocircuitry by causing a redistribution of V1aR. Syrian hamsters that are singly housed show more V1aR binding in several brain areas compared with group-housed males (Smith et al., 2001) and tend to be more aggressive (Brain, 1972). There is more VP-ir in the LS of mice bred for short-attack latencies, and more VP-ir in the BNST of mice bred for long-attack latencies (Compaan et al., 1993). While it is not surprising that the brain of a dominant animal differs from that of a subordinate animal, understanding this plasticity will continue to be an exciting area of research.

Until recently, most work examining the regulation of aggressive behavior by VP assumed action via the V1aR. However, aggression studies using V1bR knockout (KO) mice suggest that normal displays of aggressive behavior require a functional V1bR. V1bRKO mice show significant reductions in aggressive behavior as they do not attack intruders in either neutral arena or resident–intruder behavioral models (Wersinger et al., 2002). In contrast, V1bRKO mice show normal predatory aggression (Wersinger et al., 2003), suggesting that the V1bR is critical for social forms of aggressive behavior. V1bRKO mice also have reduced social motivation and spend equal time investigating clean bedding or bedding soiled either by females or males (Wersinger et al., 2004). A recent study in Syrian hamsters supports the findings of the V1bRKO studies. Hamsters administered a selective V1bR antagonist orally showed marked reductions in offensive aggression, and the authors also suggested that the V1bR may be involved in the behavioral response to stress (Blanchard et al., 2005). While the distribution of V1bR binding in mice and hamsters has yet to be determined, the behavioral findings indicate that further study of the V1bR and its role in aggressive behavior will be enlightening.

The role of OT in the mediation of aggressive behavior in males is conflicting. In OTKO mice, one group reported increases in aggressive behavior in male OTKO mice (Winslow et al., 2000) while another group reported decreases in aggressive behavior (DeVries et al., 1997; Young III et al., 1998). Since two different groups generated the OTKO mice used in the above studies and the methods employed were not identical, the precise role of OT in the modulation of male aggressive behavior remains unknown.

### 5.2.2 Social Memory

Formation of social bonds between individuals is important for the survival of many species. Throughout the animal kingdom successful reproduction requires interactions between individuals. Whether a species is social or asocial, monogamous or polygamous, the formation of social bonds is critical. Social recognition is a specific type of memory on which animals rely to recognize familiar from unfamiliar conspecifics, while partner preference refers to an individual's social attachment to a conspecific. Classical pharmacological studies as well as transgenic animal studies have been successful in deepening our understanding of the neural regulation of social recognition. However, the use of nontraditional model species, like voles, has provided valuable insight into the molecular basis of partner preference. This section will review some of

the more recent work in the field of social recognition and partner preference, although, the interested reader should refer to several recent reviews on the topic (Bielsky and Young, 2004; Insel and Fernald, 2004; Keverne and Curley, 2004; Young and Wang, 2004).

**5.2.2.1 Social Recognition** Although the sensory modality by which individuals recognize one another may differ among species, the ability to recognize individuals is essential for survival. Whether an animal is recognizing a parent, an offspring, a potential mate, or an aggressor, social recognition is important for displaying appropriate behaviors. In humans and nonhuman primates, social recognition depends mostly on visual and auditory cues, whereas in other mammals such as rodents, olfactory and pheromonal cues provide the most accurate information about others. The processing of the olfactory information relies upon OT and VP to aid in the formation of social memories. An in depth review of the neural regulation of social recognition can be found in a recent article by Bielsky and Young (2004).

The evidence that VP is critical for social recognition is compelling. Since the LS receives projections from the MeA and BNST (De Vries and Buijs, 1983; Caffé et al., 1985) and contains VP receptors (Johnson et al., 1993), it has been the focus of studies on social recognition. Injections of VP into the LS can enhance social recognition (Dantzer et al., 1987). Conversely, V1aR antagonists or antisense oligonucleotides can inhibit or reduce social recognition (Landgraf et al., 1995). Social recognition is improved when V1aR expression is artificially increased in the LS of rats using a viral vector expressing a vole V1aR gene (Landgraf et al., 2003).

The use of V1aRKO and V1bRKO mice has provided valuable insight into the role of VP in the regulation of social recognition. V1bRKO mice do not show normal chemoinvestigatory behaviors and have mild impairments in social recognition (Wersinger et al., 2002). V1bRKO mice do have normal olfaction, and there are no known differences in fos-like immunoreactivity between V1bRKO mice and WT mice following exposure to the odor of a conspecific male. These results suggest that V1bRKO mice process initial olfactory information normally (Wersinger et al., 2002). Interestingly, V1bRKO females do not show pregnancy block when exposed to a novel male (the Bruce effect); they remain pregnant, as if they do not recognize a stranger mouse as "new" (Temple et al., 2003). Studies utilizing an operant conditioning task to examine olfactory discrimination have confirmed that V1bKO mice can discriminate between male and female urine even though they do not spend more time investigating female than male bedding. It has been suggested that they lack normal social motivation based on these data. They can distinguish male from female, but it is as if they just do not care; therefore, they do not behave in a socially appropriate manner (Wersinger et al., 2002, 2004). Further exploration of this hypothesis will be interesting. It may be that V1bR and V1aR differentially regulate very specific aspects of social recognition. Recent studies examining V1aRKO mice suggest that they have much more profound impairments in social recognition than do V1bRKO mice (Bielsky et al., 2003). Upon repeated exposure to the same female, V1aRKO male mice fail to reduce their olfactory investigation. Since studies of V1aRKO mice show normal olfactory investigation and habituation, the authors suggest that the V1aR is critical for the appropriate processing of olfactory cues (Bielsky et al., 2003).

The effects of OT on social recognition are more complex. There are conflicting reports in rats, where OT has been shown to both facilitate and inhibit social recognition ( Popik et al., 1996; Dluzen et al., 1998). While high concentrations of OT injected into the LS enhance social recognition in the rat, low doses of OT injected into the MPOA are more effective (Popik and Van Ree, 1991). Interestingly, OTR antagonists infused into the LS or MPOA do not block social recognition (Popik et al., 1996). Possible explanations for the discrepancies between agonist and antagonist studies include, the use of antagonists that were not highly selective and that OT could affect social recognition via V1 receptors.

While studies in rats may not clearly point to OT for the regulation of social recognition, this is not the case in mouse studies. OTKO mice do not display normal social recognition (Ferguson et al., 2000), and comparisons between OTKO and estrogen receptor alpha and beta KO mice suggest the effects of OT on social recognition are gonadal-steroid-dependent (Choleris et al., 2003). The differences in social recognition between WT and OTKO mice are likely due to differences in the processing of olfactory information. OTKO mice have decreased c-fos activation within the MeA, BNST, and MPOA (Ferguson et al., 2001). All three of these areas process olfactory information downstream of the accessory olfactory bulb (Meredith,

1991). In fact, if OT is injected prior to a social encounter in OTKO mice, either ICV or intracerebrally directly into the CeA, social recognition is fully restored. Conversely, ICV injections of OT antagonists into WT mice reduce social recognition. OT has no effects on social memory if it is administered after the encounter (Ferguson et al., 2001), suggesting that OT is critical for memory acquisition rather than memory recall. Another example of the impaired social recognition is displayed by OTKO mice, which when exposed to their first mate or a novel mate (Bruce effect), do not remain pregnant (Temple et al., 2003). Choleris et al. (2004) have proposed a four-gene micronet for the regulation of social recognition in mice that includes estrogen receptor alpha, estrogen receptor beta, OT, and the OTR, although the reduced social recognition in the V1aRKO and V1bRKO suggests a much more complicated scenario in which numerous genes are involved.

**5.2.2.2 Pair Bonding** Pair bonding is the monogamous relationship between sexual partners. Pair bond formation is studied by measuring an animal's partner preference (the amount of time individuals spend with their respective partners versus strangers). While monogamy is rare among mammalian species, being found in fewer than 5% of species (Kleiman, 1977), understanding its neural basis continues to be an exciting area of research. One of the questions driving this field of research is why does one species demonstrate monogamy while another species does not?

The first work linking OT and VP to partner preference was completed in prairie voles (Carter et al., 1992; Winslow et al., 1993). Voles have continued to be the model species of choice since there are monogamous as well as polygamous species within the genus *Microtus*. As mentioned previously, prairie and pine voles tend to be social and monogamous while montane and meadow voles tend to be asocial and polygamous. The facilitation of partner preference by OT and VP is sex specific, with OT being more important in females and VP in males. Female prairie voles administered OT ICV develop a partner preference more rapidly, while an OT antagonist given prior to mating can block partner preference formation (Williams et al., 1994; Insel and Hulihan, 1995; Cho et al., 1999). In male prairie voles, VP and V1aR antagonists facilitates and inhibit formation of partner preference, respectively (Winslow et al., 1993; Cho et al., 1999). The reason for this sex difference remains poorly understood since there are no discernable differences in OTR and V1aR densities and distributions between male and female prairie voles. A recent study has suggested that neonatal exposure to OT can increase partner preference in adult males (Bales and Carter, 2003a).

While the exact basis for the sex difference remains to be determined, the reasons why OT and VP have differential effects in monogamous versus polygamous voles is better understood. Polygamous voles have a lower density of OTR in the caudate-putamen and nucleus accumbens compared with monogamous voles (Insel and Shapiro, 1992). They also have lower densities of V1aR in the ventral pallidum, MeA, and thalamus (Insel et al., 1994). Most of the aforementioned areas are a part of the mesolimbic dopamine reward pathway, suggesting that in certain species, pair bonding may be reinforcing (Insel and Young, 2001; Insel, 2003). This hypothesis is supported by studies that have shown that dopamine acting through $D_2$ receptors within the nucleus accumbens is necessary for partner preference formation (Gingrich et al., 2000; Aragona et al., 2003a, b; Liu and Wang, 2003).

There is increasing support for the idea that animals are polygamous or monogamous partly because of differences in the distribution of OT and VP receptors. Transgenic mice that express the prairie vole V1aR gene in a prairie vole-like pattern show increased affiliative behaviors when VP is injected ICV (Young et al., 1999). VP induces increased partner preference in polygamous meadow voles in which the prairie vole V1aR is overexpressed in the ventral pallidum via a viral vector (Lim et al., 2004b). The molecular basis of pair bonding, as reflected in the distribution patterns of V1aR expression, has been attributed to differences in microsatellite sequence length in the 5′-UTR region of the V1aR coding sequence. These microsatellite sequences are repetitive, unstable (Li et al., 2004), and can modulate gene expression levels and regional distribution (Hammock and Young, 2002; Hammock et al., 2004). It is thought that microsatellite sequences are more susceptible to mutation and may represent a mechanism for the generation of individual variation within a species (Hammock and Young, 2002; Phelps and Young, 2003; Lim et al., 2004b). For a more in depth review of pair bonding and its genetic regulation, see articles by Insel (2003), Young and Wang (2004), Aragona and Wang (2004), and Lim et al. (2004a).

## 5.3 Other Behavioral and Physiological Effects

### 5.3.1 Scent Marking and Grooming

The most extensive work examining the role of VP in scent marking has been done in Syrian hamsters. Syrian hamsters have a specialized form of scent marking known as flank marking. Flank marking is displayed for several reasons including marking territory, attracting a mate, and informing others of their dominance status (Johnson, 1973). In 1984, Ferris and colleagues made the serendipitous finding that flank marking was induced when VP was injected unilaterally into the MPOA-AH (Ferris et al., 1984). The MPOA-AH is thought to be the critical regulatory site for this behavior, because lesions of the MPOA-AH, but not other sites, result in reductions in flank marking (Ferris et al., 1986). Not only does VP induce flank marking, but also does so in a dose-dependent manner. Concentrations ranging from 0.09 μM to 90 μM induce from 3 to 80 flank marks, respectively, within a 10-min period (Ferris and Potegal, 1988). The facilitation of flank marking is also testosterone-dependent. When hamsters are castrated, there are significant declines in flank marking that can be restored following treatment with exogenous testosterone (Albers et al., 1988). The effects of VP on flank marking are mediated primarily through V1aR. Specific antagonists for the V1aR have been shown to significantly reduce levels of VP-induced flank marking and odor-induced flank marking (Ferris et al., 1985, 1988; Albers et al., 1986). In female Syrian hamsters, OT has been found to facilitate flank marking when injected into the MPOA-AH of socially dominant female hamsters. Similar to what has been found in VP-facilitated aggression, social experience is critical for OT's effects on flank marking as socially naïve females show no increases in flank marking compared with controls (Harmon et al., 2002b).

The only other species in which VP has been shown to have an effect on scent marking is in male squirrel monkeys. When VP is administered centrally during a social separation test, squirrel monkeys will increase their scent marking and grooming behavior (Winslow and Insel, 1991a). In other species, VP tends to affect grooming. In mice, VP injected in the MPOA can induce grooming (Meisenberg and Simmons, 1982; Lumley et al., 2001). In rats, OT, rather than VP, given ICV can induce self-grooming in males and females and this effect of OT is inhibited by an OTR antagonist (Delanoy et al., 1978; Caldwell et al., 1986; Drago et al., 1986, 1991). OT-induced grooming has even been used as a way to measure the sensitivity of OT receptors in OTKO mice. OTKO mice given OT ICV show increased grooming behavior compared with WT controls, suggesting that in OTKO mice, the OTR is more responsive to OT (Amico et al., 2004).

### 5.3.2 Anxiety and Depression

Anxiety[1] is one of the behavioral manifestations of stress. While OT has been consistently linked to anxiety, the role of VP has been much less clear. However, there is increasing evidence that both OT and VP are important in the modulation of anxiety in a variety of species. In two rat lines bred for high- or low-anxiety-related behavior, there is a correlation between increased VP mRNA and VP release from the PVN and the high-anxiety phenotype (Wigger et al., 2004). The increase in VP in the PVN has been attributed to an overexpression of VP due to an impaired repression of the VP promoter (Murgatroyd et al., 2004). These studies suggest that VP release from the PVN may be important to the regulation of anxiety in males. The LS is also an area demonstrated to be involved in the regulation of anxiety. Lesions of the LS and V1aR antisense oligonucleotides infused into the LS result in decreases in anxiety-like behaviors in rats (Landgraf et al., 1995; Menard and Treit, 1996). Interestingly, disruption of the V1bR in mice has not been found to affect anxiety-like behaviors (Wersinger et al., 2003). However, V1aRKO males are significantly less anxious than WT males (Bielsky et al., 2003). Therefore, it may be that the V1aR is the more critical receptor for the

[1] When anxiety is mentioned in relation to animals, one is really referring to behaviors that are affected with a similar rank order of potency by agents ("anxiolytics") that are used to treat anxiety in humans. Similar caveats are applied when anthropomorphizing any human mental disturbance.

effect of VP on anxiety. The V1bR antagonist SSR149415 shows weak anxiolytic activity but stronger activity indicative of antidepressant potential in rats (Griebel et al., 2002). This selective V1bR antagonist, when injected into the LS of rats, has antidepressant-like effects on their behavior (Stemmelin et al., 2005). Finally, a single-nucleotide polymorphism in the V1bR gene in humans has been reported to have a protective effect on recurrent major depression (van West et al., 2004).

In birds, OT injected ICV decreases food intake and pecking frequency, suggesting that their general state of arousal and anxiety may be increased (Jonaidi et al., 2003). In rats and mice, OT has been characterized as an anxiolytic (Windle et al., 1997; Neumann et al., 2000; Bale et al., 2001; McCarthy et al., 1996). During the perinatal period, rats show increased anxiety-like behavior, compared with virgin female controls. These behaviors can be enhanced following treatment with an OTR antagonist. However, in virgin female and male rats, treatment with the OTR antagonist has no effect on anxiety-like behaviors (Neumann et al., 2000; Neumann, 2001). Female OTKO mice have higher level of anxiety-like behavior than WT animals, and this anxiety-like behavior can be decreased by ICV administration of OT (Mantella et al., 2003). In contrast to females, male OTKO mice display less anxiety-like behavior (Winslow et al., 2000; Mantella et al., 2003). Overall, it appears that OT may be more important to the regulation of anxiety in females and VP in males. It will be interesting to find out if the regulation of anxiety-like behaviors is truly sexually dimorphic, using two completely different neuroanatomical circuits, or if the main difference lies in the type of neuropeptide and receptor.

### 5.3.3 Learning and Memory

Throughout the 1960s and 1970s, David De Wied dominated this field by examining the effects of VP and OT on learning and memory. His earliest study in 1965 found that in rats, removal of the posterior pituitary impaired active avoidance shuttlebox performance (De Weid, 1965). He subsequently showed that this impairment is improved by treatment with VP (De Weid, 1976). Not only did he find that VP facilitates memory processing, but that its effects are more robust during consolidation and retrieval rather than during learning. By chemically altering VP, he was able to determine which parts of the peptide (VP and OT "metabolites") were biologically active in the aspects of learning and memory he studied (De Weid et al., 1993). He also showed that OT acts as a natural amnestic agent by impairing memory consolidation and retrieval (De Weid et al., 1991). De Wied's theories on learning and memory have been challenged. Sahgal and colleagues propose that both central and peripheral VP increase baseline arousal, which in turn alters learning and memory (Sahgal, 1984; Sahgal and Wright, 1984). While this argument has yet to be settled, especially with regard to the existence of VP and OT metabolite receptors, there is still ongoing research examining the roles of OT and VP on learning and memory, including spatial memory.

While it is known that rodents that give birth have improved spatial memory, only recently has it been shown that OT has a role. In a study by Tomizawa and colleagues (2003), OT given ICV to mice that have never been pregnant increased spatial learning (reference memory only with no effect on working memory). Conversely, they showed that an OTR antagonist administered ICV to females that had delivered several litters inhibited spatial learning. Further, they suggest that OT improves spatial learning by stimulating long-lasting, long-term potentiation and phosphorylation of the cAMP responsive element binding protein in the hippocampus (Tomizawa et al., 2003). It will be interesting to see if these hypotheses are confirmed and expanded to include males.

Some studies in male rats and mice have suggested that VP acting on the V1aR is important for normal spatial memory. VP microinjected into the ventral hippocampus of rats can improve scopolamine-induced impairment of spatial memory (Fujiwara et al., 1997). Conversely V1aR agonists enhance spatial memory (Mishima et al., 2003), whereas V1aR antagonists, but not V2 antagonists, suppress this effect (Mishima et al., 2001; Egashira et al., 2004). Recent work examining spatial memory in V1aRKO male mice has supported the idea that VP may be important to spatial memory. V1aRKO mice show more errors in the eight-arm radial maze than do WT mice (Egashira et al., 2004). Interestingly, no impairments are seen in the Morris water maze. The authors suggest that this is because the eight-arm radial maze is also testing working memory, and it is this aspect of memory that may be affected by the lack of V1aR (Egashira et al.,

2004). To date no deficits in spatial memory have been observed in V1bRKO (Wersinger et al., 2002; Egashira et al., 2004).

### 5.3.4 Fever

During late pregnancy and in newborns, there is a natural suppression of fever concurrent with increases in circulating VP (Alexander et al., 1974; Stark et al., 1979). Studies examining circulating VP find no effect on fever reduction (antipyresis) (Cooper et al., 1979). However, VP infused into the septal area of the brain in several species reduces fever (Cooper et al., 1979; Naylor et al., 1985; Cooper et al., 1987). Fever is also reduced when the BNST is electrically stimulated, resulting in the release of VP into the septum (Naylor et al., 1988). These effects are thought to be mediated through the V1aR based on agonist/antagonist studies (Cooper et al., 1987; Landgraf et al., 1990). The interested reader is referred to a recent review (Roth et al., 2004).

## 6 Future Directions

The future for research into the roles of VP and OT in brain function is bright. There is ample work to be done in the continuing examination of knockout mice. This work will be considerably aided through the use of conditional knockouts and virally mediated interventions so that the possibility of developmental compensation is avoided. Obviously, the relevance of these studies to human behavior will remain a strong focus. Although not covered in this review, the development of specific and orally active pharmacologic VP and OT agents will play a critical role in sharpening this focus. Recent publications discuss some of the advances in this field of pharmacology (Serradeil-Le Gal et al., 2002; Cirillo et al., 2003; Pitt et al., 2004).

Other recent work in nonhuman animals investigates whether some human diseases might be caused by dysfunctions of the VP and OT systems. Attention has focused on autism and the OTR and V1aR given their roles in social recognition. To date, however, links have been tantalizing but not definitive (Auranen et al., 2002; Kim et al., 2002; Shao et al., 2002; Wassink et al., 2004). The V1aR has also been investigated for roles in sexual fidelity (Cherkas et al., 2004) and eating habits (Bachner-Melman et al., 2004). Although the V1bR has not been implicated in the above behaviors, a recent study has proposed a protective role against major depression (van West et al., 2004). Linkage analysis remains a promising avenue of research. Coupled with the development of better behavioral animal models, the next decade should be a rewarding one for investigators of OT and VP.

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## References

Acher R. 1990. Structure, evolution and processing adaptation of neurohypophysial hormone-neurophysin precursors. Progress in comparative endocrinology. Epple A, Scanes CG, Stetson MH, editors. New York: Wiley; pp. 1-9.

Acher R, Chauvet J. 1995. The neurohypophysial endocrine regulatory cascade: precursors, mediators, receptors, and effectors. Front Neuroendocrinol 16: 237-289.

Acher R, Chauvet J, Chauvet MT. 1995. Man and chimera: selective versus neutral oxytocin evolution. Adv Exp Med Biol 395: 615-627.

Acher R, Chauvet J, Rouille Y. 2002. Dynamic processing of neuropeptides: sequential conformation shaping of neurohypophysial preprohormones during intraneuronal secretory transport. J Mol Neurosci 18: 223-228.

Adan RA, Cox JJ, Beischlag TV, Burbach JP. 1993. A composite hormone response element mediates the tranactivation of the rat oxytocin gene by different classes of nuclear hormone receptors. Mol Endocrinol 7: 47-57.

Adan RA, Cox JJ, van Kats JP, Burbach JP. 1992. Thyroid hormone regulates the oxytocin gene. J Biol Chem 267: 3771-3777.

Adan RA, Walther N, Cox JJ, Ivell R, Burbach JP. 1991. Comparison of the estrogen responsiveness of the rat and bovine oxytocin gene promoters. Biochem Biophys Res Commun 175: 117-122.

Aguilera G, Rabadan-Diehl C. 2000. Regulation of vasopressin V1b receptors in the anterior pituitary gland. Exp Physiol 85: S19-S26.

Aguilera G, Volpi S, Rabadan-Diehl C. 2003. Transcriptional and post-transcriptional mechanism regulating the rat pituitary vasopressin V1b receptor gene. J Mol Endocrinol 30: 99-108.

Albers HE, Ferris CF. 1985. Behavioral effects of vasopressin and oxytocin within the medial preoptic area of the golden hamster. Regul Pept 12: 257-260.

Albers HE, Liou SY, Ferris CF. 1988. Testosterone alters the behavioral response of the medial preoptic-anterior hypothalamus to microinjection of arginine vasopressin in the hamster. Brain Res 456: 382-386.

Albers HE, Liou SY, Ferris CF, Stopa EG, Zoeller RT. 1991a. Neurochemistry of circadian timing. Suprachiasmatic nucleus: the mind's clock. Klein DC, Moore RY, Reppert SM, editors. New York: Oxford University Press; pp. 263-288.

Albers HE, Liou SY, Stopa EG, Zoeller RT. 1991b. Interaction of colocalized neuropeptides: functional significance in the circadian timing system. J Neurosci 11: 846-851.

Albers HE, Pollock J, Simmons WH, Ferris CF. 1986. A V1-like receptor mediates vasopressin-induced flank marking behavior in hamster hypothalamus. J Neurosci 6: 2085-2089.

Albers HE, Rowland CM, Ferris CF. 1991c. Arginine-vasopressin immunoreactivity is not altered by photoperiod or gonadal hormones in the Syrian hamster (*Mesocricetus auratus*). Brain Res 539: 137-142.

Alexander DP, Bashore RA, Britton RA, Forsling MA. 1974. Maternal and fetal arginine vasopressin in the chronically catheterized sheep. Biol Neonate 25: 242-248.

Alonso G, Szafarczyk A, Assenmacher I. 1986. Radioautographic evidence that axons from the area of supraoptic nulei in the rat project to extrahypothalamic brain regions. Neurosci Lett 66: 251-256.

Alves SE, Lopez V, McEwen BS, Weiland NG. 1998. Differential colocalization of estrogen receptor beta (ERbeta) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: an immunocytochemical study. Proc Natl Acad Sci USA 95: 3281-3286.

Amico JA, Vollmer RR, Karam JR, Lee PR, Li X, et al. 2004. Centrally administered oxytocin elicits exaggerated grooming in oxytocin null mice. Pharmacol Biochem Behav 78: 333-339.

Andersson KE. 2001. Pharmacology of penile erection. Pharmacol Rev 53: 417-450.

Antoni FA. 1984. Novel ligand specificity of pituitary vasopressin receptors in the rat. Neuroendocrinology 39: 186-188.

Antoni FA. 1993. Vasopressinergic control of pituitary adrenocorticotropin secretion comes of age. Front Neuroendocrinol 14: 76-122.

Aragona BJ, Liu Y, Cameron A, Perlman G, Wang ZX. 2003a. Opposite modulation of social attachment by D1- and D2-type dopamine receptor activation in nucleus accumbens shell. Horm Behav 44, 37. [Abstract]

Aragona BJ, Liu Y, Curtis JT, Stephan FK, Wang ZX. 2003b. A critical role for nucleus accumbens dopamine in partner preference formation of male prairie voles (*Microtus ochrogaster*). J Neurosci 23: 3483-3490.

Aragona BJ, Wang Z. 2004. The prairie vole (*Microtus ochrogaster*): an animal model for behavioral neuroendocrine research on pair bonding. ILAR J 45: 35-45.

Archer R, du Vigneaud V. 1953. La structure de la vasopressine de boeuf. Biochim Biochim Acta (Amst) 12: 487-488.

Argiolas A. 1999. Neuropeptides and sexual behaviour. Neurosci Biobehav Rev 23: 1127-1142.

Argiolas A, Collu M, Gessa GL, Melis MR, Serra G. 1988. The oxytocin antagonist d(CH2)5Tyr(Me)-Orn8-vasotocin inhibits male copulatory behaviour in rats. Eur J Pharmacol 149: 389-392.

Argiolas A, Melis MR. 2004. The role of oxytocin and the paraventricular nucleus in the sexual behaviour of male mammals. Physiol Behav 83: 309-317.

Argiolas A, Melis MR, Gessa GL. 1985. Intraventricular oxytocin induces yawning and penile erection in rats. Eur J Pharmacol 117: 395-396.

Arima H, House SB, Gainer H, Aguilera G. 2001. Direct stimulation of arginine vasopressin gene transcription by cAMP in parvocellular neurons of the paraventricular nucleus in organotypic cultures. Endocrinology 142: 5027-5030.

Arima H, House SB, Gainer H, Aguilera G. 2002. Neuronal activity is required for the circadian rhythm of vasopressin gene transcription in the suprachiasmatic nucleus in vitro. Endocrinology 143: 4165-4171.

Arsenijevic Y, Dubois-Dauphin M, Tribollet E, Manning M, Sawyer WH, et al. 1994. Vasopressin-binding sites in the pig pituitary gland: competition by novel vasopressin antagonists suggests the existence of an unusual receptor subtype in the anterior lobe. J Endocrinol 141: 383-391.

Auranen M, Vanhala R, Varilo T, Ayers K, Kempas E, et al. 2002. A genomewide screen for autism-spectrum disorders: evidence for a major susceptibility locus on chromosome 3q25-27. Am J Hum Genet 71: 777-790.

Bachner-Melman R, Zohar AH, Elizur Y, Nemanov L, Gritsenko I, et al. 2004. Association between a vasopressin receptor AVPR1A promoter region microsatellite and eating behavior measured by a self-report questionnaire (Eating Attitudes Test) in a family-based study of a nonclinical population. Int J Eat Disord 36: 451-460.

Bale TL, Davis AM, Auger AP, Dorsa DM, McCarthy MM. 2001. CNS region-specific oxytocin receptor expression: importance in regulation of anxiety and sex behavior. J Neurosci 21: 2546-2552.

Bale TL, Dorsa DM. 1995. Regulation of oxytocin receptor messenger ribonucleic acid in the ventromedial hypothalamus by testosterone and its metabolites. Endocrinology 136: 5135-5138.

Bale TL, Dorsa DM. 1997. Cloning, novel promoter sequence, and estrogen regulation of a rat oxytocin receptor gene. Endocrinology 138: 1151-1158.

Bale TL, Dorsa DM. 1998. NGF, cyclic AMP, and phorbol esters regulate oxytocin receptor gene transcription in SK-N-SH and MCF7 cells. Brain Res Mol Brain Res 53: 130-137.

Bale TL, Pedersen CA, Dorsa DM. 1995. Oxytocin receptor mRNA expression in the ventromedial hypothalamus during the estrous cycle. J Neurosci 15: 5058-5064.

Bales KL, Carter CS. 2003a. Developmental exposure to oxytocin facilitates partner preferences in male prarie voles (*Microtus ochrogaster*). Behav Neurosci 117: 854-859.

Bales KL, Carter CS. 2003b. Sex differences and developmental effects of oxytocin on aggression and social behavior in prairie voles (*Microtus ochrogaster*). Horm Behav 44: 178-184.

Bankir L. 2001. Antidiuretic action of vasopressin: quantitative aspects and interaction between V1a and V2 receptor-mediated effects. Cardiovasc Res 51: 372-390.

Barat C, Simpson L, Breslow E. 2004. Properties of human vasopressin precursor constructs: inefficient monomer folding in the absence of copeptin as a potential contributor to diabetes insipidus. Biochemistry 43: 8191-8203.

Barberis C, Morin D, Durroux T, Mouillac B, Guillon G, et al. 1999. Molecular pharmacology of AVP and OT receptors and therapeutic potential. Drug News Perspect 12: 279-292.

Benelli A, Poggioli R, Luppi P, Ruini L, Bertolini A, et al. 1994. Oxytocin enhances, and oxytocin antagonism decreases, sexual receptivity in intact female rats. Neuropeptides 27: 245-250.

Bielsky IF, Hu S.-B, Szegda KL, Westphal H, Young LJ. 2003. Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. Neuropsychopharmacology 29: 483-493.

Bielsky IF, Young LJ. 2004. Oxytocin, vasopressin, and social recognition in mammals. Peptides 25: 1565-1574.

Blanchard RJ, Griebel G, Farrokhi C, Markham C, Yang M, et al. 2005. AVP V(1b) selective antagonist SSR149415 blocks aggressive behaviors in hamsters. Pharmacol Biochem Behav 80: 189-194.

Boyd SK, Moore FL. 1992. Sexually dimorphic concentrations of arginine vasotocin in sensory regions of the amphibian brain. Brain Res 588: 304-306.

Brain PF. 1972. Effects of isolation/grouping on endocrine function and fighting behavior in male and female golden hamsters (*Mesocricetus auratus* Waterhouse). Behav Biol 7: 349-357.

Breton C, Nuculcea J, Zingg HH. 1996. Renal oxytocin receptor messenger ribonucleic acid: characterization and regulation during pregnancy and in response to ovarian steroid treatment. Endocrinology 137: 2711-2717.

Breton C, Zingg HH. 1997. Expression and region-specific regulation of the oxytocin receptor gene in rat brain. Endocrinology 138: 1857-1862.

Brot MD, De Vries GJ, Dorsa DM. 1993. Local implants of testosterone metabolites regulate vasopressin mRNA in sexually dimorphic nuclei of the rat brain. Peptides 14: 933-940.

Brownstein MJ, Russel JT, Gainer H. 1980. Synthesis, transport, and release of posterior pituitary hormones. Science 207: 373-378.

Buijs RM. 1978. Intra- and extrahypothalamic vasopressin and oxytocin pathways in the rat. Pathways to the limbic system, medulla oblongata and spinal cord. Cell Tissue Res 192: 423-435.

Buijs RM. 1987. Vasopressin localization and putative functions in the brain. Vasopressin: principles and properties. Gash DM, Boer GJ, editors. New York: Plenum Press; pp. 91-115.

Buijs RM, De Vries GJ, Van Leeuwen FW, Swaab DF. 1983. Vasopressin and oxytocin: distribution and putative functions in the brain. Prog Brain Res 60: 115-122.

Burbach JP, Adan RA. 1993. The rat oxytocin gene. Physiological changes in expression in the hypothalamo-neurohypophysial system and responsiveness of promoter activity. Ann N Y Acad Sci 689: 34-49.

Burbach JP, Adan RA, de Bree FM. 1992. Regulation of oxytocin gene expression and forms of oxytocin in the brain. Ann N Y Acad Sci 652: 1-13.

Burbach JP, Adan RA, Cox JJ, da Silva SL. 1993. Transactivation of the rat oxytocin and vasopressin promoters by nuclear hormone receptors. Regul Pept 45: 31-35.

Burbach JP, Adan RA, Van Tol HH, Verbeeck MA, Axelson JF, et al. 1990. Regulation of the rat oxytocin gene by estradiol:

examination of promoter activity in transfected cells and of messenger ribonucleic acid and peptide levels in the hypothalamo-neurohypophyseal system. J Neuroendocrinol 2: 639.

Burbach JP, Liu B, Voorhuis TA, Van Tol HH. 1988. Diurnal variation in vasopressin and oxytocin messenger RNAs in hypothalamic nuclei of the rat. Brain Res 464: 157-160.

Burbach JP, Luckman SM, Murphy D, Gainer H. 2001. Gene regulation in the magnocellular hypothalamo-neurohypophysial system. Physiol Rev 81: 1197-1267.

Burnatowska-Hledin MA, Spielman WS, Smith WL, Shi P, Meyer JM, et al. 1995. Expression of an AVP-activated, calcium mobilizing receptor from rabbit kidney medulla. Am J Physiol 268: F1198-F1210.

Caffè AR, Van Leeuwen FW. 1983. Vasopressin-immunoreactive cell bodies in the dorsomedial hypothalamic region, medial amygdaloid nucleus, and locus coeruleus of the rat. Cell Tissue Res 233: 23-33.

Caffè AR, Van Leeuwen FW, Buijs RM, De Vries GJ, Geffard M. 1985. Vasopressin cells in the medial amygdala of the rat project to the lateral septum and ventral hippocampus. Brain Res 338: 160-164.

Caldwell HK, Albers HE. 2003. Short-photoperiod exposure reduces vasopressin (V1a) receptor binding but not arginine-vasopressin-induced flank marking in male Syrian hamsters. J Neuroendocrinol 15: 971-977.

Caldwell HK, Albers HE. 2004a. Effect of photoperiod on vasopressin-induced aggression in Syrian hamsters. Horm Behav 46: 444-449.

Caldwell HK, Albers HE. 2004b. Photoperiodic regulation of vasopressin receptor binding in female Syrian hamsters. Brain Res 1002: 136-141.

Caldwell JD, Drago F, Prange AJ Jr, Pedersen CA. 1986. A comparison of grooming behavior potencies of neurohypophysial nonapeptides. Regul Pept 14: 261-271.

Caldwell JD, Jirikowski GF, Greer ER, Pedersen CA. 1989. Medial preoptic area oxytocin and female sexual receptivity. Behav Neurosci 103: 655-662.

Caldwell JD, Moe BD. 1999. Conjugated estradiol increases female sexual receptivity in response to oxytocin infused into the medial preoptic area and the medial basal hypothalamus. Horm Behav 35: 38-46.

Carmichael MS, Humbert R, Dixon J, Palmisano G, Greenleaf W, et al. 1987. Plasma oxytocin increases in the human sexual response. J Clin Endocrinol Metab 64: 27-31.

Carrazana EJ, Pasieka KB, Majzoub JA. 1988. The vasopressin mRNA poly(A) tract is unusually long and increases during stimulation of vasopressin gene expression in vivo. Mol Cell Biol 8: 2267-2274.

Carter DA, Murphy D. 1989. Independent regulation of neuropeptide mRNA level and poly(A) tail length. J Biol Chem 264: 6601-6603.

Carter DA, Murphy D. 1990. Regulation of c-fos and c-jun expression in the rat supraoptic nucleus. Cell Mol Neurobiol 10: 435-445.

Carter CS, Williams JR, Witt DM, Insel TR. 1992. Oxytocin and social bonding. Ann N Y Acad Sci 652: 204-211.

Castel M, Morris JF. 1988. The neurophysin-containing innervation of the forebrain of the mouse. Neuroscience 24: 937-966.

Ceremuga TE, Yao XL, McCabe JT. 2003a. Cullin-5 is ubiquitous in the rat brain. Neurosci Lett 345: 121-125.

Ceremuga TE, Yao XL, Xia Y, Mukherjee D, McCabe JT. 2003b. Osmotic stress increases cullin-5 (cul-5) mRNA in the rat cerebral cortex, hypothalamus and kidney. Neurosci Res 45: 305-311.

Champagne F, Diorio J, Sharma S, Meaney MJ. 2001. Naturally occuring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. Proc Natl Acad Sci USA 98: 12736-12741.

Cherkas LF, Oelsner EC, Mak YT, Valdes A, Spector TD. 2004. Genetic influences on female infidelity and number of sexual partners in humans: a linkage and association study of the role of the vasopressin receptor gene (AVPR1A). Twin Res 7: 649-658.

Cho MM, De Vries AC, Williams JR, Carter CS. 1999. The effects of oxytocin and vasopressin on partner preferences in male and female prairie voles (*Microtus ochrogaster*). Behav Neurosci 113: 1079.

Choleris E, Gustafsson JA, Korach KS, Muglia LJ, Pfaff DW, et al. 2003. An estrogen-dependent four-gene micronet regulating social recognition: a study with oxytocin and estrogen receptor-alpha and -beta knockout mice. Proc Natl Acad Sci USA 100: 6192-6197.

Choleris E, Kavaliers M, Pfaff DW. 2004. Functional genomics of social recognition. J Neuroendocrinol 16: 383-389.

Chooi KF, Carter DA, Murphy D. 1992. Decrease in hypothalamic vasopressin mRNA poly(A) tail length following physiological stimulation. Cell Mol Neurobiol 12: 557-567.

Ciosek J. 2002. Vasopressin and oxytocin release as influenced by thyrotropin-releasing hormone in euhydrated and dehydrated rats. J Physiol Pharmacol 53: 423-437.

Cirillo R, Gillio Tos E, Schwarz MK, Quattropani A, Scheer A, et al. 2003. Pharmacology of (2S,4Z)-*N*-[(2s)-2-hydroxy-2-phenylethyl]-4-(methoxyimino)-1-[(2′-methyl[1,1′-biphenyl]-4-yl)carbonyl]-2-pyrrolidinecarboxamide, a new potent and selective nonpeptide antagonist of the oxytocin receptor. J Pharmacol Exp Ther 306: 253-261.

Coirini H, Johnson AE, McEwen BS. 1989. Estradiol modulation of oxytocin binding in the ventromedial hypothalamic nucleus of male and female rats. Neuroendocrinology 50: 193-198.

Compaan JC, Buijs RM, Pool CW, De Ruiter AJH, Koolhaas JM. 1993. Differential lateral septal vasopressin innervation in aggressive and nonaggressive male mice. Brain Res Bull 30: 1-6.

Cooper KE, Kasting NW, Lederis K, Veale WL. 1979. Evidence supporting a role for endogenous vasopressin in natural suppression of fever in the sheep. J Physiol 295: 33-45.

Cooper KE, Naylor AM, Veale WL. 1987. Evidence supporting a role for endogenous vasopressin in fever suppression in the rat. J Physiol 387: 163-172.

Coulson JM. 2002. Positive and negative regulators of the vasopressin gene promoter in small cell lung cancer. Prog Brain Res 139: 329-343.

Crowley RS, Amico JA. 1993. Gonadal steroid modulation of oxytocin and vasopressin gene expression in the hypothalamus of the osmotically stimulated rat. Endocrinology 133: 2711-2718.

Dale HH. 1906. On some physiological actions of ergot. J Physiol (Lond) 34: 163-206.

Dantzer R, Bluthe RM, Koob GF, Le Moal M. 1987. Modulation of social memory in male rats by neurohypophyseal peptides. Psychopharmacology (Berl) 91: 363-368.

de Bree FM. 2000. Trafficking of the vasopressin and oxytocin prohormone through the regulated secretory pathway. J Neuroendocrinol 12: 589-594.

de Kloet ER, Voorhuis DA, Boschma Y, Elands J. 1986. Estradiol modulates density of putative 'oxytocin receptors' in discrete rat brain regions. Neuroendocrinology 44: 415-421.

de Kloet ER, Voorhuis DA, Elands J. 1985. Estradiol induces oxytocin binding sites in rat hypothalamic ventromedial nucleus. Eur J Pharmacol 118: 185-186.

De Vries GJ, Best W, Sluiter AA. 1983. The influence of androgens on the development of a sex difference in the vasopressinergic innervation of the rat lateral septum. Brain Res 284: 377-380.

De Vries GJ, Buijs RM. 1983. The origin of the vasopressinergic and oxytocinergic innervation of the rat brain with special reference to the lateral septum. Brain Res 273: 307-317.

De Vries GJ, Buijs RM, Sluiter AR. 1984. Gonadal hormone actions on the morphology of the vasopressinergic innervation of the adult rat brain. Brain Res 298: 141-145.

De Vries GJ, Buijs RM, Swaab DF. 1985. The vasopressinergic innervation of the brain in normal and castrated rats. J Comp Neurol 233: 236-254.

De Vries GJ, Duetz W, Buijs RM, Van Heerikhuize J, Vreeburg JTM. 1986. Effects of androgens and estrogens on the vasopressin and oxytocin innervation of the adult rat brain. Brain Res 399: 296-302.

De Vries GJ, Wang Z, Bullock NA, Numan S. 1994. Sex differences in the effects of testosterone and its metabolites on vasopressin messenger RNA levels in the bed nucleus of the stria terminalis of rats. J Neurosci 14: 1789-1794.

De Weid D. 1965. The influence of the posterior and intermediate lobe of the pituitary and the pituitary peptides on the maintenance of a conditioned avoidance response in rats. Int J Pharmacol 16: 157-167.

De Weid D. 1976. Behavioral effects of intraventricularly administered vasopressin and vasopressin fragments. Life Sci 19: 685-690.

De Weid D, Diamant M, Fodor M. 1993. Central nervous system effects of the neurohypophyseal hormones and related peptides. Front Neuroendocrinol 14: 251-302.

De Weid D, Elands J, Kovács K. 1991. Interactive effects of neurohypophyseal neuropeptides with receptor antagonist on passive avoidance behaivor: mediation by a cerebral neurohypophyseal hormone receptor? Proc Natl Acad Sci USA 88: 1494-1498.

Delanoy RL, Dunn AJ, Tintner R. 1978. Behavioral responses to intracerebroventricularly administered neurohypophyseal peptides in mice. Horm Behav 11: 348-362.

Dellovade TL, Zhu YS, Pfaff DW. 1999. Thyroid hormones and estrogen affect oxytocin gene expression in hypothalamic neurons. J Neuroendocrinol 11: 1-10.

Delville Y, Conklin LS, Ferris CF. 1995. Differential expression of vasopressin receptor binding in the hypothalamus during lactation in golden hamsters. Brain Res 689: 147-150.

Delville Y, De Vries GJ, Ferris CF. 2000. Neural connections of the anterior hypothalamus and agonistic behavior in golden hamsters. Brain Behav Evol 55: 53-76.

Delville Y, Mansour KM, Ferris CF. 1996. Testosterone facilitates aggression by modulating vasopressin receptors in the hypothalamus. Physiol Behav 60: 25-29.

Desmedt A, Garcia R, Jaffard R. 1999. Vasopressin in the lateral septum promotes elemental conditioning to the detriment of contextual fear conditioning in mice. Eur J Neurosci 11: 3913-3921.

DeVries AC, Young WS III, Nelson RJ. 1997. Reduced aggressive behaviour in mice with targeted disruption of the oxytocin gene. J Neuroendocrinol 9: 363-368.

Dluzen DE, Muraoka S, Engelmann M, Landgraf R. 1998. The effects of infusion of arginine vasopressin, oxytocin, or their antagonists into the olfactory bulb upon social recognition responses in male rats. Peptides 19: 999-1005.

Drago F, Pedersen CA, Caldwell JD, Prange AJ. 1986. Oxytocin potently enhances novelty-induced grooming behavior in the rat. Brain Res 368: 287-295.

Drago F, Sarnyaizi Z, D'Agata V. 1991. The inhibition of oxytocin-induced grooming by a specific receptor antagonist. Physiol Behav 50: 533-536.

du Vigneaud V. 1952. A trail of research. Ithaca: Cornell University Press.

du Vigneaud V, Gish DT, Katsoyannis PG. 1954a. A synthetic preparation possessing biological properties associated with arginine–vasopressin. J Am Chem Soc 76: 4751-4752.

du Vigneaud V, Lawler HC, Popenoe EA. 1953a. Enzymic cleavage of glycinamide from vasopressin and a proposed structure for this pressor-antidiuretic hormone of the posterior pituitary. J Am Chem Soc 75: 4880-4881.

du Vigneaud V, Ressler C, Swan JM, Roberts CW, Katsoyannis PG. 1954b. The synthesis of oxytocin. J Am Chem Soc 76: 3115-3121.

du Vigneaud V, Ressler C, Trippett S. 1953b. The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin. J Biol Chem 205: 949-957.

Dubois-Dauphin M, Theler JM, Ouarour A, Pevet P, Barberis C, et al. 1994. Regional differences in testosterone effects on vasopressin receptors and on vasopressin immunoreactivity in intact and castrated Siberian hamsters. Brain Res 638: 267-276.

Dutil J, Moujahidine M, Lemieux C, Jankowski M, Gutkowska J, et al. 2001. Chromosomal and comparative mapping of rat oxytocin, oxytocin receptor and vasopressin genes. Cytogenet Cell Genet 93: 57-59.

Egashira N, Tanoue A, Higashihara F, Mishimi K, Fukue Y, et al. 2004. V1a receptor knockout mice exhibit impairment of spatial memory in an eight-arm radial maze. Neurosci Lett 356: 195-198.

Elliott JC, Lubin DA, Walker CH, Johns JM. 2001. Acute cocaine alters oxytocin levels in the medial preoptic area and amygdala in lactating rat dams: implication for cocaine-induced changes in maternal behavior and maternal aggression. Neuropeptides 35: 127-134.

Everts HGJ, Koolhaas JM. 1999. Differential modulation of lateral septal vasopressin receptor blockade in spatial learning, social recognition, and anxiety-related behaviors in rats. Behav Brain Res 99: 7-16.

Everts HGJ, De Ruiter AJH, Koolhaas JM. 1997. Differential lateral septal vasopressin in wild-type rats: correlation with aggression. Horm Behav 31: 136-144.

Fahrbach SE, Morrell JI, Pfaff DW. 1985. Possible role for endogenous oxytocin in estrogen-facilitated maternal behavior in rats. Neuroendocrinology 40: 526-532.

Farini F. 1913. Diabete insipido ed opoterapia. Gazz Osped Clin 34: 1135-1139.

Ferguson JN, Aldag JM, Insel TR, Young LJ. 2001. Oxytocin in the medial amygdala is essential for social recognition in the mouse. J Neurosci 21: 8278-8285.

Ferguson JN, Young LJ, Hearn EF, Matzuk MM, Insel TR, et al. 2000. Social amnesia in mice lacking the oxytocin gene. Nat Genet 25: 284-288.

Ferris CF, Albers HE, Wesolowski SM, Goldman BD, Luman SE. 1984. Vasopressin injected into the hypothalamus triggers a complex stereotypic behavior in golden hamsters. Science 224: 521-523.

Ferris CF, Delville Y. 1994. Vasopressin and serotonin interactions in the control of agonistic behavior. Psychoneuroendocrinology 19: 593-601.

Ferris CF, Foote KB, Meltser HM, Plenby MG, Smith KL, et al. 1992. Oxytocin in the amygdala facilitates maternal aggression. Ann N Y Acad Sci 652: 456-457.

Ferris CF, Meenan DM, Axelson JF, Albers HE. 1986. A vasopressin antagonist can reverse dominant/subordinate behavior in hamsters. Physiol Behav 38: 135-138.

Ferris CF, Melloni RJ Jr, Koppel G, Perry KW, Fuller RW, et al. 1997. Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in golden hamsters. J Neurosci 17: 4331-4340.

Ferris CF, Pollock J, Albers HE, Leeman SE. 1985. Inhibition of flank-marking behavior in golden hamsters by microinjection of a vasopressin antagonist into the hypothalamus. Neurosci Lett 55: 239-243.

Ferris CF, Potegal M. 1988. Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. Physiol Behav 44: 235-239.

Ferris CF, Singer EA, Meenan DM, Albers HE. 1988. Inhibition of vasopressin-stimulated flank marking behavior by $V_1$-receptor antagonists. Eur J Pharmacol 154: 153-159.

Fields RL, House SB, Gainer H. 2003 Regulatory domains in the intergenic region of the oxytocin and vasopressin genes that control their hypothalamus-specific expression *in vitro.* J Neurosci 23: 7801-7809.

Floody OR, Comerci JT, Lisk RD. 1987. Hormonal control of sex differences in ultrasound production by hamsters. Horm Behav 21: 17-35.

Foletta VC, Brown FD, Young WS III. 2002. Cloning of rat ARHGAP4/C1, a RhoGAP family member expressed in the nervous system that colocalizes with the Golgi complex and microtubules. Brain Res Mol Brain Res 107: 65-79.

Francis DD, Young LJ, Meaney MJ, Insel TR. 2002. Naturally occurring differences in maternal care are associated with the expression of oxytocin and vasopressin (V1a) receptors: gender differences. J Neuroendocrinol 14: 349-353.

Fujiwara M, Ohgami Y, Inada K, Iwasaki K. 1997. Effect of active fragments of arginine–vasopressin on the disturbance of spatial cognition in rats. Brain Res 83: 91-96.

Funabashi T, Shinohara K, Mitsushima D, Kimura F. 2000. Estrogen increases arginine–vasopressin V1a receptor mRNA in the preoptic area of young but not of middle-ages female rats. Neurosci Lett 285: 205-208.

Gainer H, Field RL, House SB. 2001. Vasopressin gene expression: experimental models and strategies. Exp Neurol 171: 190-199.

Gainer H, Wray S. 1994. Oxytocin and vasopressin cell biology. The physiology of reproduction. Knobil E, Neill JD, editors. New York: Raven Press; pp. 1099-1129.

Gimpl G, Fahrenholz F. 2001. The oxytocin receptor system: structure, function, and regulation. Physiol Rev 81: 629-683.

Gingrich B, Liu Y, Cascio C, Wang Z, Insel TR. 2000. Dopamine D2 receptors in the nucleus accumbens are important for social attachment in female prairie voles (*Microtus ochrogaster*). Behav Neurosci 114: 183.

Giovannelli L, Shiromani PJ, Jirikowski GF, Bloom FE. 1992. Expression of c-fos protein by immunohistochemically identified oxytocin neurons in the rat hypothalamus upon osmotic stimulation. Brain Res 588: 41-48.

Giovenardi M, Padoin MJ, Cadore LP, Lucion AB. 1998. Hypothalamic paraventricular nucleus modulates maternal aggression in rats: effects of ibotenic acid lesion and oxytocin antisense. Physiol Behav 63: 351-359.

Glasgow E, Kusano K, Chin H, Mezey E, Young WS III, et al. 1999. Single cell reverse transcription-polymerase chain reaction analysis of rat supraoptic magnocellular neurons: neuropeptide phenotypes and high voltage-gated calcium channel subtypes. Endocrinology 140: 5391-5401.

Goodson JL, Adkins-Regan E. 1999. Effect of intraseptal vasotocin and vasoactive intestinal polypeptide infusions on courtship song and aggression in the male zebra finch (*Taeniopygia guttata*). J Neuroendocrinol 11: 19-25.

Gould BR, Zingg HH. 2003. Mapping oxytocin receptor gene expression in the mouse brain and mammary gland using an oxytocin receptor-LacZ reporter mouse. Neuroscience 122: 155-167.

Grace CO, Fink G, Quinn JP. 1999. Characterization of potential regulatory elements within the rat arginine vasopressin proximal promoter. Neuropeptides 33: 81-89.

Grelk DF, Papson BA, Cole JE, Rowe FA. 1974. The influence of caging conditions and hormone treatments on fighting in male and female hamsters. Horm Behav 5: 355-366.

Griebel G, Simiand J, Serradeil-Le Gal C, Wagnon J, Pascal M, et al. 2002. Anxiolytic- and antidepressant-like effects of the non-peptide vasopressin V1b receptor antagonist, SSR149415, suggest an innovative approach for the treatment of stress-related disorders. Proc Natl Acad Sci USA 99: 6370-6375.

Grimmelikhuijzen CJP. 1984. Peptides in the nervous system of coelenterates. Evolution and tumour pathology of the neuroendocrine system. Falkmer S, Håkanson R, Sundler S, editors. Amsterdam, New York: Elsevier; pp. 39-58.

Hallbeck M, Hermanson O, Blomqvist A. 1999. Distribution of preprovasopressin mRNA in the rat central nervous system. J Comp Neurol 411: 181-200.

Hammock EAD, Lim MM, Young LJ. (2004) Microsatellite polymorphism predicts vasopressin 1a receptor gene expression and behavior in prairie voles. Annu Meet Soc Neurosci Abstract.

Hammock EAD, Young LJ. 2002. Variation in the vasopressin V1a receptor promoter and expression: implications for inter- and intraspecific variation in social behaviour. Eur J Neurosci 16: 399-402.

Han TM, De Vries GJ. 2003. Organization effects of testosterone, estradiol, and dihydrotestosterone on vasopressin mRNA expression in the bed nucleus of the stria terminalis. J Neurobiol 54: 502-510.

Hara Y, Battey J, Gainer H. 1990. Structure of mouse vasopressin and oxytocin genes. Brain Res Mol Brain Res 8: 319-324.

Harmon AC, Huhman KL, Moore TO, Albers HE. 2002a. Oxytocin inhibits aggression in female Syrian hamsters. J Neuroendocrinol 14: 963-969.

Harmon AC, Moore TO, Huhman KL, Albers HE. 2002b. Social experience and social context alter the behavioral response to centrally administered oxytocin in female Syrian hamsters. Neuroscience 109: 767-772.

Hastings MH, Herzog ED. 2004. Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. J Biol Rhythms 19: 400-413.

Hernando F, Schoots O, Lolait SJ, Burbach JP. 2001. Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: anatomical support for its involvement in the central effects of vasopressin. Endocrinology 142: 1659-1668.

Hillegaart V, Alster P, Uvnas-Moberg K, Ahlenius S. 1998. Sexual motivation promotes oxytocin secretion in male rats. Peptides 19: 39-45.

Hirasawa M, Schwab Y, Natah S, Hillard CJ, Mackie K, et al. 2004. Dedritically release transmitters cooperate via autocrine and retrograde actions to inhibit afferent excitation in rat brain. J Physiol 559: 611-624.

Ho M-Y, Murphy D. 2002. The vasopressin gene non-canonical Hogness box: effect on protein binding and promoter function. Mol Cell Endocrinol 186: 17-25.

Holekamp KE, Smale L. 2000. Feisty females and meek males: reproductive strategies in the spotted hyena. Reproduction in context. Wallen K, Schneider JE, editors. Cambridge: The MIT Press; pp. 257-285.

Hou-Yu A, Lamme AT, Zimmerman EA, Silverman AJ. 1986. Comparative distribution of vasopressin and oxytocin neurons in the rat brain using a double-label procedure. Neuroendocrinology 44: 235-246.

Hruby VJ, Chow M, Smith DD. 1990. Conformational and structural considerations in oxytocin-receptor binding and biological activity. Annu Rev Pharmacol Toxicol 30: 501-534.

Hurbin A, Orcel H, Ferraz C, Moos FC, Rabie A. 2000. Expression of genes encoding the vasopressin-activated

calcium-mobilizing receptor and the dual angiotensin II/vasopressin receptor in the rat central nervous system. J Neuroendocrinol 12: 677-684.

Inoue T, Kimura T, Azuma C, Inazawa J, Takemura M, et al. 1994. Structural organization of the human oxytocin receptor gene. J Biol Chem 269: 32451-32456.

Inoue T, Nonoguchi H, Tomita K. 2001. Physiological effects of vasopressin and atrial natriuretic peptide in the collecting duct. Cardiovasc Res 51: 470-480.

Insel TR. 2003. Is social attachment an addictive disorder? Physiol Behav 79: 351-357.

Insel TR, Fernald RD. 2004. How the brain processes social information: searching for the social brain. Annu Rev Neurosci 27: 697-722.

Insel TR, Harbaugh CR. 1989. Lesions of the hypothalamic paraventricular nucleus disrupt the initiation of maternal behavior. Physiol Behav 45: 1033-1041.

Insel TR, Hulihan TA. 1995. A gender-specific mechanism for pair bonding: oxytocin and partner preference formation in monogamous voles. Behav Neurosci 109: 782-789.

Insel TR, Shapiro LE. 1992. Oxytocin receptor distribution relects social organization in monogamous and polygamous voles. Proc Natl Acad Sci USA 89: 5981-5985.

Insel TR, Wang ZX, Ferris CF. 1994. Patterns of brain vasopressin receptor distribution associated with social organization in microtine rodents. J Neurosci 14: 5381-5392.

Insel TR, Young L, Wang Z. 1997. Central oxytocin and reproductive behaviours. Rev Reprod 2: 28-37.

Insel TR, Young L, Witt DM, Crews D. 1993. Gonadal steroids have paradoxical effects on brain oxytocin receptors. J Neuroendocrinol 5: 619-628.

Insel TR, Young LJ. 2001. The neurobiology of attachment. Nat Rev Neurosci 2: 129-136.

Itoi K, Jiang YQ, Iwasaki Y, Watson SJ. 2004. Regulatory mechanisms of corticotropin-releasing hormone and vasopressin gene expression in the hypothalamus. J Neuroendocrinol 16: 348-355.

Ivell R, Richter D. 1984. Structure and comparison of the oxytocin and vasopressin genes from rat. Proc Natl Acad Sci USA 81: 2006-2010.

Iwasaki Y, Oiso Y, Saito H, Majzoub JA. 1997. Positive and negative regulation of the rat vasopressin gene promoter. Endocrinology 138: 5266-5274.

Jard S, Barberis C, Audigier S, Tribollet E. 1987. Neurohypophyseal hormone receptor systems in brain and periphery. Prog Brain Res 72: 173-187.

Jard S, Gaillard RC, Guillon G, Marie J, Schoenenberg P, et al. 1986. Vasopressin antagonists allow demonstration of a novel type of vasopressin receptor in the rat adenohypophysis. Mol Pharmacol 30: 171-177.

Jin X, Shearman LP, Weaver DR, Zylka MJ, De Vries GJ, et al. 1999. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell 96: 57-68.

Jirikowski GF, Caldwell JD, Pedersen CA, Stumpf WE. 1988. Estradiol influences oxytocin-immunoreactive brain systems. Neuroscience 25: 237-248.

Jirikowski GF, Caldwell JD, Stumpf WE, Pedersen CA. 1990. Topography of oxytocinergic estradiol target neurons in the mouse hypothalamus. Folia Histochem Cytobiol 28: 3-9.

Johns JM, Lubin DA, Walker CH, Joyner P, Middleton C, et al. 2004. Gestational treatment with cocaine and fluoxetine alters oxytocin receptor number and binding affinity in lactating rat dams. Int J Dev Neurosci 22: 321-328.

Johns JM, Lubin DA, Walker CH, Meter KE, Mason GA. 1997. Chronic gestational cocaine treatment decreases oxytocin levels in the medial preoptic area, ventral tegmental area and hippocampus in Sprague-Dawley rats. Neuropeptides 31: 439-443.

Johnson AE, Audigier S, Rossi F, Jard S, Tribollet E, et al. 1993. Localization and characterization of vasopressin binding sites in the rat brain using an iodinated linear AVP antagonist. Brain Res 622: 9-16.

Johnson AE, Barberis C, Albers HE. 1995. Castration reduces vasopressin receptor binding in the hamster hypothalamus. Brain Res 674: 153-158.

Johnson AE, Coirini H, Insel TR, McEwen BS. 1991. The regulation of oxytocin receptor binding in the ventromedial hypothalamic nucleus by testosterone and its metabolites. Endocrinology 128: 891-896.

Johnson RP. 1973. Scent marking in mammals. Anim Behav 21: 521-535.

Jonaidi H, Oloumi MM, Denbow DM. 2003. Behavioral effects of intracerebroventricular injection of oxytocin in birds. Physiol Behav 79: 725-729.

Kendrick KM, Da Costa APC, Broad KD, Ohkura S, Guevara R, et al. 1997. Neural control of maternal behaviour and olfactory recognition of offspring. Brain Res Bull 44: 383-395.

Keverne EB, Curley JP. 2004. Vasopressin, oxytocin and social behaviour. Curr Opin Neurobiol 14: 777-783.

Keverne EB, Kendrick KM. 1994. Maternal behaviour in sheep and its neuroendocrine regulation. Acta Paediatr Suppl 397: 47-56.

Kim JK, Summer SN, Wood WM, Schrier RW. 2001. Role of glucocorticoid hormones in arginine vasopressin gene regulation. Biochem Biophys Res Commun 289: 1252-1256.

Kim SJ, Young LJ, Gonen D, Veenstra-Vander Weele J, Courchesne R, et al. 2002. Transmission disequilibrium testing of arginine vasopressin receptor 1A (AVPR1A) polymorphisms in autism. Mol Psychiatry 7: 503-507.

Kimura T, Saji F, Nishimori K, Ogita K, Nakamura H, et al. 2003. Molecular regulation of the oxytocin receptor in peripheral organs. J Mol Endocrinol 30: 109-115.

Kimura T, Tanizawa O, Mori K, Brownstein MJ, Okayama H. 1992. Structure and expression of a human oxytocin receptor. Nature 356: 526-529.

Kiss JZ, Mezey E, Skirboll L. 1984. Corticotropin-releasing factor-immunoreactive neurons of the paraventricular nucleus become vasopressin positive after adrenalectomy. Proc Natl Acad Sci USA 81: 1854-1858.

Kiyama H, Emson PD. 1990. Evidence for coexpression of oxytocin and vasopressin messenger ribonucleic acids in magnocellular neurosecretory cells: simultaneous demonstration of two neurophysin messenger ribonucleic acids by hybridization histochemistry. J Neuroendocrinol 2: 257-259.

Kleiman DG. 1977. Monogamy in mammals. Q Rev Biol 52: 39-69.

Koolhaas JM, Moor E, Hiemstra Y. 1991. The testosterone-dependent vasopressinergic neurons in the medial amygdala and lateral septum: involvement in social behaviour in male rats. Proceedings of the Third International Vasopressin Conference. Jard S, Jamison R, editors. Paris, London: Colloque INSERM/John Libbey Eurotext Ltd; pp. 213-219.

Kremarik P, Freund-Mercier MJ, Stoeckel ME. 1993. Histoautoradiographic detection of oxytocin- and vasopressin-binding sites in the telencephalon of the rat. J Comp Neurol 333: 343-359.

Ku CY, Qian A, Wen Y, Anwer K, Sanborn BM. 1995. Oxytocin stimulates myometrial guanosine triphosphatase and phospholipase-C activities via coupling to G alpha q/11. Endocrinology 136: 1509-1515.

Kuwahara S, Arima H, Banno R, Sato I, Kondo N, et al. 2003. Regulation of vasopressin gene expression by cAMP and glucocorticoids in parvocellular neurons of the paraventricular nucleus in rat hypothalamic organotypic cultures. J Neurosci 23: 10231-10237.

Landgraf R, Frank E, Aldag JM, Neumann ID, Sharer CA, et al. 2003. Viral vector-mediated gene transfer of the vole V1a vasopressin receptor in the rat septum: improved social discrimination and active social behaviour. Eur J Neurosci 18: 403-411.

Landgraf R, Gerstberger R, Montkowski A, Probst JC, Wotjak CT, et al. 1995. V1 vasopressin receptor antisense oligodeoxynucleotide into septum reduces vasopressin binding, social discrimination abilities, and anxiety-related behavior in rats. J Neurosci 15: 4250-4258.

Landgraf R, Malkinson TJ, Veale WL, Lederis K, Pittman QJ. 1990. Vasopressin and oxytocin in the rat brain in response to prostaglandin fever. Am J Physiol 259: R1056-R1062.

Landgraf R, Neumann ID. 2004. Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. Front Neuroendocrinol 25: 150-176.

Larcher A, Neculcea J, Breton C, Arslan A, Rozen F, et al. 1995. Oxytocin receptor gene expression in the rat uterus during pregnancy and the estrous cycle in response to gonadal steroid treatment. Endocrinology 136: 5350-5356.

Legros JJ, Geenen V. 1996. Neurophysins in central diabetes insipidus. Horm Res 45: 182-186.

Li YC, Korol AB, Fahima T, Nevo E. 2004. Microsatellites within genes: structure, function, and evolution. Mol Biol Evol 21: 991-1007.

Lim MM, Hammock EA, Young LJ. 2004a. The role of vasopressin in the genetic and neural regulation of monogamy. J Neuroendocrinol 16: 325-332.

Lim MM, Wang Z, Olazabal DE, Ren X, Terwilliger EF, et al. 2004b. Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene. Nature 429: 754-757.

Lin SH, Kiyohara T, Sun B. 2003. Maternal behavior: activation of the central oxytocin receptor system in parturient rats? Neuroreport 14: 1439-1444.

Lipkin SM, Nelson CA, Glass CK, Rosenfeld MG. 1992. A negative retinoic acid response element in the rat oxytocin promoter restricts transcriptional stimulation by heterologous transactivation domains. Proc Natl Acad Sci USA 89: 1209-1213.

Liu Y, Curtis JT, Wang ZX. 2001. Vasopressin in the lateral septum regulates pair bond formation in male prairie voles (*Microtus ochrogaster*). Behav Neurosci 115: 910-919.

Liu Y, Wang ZX. 2003. Nucleus accumbens oxytocin and dopamine interact to regulate pair bond formation in female prairie voles. Neuroscience 121: 537-544.

Lolait SJ, O'Carroll AM, Mahan LC, Felder CC, Button DC, et al. 1995. Extrapituitary expression of the rat V1b vasopressin receptor gene. Proc Natl Acad Sci USA 92: 6783-6787.

Lubin DA, Elliott JC, Black MC, Johns JM. 2003. An oxytocin antagonist infused into the central nucleus of the amygdala increases maternal aggressive behavior. Behav Neurosci 117: 195-201.

Ludwig M. 1998. Dendritic release of vasopressin and oxytocin. J Neuroendocrinol 10: 881-895.

Lumley LA, Robison CL, Chen WK, Mark B, Meyerhoff JL. 2001. Vasopressin into the preoptic area increases grooming behavior in mice. Physiol Behav 73: 451-455.

Manning M, Sawyer WH. 1991. Antagonists of vasopressin and oxytocin: current status and future perspective. Vasopressin. Jard S, Jamison R, editors. France: John Libbey Eurotext Ltd; pp. 297-309.

Mantella RC, Vollmer RR, Li X, Amico JA. 2003. Female oxytocin-deficient mice display enhanced anxiety-related behavior. Endocrinology 144: 2291-2296.

Mason WT, Ho YW, Hatton GI. 1984. Axon collaterals of supraoptic neurones: anatomical and electrophysiological

evidence for their existence in the lateral hypothalamus. Neuroscience 11: 169-182.

McCarthy MM. 1990. Oxytocin inhibits infanticide in female house mice (*Mus domesticus*). Horm Behav 24: 365-375.

McCarthy MM, Kleopoulous SP, Mobbs CV, Pfaff DW. 1994. Infusion of antisense oligodeoxynucleotides to the oxytocin receptor in the ventromedial hypothalamus reduces estrogen-induced sexual receptivity and oxytocin receptor binding in the female rat. Neuroendocrinology 59: 432-440.

McCarthy MM, McDonald CH, Brooks PJ, Goldman D. 1996. An anxiolytic action of oxytocin is enhanced by estrogen in the mouse. Physiol Behav 60: 1209-1215.

Meeker RB, Fernandes A. 2002. Sustained increases in activating transcription factor-2 and activator protein-2 in the rat supraoptic nucleus during water deprivation. Neuroendocrinology 76: 111-120.

Meisenberg G, Simmons WH. 1982. Behavioral effects of intracerebroventricularly administered neurohypophyseal hormone analogs in mice. Pharmacol Biochem Behav 16: 819-825.

Menard J, Treit D. 1996. Lateral and medial septal lesions reduce anxiety in the plus-maze and probe-burying tests. Physiol Behav 60: 845-853.

Meredith M. 1991. Sensory processing in the main and accessory olfactory systems: comparisons and contrasts. J Steroid Biochem Mol Biol 39: 601-614.

Mezey E, Kiss JZ. 1991. Coexpression of vasopressin and oxytocin in hypothalamic supraoptic neurons of lactating rats. Endocrinology 129: 1814-1820.

Michell RH, Kirk CJ, Billah MM. 1979. Hormonal stimulation of phosphotidylinositol breakdown with particular reference to the hepatic effects of vasopressin. Biochem Soc Trans 7: 861-865.

Millan MJ, Millan MH, Czlonkowski A, Herz A. 1984. Vasopressin and oxytocin in the rat spinal cord: distribution and origins in comparison to [Met]enkephalin, dynorphin and related opioids and their irresponsiveness to stimuli modulating neurohypophyseal secretion. Neuroscience 13: 179-187.

Miller MA, Ferris CF, Kolb PE. 1999. Absence of vasopressin expression by galanin neurons in the golden hamster: implications for species differences in extrahypothalamic vasopressin pathways. Brain Res Mol Brain Res 67: 28-35.

Miller MA, Urban JH, Dorsa DM. 1989. Steroid dependency of vasopressin neurons in the bed nucleus of the stria terminalis by in situ hybridization. Endocrinology 125: 2335-2340.

Miller MA, De Vries GJ, Al-Shamma HA, Dorsa DM. 1992. Decline of vasopressin immunoreactivity and mRNA levels in the bed nucleus of the stria terminalis following castration. J Neurosci 12: 2881-2887.

Mishima K, Tsukikawa H, Inada K, Fujiwara M, Iwasaki K, et al. 2001. Ameliorative effect of vasoporessin-(4-9) through vasopressin V1A receptor on scopolamne-induced impairments of rat spatial memory in the eight-arm radial maze. Eur J Pharmacol 427: 43-52.

Mishima K, Tsukikawa H, Miura I, Inada K, Abe K, et al. 2003. Ameliorative effect of NC-1900, a new AVP4-9 analog, through vasopressin V1A receptor on scopolamine-induced impairments of spatial memory in the 8-arm radial maze. Neuropsychopharmacology 44: 541-552.

Mohr E, Richter D. 1990. Sequence analysis of the promoter region of the rat vasopressin gene. FEBS Lett 260: 305-308.

Mohr E, Richter D. 2004. Subcellular vasopressin mRNA trafficking and local translation in dendrites. J Neuroendocrinol 16: 333-339.

Mohr E, Schmitz E. 1991. Functional characterization of estrogen and glucocorticoid responsive elements in the rat oxytocin gene. Brain Res Mol Brain Res 9: 293-298.

Mohr E, Schmitz E, Richter D. 1998. A single rat genomic DNA fragment encodes both the oxytocin and vasopressin genes separated by 11 kilobases and oriented in opposite transcriptional directions. Biochimie 70: 649-654.

Moore FL. 1983. Behavioral endocrinology of amphibian reproduction. Bioscience 33: 557-561.

Moore RY. 1992. The organization of the human circadian timing system. Prog Brain Res 93: 101-115.

Morris JF, Ludwig M. 2004. Magnocellular dendrites: prototypic receiver/transmitters. J Neuroendocrinol 16: 403-408.

Muñoz E, Brewer M, Baler R. 2002. Circadian transcription: thinking outside the E-Box. J Biol Chem 277: 36009-36017.

Murgatroyd C, Wigger A, Frank E, Singewald N, Bunck M, et al. 2004. Impaired repression at a vasopressin promoter polymorphism underlies overexpression of vasopressin in a rat model of trait anxiety. J Neurosci 24: 7762-7770.

Murphy MR, Seckl JR, Burton S, Checkley SA, Lightman SL. 1987. Changes in oxytocin and vasopressin secretion during sexual activity in men. J Clin Endocrinol Metab 65: 738-741.

Naylor AM, Pittman QJ, Veale WL. 1988. Stimulation of vasopressin release in the ventral septum of the brain suppresses prostaglandin E1 fever. J Physiol 399: 177-189.

Naylor AM, Ruwe WD, Kohut AF, Veale WL. 1985. Perfusion of vasopressin within the ventral septum of the rabbit suppresses endotoxin fever. Brain Res Bull 15: 209-213.

Neumann ID. 2001. Alterations in behavioral and neuroendocrine stress coping strategies in pregnant, parturient and lactating rats. Prog Brain Res 133: 143-152.

Neumann ID, Torner L, Wigger A. 2000. Brain oxytocin: differential inhibition of neuroendocrine stress responses and anxiety-related behaviour in virgin, pregnant and lactating rats. Neuroscience 95: 567-575.

Nishimori K, Young LJ, Guo Q, Wang Z, Insel TR, et al. 1996. Oxytocin is required for nursing but is not essential for parturition or reproductive behavior. Proc Natl Acad Sci USA 93: 11699-11704.

Nomura A, Iwasaki Y, Saito M, Aoki Y, Yamamori E, et al. 2001. Involvement of upstream open reading frames in regulation of rat V(1b) receptor expression. Am J Physiol Endocrinol Metab 280: E780-E787.

Nomura M, McKenna E, Korach KS, Pfaff DW, Ogawa S. 2002. Estrogen receptor-beta regulates transcript levels for oxytocin and arginine vasopressin in the hypothalamic paraventricular nucleus of male mice. Brain Res Mol Brain Res 109: 84-94.

North WG. 2000. Gene regulation of vasopressin and vasopressin receptors in cancer. Exp Physiol 85: 26S-40S.

O'Keefe JA, Crowley RS, Hrivnak P, Kim NB, Amico JA. 1995. Effect of testosterone and its metabolites upon the level of vasopressin messenger ribonucleic acid in the hypothalamus of the hyperosmotically stimulated male rat. Neuroendocrinology 61: 405-411.

Oliver MD, Schäfer FRS. 1895. On the physiological action of extracts of pituitary body and certain other glandular organs. J Physiol 18: 277-279.

Ostrowski NL, Lolait SJ, Bradley DJ, O'Carroll A, Brownstein MJ, et al. 1992. Distribution of V1a and V2 vasopressin receptor messenger ribonucleic acids in rat liver, kidney, pituitary and brain. Endocrinology 131: 533-535.

Ostrowski NL, Lolait SJ, Young WS III. 1994. Cellular localization of vasopressin V1a receptor messenger ribonucleic acid in adult male rat brain, pineal, and brain vasculature. Endocrinology 135: 1511-1528.

Ostrowski NL, Young WS III, Lolait SJ. 1995. Estrogen increases renal oxytocin receptor gene expression. Endocrinology 136: 1801-1804.

Ott I, Scott JC. 1910. The action of infundibulin upon the mammary secretion. Proc Soc Exp Biol (NY) 8: 48-49.

Patchev VK, Schlosser SF, Hassan AHS, Almeida OFX. 1993. Oxytocin binding sites in rat limbic and hypothalamic structures: site-specific modulation by adrenal and gonadal steroids. Neuroscience 57: 537-543.

Patisaul HB, Scordalakes EM, Young LJ, Rissman EF. 2003. Oxytocin, but not oxytocin receptor, is regulated by oestrogen receptor beta in the female mouse hypothalamus. J Neuroendocrinol 15: 787-793.

Payne AP, Swanson HH. 1970. Agonistic behaviour between pairs of hamsters of the same and opposite sex in a neutral observation area. Behaviour 36: 260-269.

Pedersen CA. 1997. Oxytocin control of maternal behavior. Regulation by sex steroids and offspring stimuli. Ann N Y Acad Sci 807: 126-145.

Pedersen CA, Ascher JA, Monroe YL, Prange AJ Jr. 1982. Oxytocin induces maternal behavior in virgin female rats. Science 216: 648-649.

Pedersen CA, Prange AJ. 1979. Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin. Proc Natl Acad Sci USA 76: 6661-6665.

Phelps SM, Young LJ. 2003. Extraordinary diversity in vasopressin (V1a) receptor distributions among wild prairie voles (*Microtus ochrogaster*): patterns of variation and covariation. J Comp Neurol 466: 564-576.

Pitt GR, Batt AR, Haigh RM, Penson AM, Robson PA, et al. 2004. Non-peptide oxytocin agonists. Bioorg Med Chem Lett 14: 4585-4589.

Planas B, Kolb PE, Raskind MA, Miller MA. 1995. Vasopressin and galanin mRNAs coexist in the nucleus of the horizontal diagonal band: a novel site of vasopressin gene expression. J Comp Neurol 361: 48-56.

Plumari L, Viglietti-Panzica C, Allieri F, Honda S, Harada N, et al. 2002. Changes in the arginine–vasopressin immunoreactive systems in male mice lacking a functional aromatase gene. J Neuroendocrinol 14: 971-978.

Popik P, Van Ree JM. 1991. Oxytocin but not vasopressin facilitates social recognition following injection into the medial preoptic area of the rat brain. Eur Neuropsychopharmacol 1: 555-560.

Popik P, Vetulani J, Van Ree JM. 1996. Facilitation and attenuation of social recognition in rats by different oxytocin-related peptides. Eur J Pharmacol 308: 113-116.

Qahwash IM, Cassar CA, Radcliff RP, Smith GW. 2002. Bacterial lipopolysaccharide-induced coordinate downregulation of arginine vasopressin receptor V3 and corticotropin-releasing factor receptor 1 messenger ribonucleic acids in the anterior pituitary of endotoxemic steers. Endocrine 18: 13-20.

Quinones-Jenab V, Jenab S, Ogawa S, Adan RA, Burbach JP, et al. 1997. Effects of estrogen on oxytocin receptor messenger ribonucleic acid expression in the uterus, pituitary, and forebrain of the female rat. Neuroendocrinology 65: 9-17.

Rabadan-Diehl C, Aguilera G. 1998. Glucocorticoids increase vasopressin V1b receptor coupling to phospholipase C. Endocrinology 139: 3220-3226.

Rabadan-Diehl C, Lolait SJ, Aguilera G. 1995. Regulation of pituitary vasopressin V1b receptor mRNA during stress in the rat. J Neuroendocrinol 7: 903-910.

Rabadan-Diehl C, Makara G, Kiss A, Lolait S, Zelena D, et al. 1997. Regulation of pituitary V1b vasopressin receptor messenger ribonucleic acid by adrenalectomy and glucocorticoid administration. Endocrinology 138: 5189-5194.

Ragnauth AK, Goodwillie A, Brewer C, Muglia LJ, Pfaff DW, et al. 2004. Vasopressin stimulates ventromedial

hypothalamic neurons via oxytocin receptors in oxytocin gene knockout male and female mice. 80 (2): 99.

Richard S, Zingg HH. 1990. The human oxytocin gene promoter is regulated by estrogens. J Biol Chem 265: 6098-6103.

Rinaman L. 1998. Oxytocinergic inputs to the nucleus of the solitary tract and dorsal motor nucleus of the vagus in neonatal rats. J Comp Neurol 399: 101-109.

Roth J, Zeisberger E, Vybíral S, Janský L. 2004. Endogenous antipyretics: neuropeptides and glucocorticoids. Front Biosci 9: 816-826.

Rozen F, Russo C, Banville D, Zingg HH. 1995. Structure, characterization, and expression of the rat oxytocin receptor gene. Proc Natl Acad Sci USA 92: 200-204.

Ruiz-Opazo N, Akimoto K, Herrera VL. 1995. Identification of a novel dual angiotensin II/vasopressin receptor on the basis of molecular recognition theory. Nat Med 1: 1074-1081.

Ruppert S, Scherer G, Schütz G. 1984. Recent gene conversion involving bovine vasopressin and oxytocin precursor genes suggested by nucleotide sequence. Nature 308: 554-557.

Sahgal A. 1984. A critique of the vasopressin-memory hypothesis. Psychopharmacology 83: 215-228.

Sahgal A, Wright C. 1984. Choice, as opposed to latency, measures in avoidance suggest that vasopressin and oxytocin do not affect memory in rats. Neurosci Lett 48: 299-304.

Saito M, Sugimoto T, Tahara A, Kawashima H. 1995. Molecular cloning and characterization of rat V1b vasopressin receptor: evidence for its expression in extra-pituitary tissues. Biochem Biophys Res Commun 212: 751-757.

Sanchez R, Nguyen D, Rocha W, White JH, Mader S. 2002. Diversity in the mechanisms of gene regulation by estrogen receptors. Bioessays 24: 244-254.

Sausville E, Carney D, Battey J. 1985. The human vasopressin gene is linked to the oxytocin gene and is selectively expressed in a cultured lung cancer cell line. J Biol Chem 260: 10236-10241.

Sawchenko PE, Swanson LW. 1982. Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. J Comp Neurol 205: 260-272.

Sawchenko PE, Swanson LW, Vale WW. 1984. Co-expression of corticotropin-releasing factor and vasopressin immunoreactivity in parvocellular neurosecretory neurons of the adrenalectomized rat. Proc Natl Acad Sci USA 81: 1883-1887.

Schipper J, Werkman TR, Tilders FJ. 1984. Quantitative immunocytochemistry of corticotropin-releasing factor (CRF). Studies on nonbiological models and on hypothlamic tissues of rats after hypophysectomy, adrenalectomy and dexamethasone treatment. Brain Res 293: 111-118.

Schulze HG, Gorzalka BB. 1992. Low concentrations of oxytocin suppress lordosis when infused into the lateral ventricle of female rats. Endocr Regul 26: 23-27.

Schwartz WJ, Carpina A Jr, de la Iglesia HO, Baler R, Klein DC, et al. 2000. Differential regulation of fos family genes in the ventrolateral and dorsomedial subdivisions of the rat suprachiasmatic nucleus. Neuroscience 98: 535-547.

Scordalakes EM, Rissman EF. 2004. Aggression and arginine vasopressin immunoreactivity regulation by androgen receptor and estrogen receptor alpha. Genes Brain Behav 3: 20-26.

Semsar K, Kandel FL, Godwin J. 2001. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. Horm Behav 40: 21-31.

Serradeil-Le Gal C, Wagnon J, Valette G, Garcia G, Pascal M, et al. 2002. Nonpeptide vasopressin receptor antagonists: development of selective and orally active V1a, V2 and V1b receptor ligands. Prog Brain Res 139: 197-210.

Shao Y, Wolpert CM, Raiford KL, Menold MM, Donnelly SL, et al. 2002. Genomic screen and follow-up analysis for autistic disorder. Am J Med Genet 114: 99-105.

Shapiro RA, Xu C, Dorsa DM. 2000. Differential transcriptional regulation of rat vasopressin gene expression by estrogen receptor alpha and beta. Endocrinology 141: 4056-4064.

Sladek CD, Gallagher MJ. 1993. The stimulation of vasopressin gene expression in cultured hypothalamic neurons by cyclic adenosine 3′,5′-monophosphate is reversible. Endocrinology 133: 1320-1330.

Sladek CD, Somponpun S. 2004. Oestrogen receptor beta: role in neurohypophyseal neurones. J Neuroendocrinol 16: 365-371.

Smith DA, Karom M, Caldwell HK, Moore TO, Albers HE. 2001. The effects of housing and gender on V1a vasopressin receptor binding from weaning to sexual maturity in Syrian hamsters. Annu Meet Soc Neurosci Abstract. 2001.

Sofroniew MV. 1983. Morphology of vasopressin and oxytocin neurones and their central and vascular projections. Prog Brain Res 60: 101-114.

Sofroniew MV. 1985a. Vasopressin, oxytocin and their related neurophysins. Handbook of chemical neuroanatomy. Bjorklund A, Hökfelt T, editors. Amsterdam: Elsevier; pp. 93-165.

Sofroniew MV. 1985b. Vasopressin- and neurophysin-immunoreactive neurons in the septal region, medial amygdala, and locus coeruleus in colchicine-treated rats. Neuroscience 15: 347-358.

Stark RI, Daniel SS, Husain KM, James LS, Van de Wiele RL. 1979. Arginine vasopressin during gestation and parturition in sheep fetus. Biol Neonate 35: 235-241.

Stedronsky K, Telgmann R, Tillmann G, Walther N, Ivell R. 2002. The affinity and activity of the multiple hormone response element in the proximal promoter of the human oxytocin gene. J Neuroendocrinol 14: 472-485.

Stemmelin J, Lukovic L, Salome N, Griebel G. 2005. Evidence that the lateral septum is involved in the antidepressant-like effects of the vasopressin V(1b) receptor antagonist SSR149415. Neuropsychopharmacology 30: 35-42.

Stevenson KR, Riley PR, Stewart HJ, Flint AP, Wathes DC. 1994. Localization of oxytocin receptor mRNA in the ovine uterus during the oestrous cycle and early pregnancy. J Mol Endocrinol 12: 93-105.

Sukhov RR, Walker LC, Rance NE, Price DL, Young WS III. 1993. Vasopressin and oxytocin gene expression in the human hypothalamus. J Comp Neurol 337: 295-306.

Szmydynger-Chodobska J, Chung I, Kozniewska E, Tran B, Harrington FJ, et al. 2004. Increased expression of vasopressin v1a receptors after traumatic brain injury. Neurotrauma 21: 1090-1102.

Szot P, Bale TL, Dorsa DM. 1994. Distribution of messenger RNA for the vasopressin V1a receptor in the CNS of male and female rats. Brain Res Mol Brain Res 24: 1-10.

Telleria-Diaz A, Grinevich VV, Jirikowski GF. 2001. Colocalization of vasopressin and oxytocin in hypothalamic magnocellular neurons in water-deprived rats. Neuropeptides 35: 162-167.

Temple JL, Young WS III, Wersinger SR. (2003) Disruption of the genes for either oxytocin or the vasopressin 1B receptor alters male-induced pregnancy block (the Bruce effect). Annu Meet Soc Neurosci Abstract.

Thomas A, Shughrue PL, Merchenthaler I, Amico J. 1999. The effects of progesterone on oxytocin mRNA levels in the paraventricular nucleus of the female rat can be altered by the administration of diazepam or RU486. J Neuroendocrinol 11: 137-144.

Tomizawa K, Iga N, Lu Y-F, Moriwaki A, Matsushita M, Li S-T, et al. 2003. Oxytocin improves long-lasting spatial memory during motherhood through MAP kinase cascade. Nat Neurosci 6: 384-390.

Tramu G, Croix C, Pillez A. 1983. Ability of the CRF immunoreactive neurons of the paraventricular nucleus to produce a vasopressin-like material. Immunohistochemical demonstration in adrenalectomized guinea pigs and rats. Neuroendocrinology 37: 467-469.

Trembleau A, Morales M, Bloom FE. 1996. Differential compartmentalization of vasopressin messenger RNA and neuropeptide within the rat hypothalamo-neurohypophysial axonal tracts: light and electron microscopic evidence. Neuroscience 70: 113-125.

Tribollet E, Audigier S, Dubois-Dauphin M, Dreifuss J. 1990. Gonadal steroids regulate oxytocin receptors but not vasopressin receptors in the brain of male and female rats. An autoradiographical study. Brain Res 511: 129-140.

Tribollet E, Barberis C, Arsenijevic Y. 1997. Distribution of vasopressin and oxytocin receptors in the rat spinal cord: sex-related differences and effect of castration in pudendal motor nuclei. Neuroscience 78: 499-509.

Tuppy H. 1953. The amino-acid sequence in oxytocin. Biochim Biochim Acta (Amst) 11: 449-450.

Turner RA, Pierce JG, Du Vigneaud V. 1951. The purification and the amino acid content of vasopressin preparations. J Biol Chem 191: 21-28.

Uhl GR, Reppert SM. 1986. Suprachiasmatic nucleus vasopressin messenger RNA: circadian variation in normal and Brattleboro rats. Science 232: 390-393.

Urban JH, Miller MA, Drake CT, Dorsa DM. 1990. Detection of vasopressin mRNA in cells of the medial amygdala but not the locus coeruleus by in situ hybridization. J Chem Neuroanat 3: 277-283.

Vaccari C, Lolait SJ, Ostrowski NL. 1998. Comparative distribution of vasopressin V1b and oxytocin receptor messenger ribonucleic acids in brain. Endocrinology 139: 5015-5033.

van Leengoed E, Kerker E, Swanson HH. 1987. Inhibition of post-partum maternal behavior in the rat by injecting an oxytocin antagonist into the cerebral ventricles. J Endocrinol 112: 275-282.

Van Leeuwen FW, Caffè SR, De Vries GJ. 1985. Vasopressin cells in the bed nucleus of the stria terminalis of the rat: sex differences and the influence of androgens. Brain Res 325: 391-394.

van West D, Del-Favero J, Aulchenko Y, Oswald P, Souery D, et al. 2004. A major SNP haplotype of the arginine vasopressin 1B receptor protects against recurrent major depression. Mol Psychiatry 9: 287-292.

Vasudevan N, Davidkova G, Zhu YS, Koibuchi N, Chin WW, et al. 2001. Differential interaction of estrogen receptor and thyroid hormone receptor isoforms on the rat oxytocin receptor promotor leads to differences in transcriptional regulation. Neuroendocrinology 74: 309-324.

Veinante P, Freund-Mercier MJ. 1997. Distribution of oxytocin- and vasopressin-binding sites in the rat extended amygdala: a histoautoradiographic study. J Comp Neurol 383: 305-325.

Volpi S, Rabadan-Diehl C, Aguilera G. 2002. Transcriptional regulation of the pituitary vasopressin V1b receptor involves a GAGA-binding protein. J Biol Chem 277: 27829-27838.

Volpi S, Rabadan-Diehl C, Aguilera G. 2004a. Regulation of vasopressin V1b receptors and stress adaptation. Ann N Y Acad Sci 1018: 293-301.

Volpi S, Rabadan-Diehl C, Aguilera G. 2004b. Vasopressinergic regulation of the hypothalamic pituitary adrenal axis and stress adaptation. Stress 7: 75-83.

von Eggelkraut-Gottanka R, Beck-Sickinger AG. 2004. Biosynthesis of peptide hormones derived from precursor sequences. Curr Med Chem 11: 2651-2665.

Vongraven D. 1913. Die nierenwirkung von hypophysenextrakten meschen. Berl Klin Wochenscgr 50: 2083-2086.

Wang Z, Ferris CF, De Vries GJ. 1994. Role of septal vasopressin innervation in paternal behavior in prairie voles (*Microtus ochrogaster*). Proc Natl Acad Sci USA 91: 400-404.

Wang Z, De Vries GJ. 1995. Androgen and estrogen effects on vasopressin messenger RNA expression in the medial amygdaloid nucleus in male and female rats. J Neuroendocrinol 7: 827-831.

Wang Z, Zhou L, Hulihan TJ, Insel TR. 1996. Immunoreactivity of central vasopressin and oxytocin pathways in microtine rodents: a quantitative comparative study. J Comp Neurol 366: 726-737.

Wassink TH, Piven J, Vieland VJ, Pietila J, Goedken RJ, et al. 2004. Examination of AVPR1a as an autism susceptibility gene. Mol Psychiatry 9: 968-972.

Watters JJ, Poulin P, Dorsa DM. 1998. Steroid homone regulation of vasopressinergic neurotransmission in the central nervous system. Prog Brain Res 119: 247-261.

Watters JJ, Wilkinson CW, Dorsa DM. 1996. Glucocorticoid regulation of vasopressin V1a receptors in rat forebrain. Brain Res Mol Brain Res 38: 276-284.

Wersinger SR, Christiansen M, Hu SB, Gold PW, Young WS III. (2003) Aggression is reduced in vasopressin 1b receptor null mice but is elevated in vasopressin 1a receptor null mice. Annu Meet Soc Neurosci Abstract.

Wersinger SR, Ginns EI, O'Carroll AM, Lolait SJ, Young WS III. 2002. Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. Mol Psychiatry 7: 975-984.

Wersinger SR, Kelliher KR, Zufall F, Lolait SJ, O'Carroll A, et al. 2004. Social motivation is reduced in vasopressin 1b receptor null mice despite normal performance in an olfactory discrimination task. Horm Behav 46: 638-645.

Whitman DC, Albers HE. 1995. Role of oxytocin in the hypothalamic regulation of sexual receptivity in hamsters. Brain Res 680: 73-79.

Wigger A, Sanchez MM, Mathys KC, Ebner K, Frank E, et al. 2004. Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. Neuropsychopharmacology 29: 1-14.

Williams JR, Insel TR, Harbaugh CR, Carter CS. 1994. Oxytocin administered centrally facilitates formation of a partner preference in prairie voles (*Microtus ochrogaster*). J Neuroendocrinol 6: 247-250.

Windle RJ, Shanks N, Lightman SL, Ingram CD. 1997. Central oxytocin administration reduces stress-induced corticosterone release and anxiety behavior in rats. Endocrinology 138: 2829-2834.

Winslow J, Insel TR. 1991a. Vasopressin modulates male squirrel monkeys' behavior during social separation. Eur J Pharmacol 200: 95-101.

Winslow JT, Hastings N, Carter CS, Harbaugh CR, Insel TR. 1993. A role for central vasopressin in pair bonding in monogamous prairie voles. Nature 365: 545-548.

Winslow JT, Hearn EF, Ferguson J, Young LJ, Matzuk MM, et al. 2000. Infant vocalization, adult aggression, and fear behavior in an oxytocin null mutant mouse. Horm Behav 37: 145-155.

Winslow JT, Insel TR. 1991b. Social status in pairs of male squirrel monkeys determines the behavioral response to central oxytocin administration. J Neurosci 11: 2032-2038.

Winslow JT, Insel TR. 2002. The social deficits of the oxytocin knockout mouse. Neuropeptides 26: 221-229.

Xi D, Kusano K, Gainer H. 1999. Quantitative analysis of oxytocin and vasopressin messenger ribonucleic acids in single mangocellular neurons isolated from supraoptic nucleus of rat hypothalamus. Endocrinology 140: 4677-4682.

Young LJ, Muns S, Wang Z, Insel TR. 1997a. Changes in oxytocin receptor mRNA in rat brain during pregnancy and the effects of estrogen and interleukin-6. J Neuroendocrinol 9: 859-865.

Young LJ, Nilsen R, Waymire KG, Mac Gregor GR, Insel TR. 1999. Increased affiliative response to vasopressin in mice expressing the V1a receptor from a monogamous vole. Nature 400: 766-768.

Young LJ, Wang Z. 2004. The neurobiology of pair bonding. Nat Neurosci 7: 1048-1054.

Young LJ, Wang Z, Cooper TT, Albers HE. 2000. Vasopressin ($V_{1a}$) receptor binding, mRNA expression and transcriptional regulation by androgen in the Syrian hamster brain. J Neuroendocrinol 12: 1179-1185.

Young LJ, Winslow JT, Nilsen R, Insel TR. 1997b. Species differences in V1a receptor gene expression in monogamous and nonmonogamous voles: behavioral consequences. Behav Neurosci 111: 599-605.

Young WS III, Gainer H. 2003. Transgenesis and the study of expression, cellular targeting and function of oxytocin, vasopressin, and their receptors. Neuroendocrinology 78: 185-203.

Young WS III, Kovács K, Lolait SJ. 1993. The diurnal rhythm in vasopressin V1a receptor expression in the suprachiasmatic nucleus is not dependent on vasopressin. Endocrinology 133: 585-590.

Young WS III, Shepard E, De Vries AC, Zimmer A, LaMarca ME, et al. 1998. Targeted reduction of oxytocin expression

provides insights into its physiological roles. Adv Exp Med Biol 449: 231-240.

Young WS III, Shepard EA, Burch RM. 1987. Plasma hyperosmolality increases G protein and 3′,5′-cyclic adenosine monophosphate synthesis in the paraventricular and supraoptic nuclei. Mol Endocrinol 1: 884-888.

Zhou L, Blaustein JD, De Vries GJ. 1994. Distribution of androgen receptor immunoreactivity in vasopressin- and oxytocin-immunoreactive neurons in the male rat brain. Endocrinology 134: 2622-2627.

Zingg HH, Laporte SA. 2003. The oxytocin receptor. Trends Endocrinol Metab 14: 222-227.

Zingg HH, Lefebvre DL. 1989. Oxytocin mRNA: increase of polyadenylate tail size during pregnancy and lactation. Mol Cell Endocrinol 65: 59-62.

Zingg HH, Lefebvre DL, Almazan G. 1988. Regulation of poly(A) tail size of vasopressin mRNA. J Biol Chem 263: 11041-11043.

# 26 Corticotropin-Releasing Factor in Brain Function

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**Abstract:** The corticotropin-releasing factor (CRF) has been associated with two major functions since its discovery in the early 1980's. The first major role of CRF is to function as a hormone released from the hypothalamus to activate the hypothalamic–pituitary–adrenal (HPA) axis during stress. The other important role of CRF, which is also known as the extrahypothalamic function, is to mediate the response to stressful conditions of many other brain regions, involved in emotional and cognitive processes, such as dorsal raphe nucleus, hippocampus, prefrontal cortex, amygdala, and ventral tegmental area. Since the HPA axis has been well studied and the extrahypothalamic function of CRF is relatively new, this chapter focuses more on the CRF acting as a neuropeptide, especially in cortical and subcortical limbic areas. Understanding the physiology and pathology of CRF in the central nervous system (CNS) could provide more opportunities to find treatment for the stress-related neuropsychiatric disorders, including depression and anxiety.

**List of Abbreviations:** 5-HT, serotonin; ACTH, adrenocorticotropic hormone; AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionic acid; AVP, vasopressin; BLA, basolateral, and basomedial amygdaloid nuclei; CaMKII, calcium/calmodulin-dependent protein kinase II; cAMP, cyclic adenosine monophosphate; CeA, central nucleus of amygdala; CF, climbing fiber; CNS, central nervous system; CREB, cAMP response element-binding protein; CRF, corticotropin-releasing factor; CRF-BP, CRF binding protein; CRFR, CRF receptors; CSF, cerebrospinal fluid; DA, dopamine; DR, dorsal raphe nucleus; EPSC, excitatory postsynaptic current; ERK, extracellular signal-regulated kinase; GABA, γ-amino butyric acid; GPCR, G-protein-coupled receptor; GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; HPG, hypothalamic–pituitary–gonadal; IPSP, inhibitory postsynaptic potential; JNK, c-jun N-terminal kinase; KO, knockout; LC, locus coeruleus; LH, luteinizing hormone; LSMLN, lateral septum mediolateral nucleus; LTD, long-term depression; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; MDD, major depressive disorder; MR, mineralocorticoid receptor; NE, norepinephrine; NMDA, *N*-methyl-D-aspartic acid; OE, overexpression; PFC, prefrontal cortex; PI3K, phosphatidylinositol-3 kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; POMC, proopiomelanocortin; PS-LTP, long-term potentiation of population spikes; PTSD, posttraumatic stress disorder; PVN, paraventricular nucleus; SAPK, stress-activated protein kinase; sIPSC, spontaneous inhibitory postsynaptic current; UcnI, urocortin I; UcnII, stresscopin-related peptide; UcnIII, stresscopin; VTA, ventral tegmental area

## 1 Introduction

Corticotropin-releasing factor (CRF) was first characterized in 1981 (Vale et al., 1981). It was first known for its important regulatory effect in the endocrine stress response. During a stress response, CRF released from the hypothalamus activates the hypothalamic–pituitary–adrenal (HPA) axis through CRF receptors (CRFR) on the anterior pituitary corticotropes to stimulate the release of adrenocorticotropic hormone (ACTH). ACTH then enters the blood stream and stimulates the synthesis and release of glucocorticoids at the adrenal gland cortex. The glucocorticoids, through a negative feedback system, can inhibit CRF or ACTH production (Tsigos and Chrousos, 2002).

Two decades since its discovery, many studies have shown that in addition to its original role as hormones, the CRF ligand family also plays a critical role as neuropeptides in the regulation of stress response and behaviors associated with these responses. While acute responses to stress are necessary in order to maintain homeostasis in the organism, chronic stress, exaggerated responses to stress, or inadequate termination of the stress response can lead to mental diseases. The extrahypothalamic functions of CRF family members are heavily involved in the development of psychological disorders related to heightened stress sensitivity and dysregulation of stress-coping mechanisms (Bale and Vale, 2004).

## 2 CRF and CRF-Associated Proteins

### 2.1 CRF Ligand Family

There are four known molecules that belong to the CRF ligand family so far (*Figure 26-1*): CRF, urocortin I (UcnI), stresscopin-related peptide (UcnII), and stresscopin (UcnIII). These ligands are expressed in different regions of the body and have different affinities toward different CRFR subtypes.

**Figure 26-1**

**Alignment of the corticotropin-releasing factor (CRF) peptide family members. The amino acids that are homologous between the CRF peptides are *boxed*. *h*, human; *m*, mouse; *o*, ovine; UCN 1, urocortin 1; UCN 2, urocortin 2; UCN 3, urocortin 3 (From Pharmacol Rev. 2003, 55:21-26).**

| Peptide | Sequence | Length | Identity(%) |
|---|---|---|---|
| hCRF | SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII | 41 | 100 |
| oCRF | SQEPPISLDLTFHLLREVLEMTKADQLAEQAHSNRKLLDIA | 41 | 83 |
| hUCN 1 | DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV | 40 | 43 |
| hUCN 2 | IVLSLDVPIGLLQILLEQARARAAREQATTNARILARV | 38 | 34 |
| mUCN 2 | VILSLDVPIGLLRILLEQARYKAARNQAATNAQILAHV | 38 | 34 |
| hUCN 3 | FTLSLDVPTNIMNLLFNIAKAKNLRAQAAANAHLMAQI | 38 | 32 |
| mUCN 3 | FTLSLDVPTNIMNILFNIDKAKNLRAKAAANAQLMAQI | 38 | 26 |

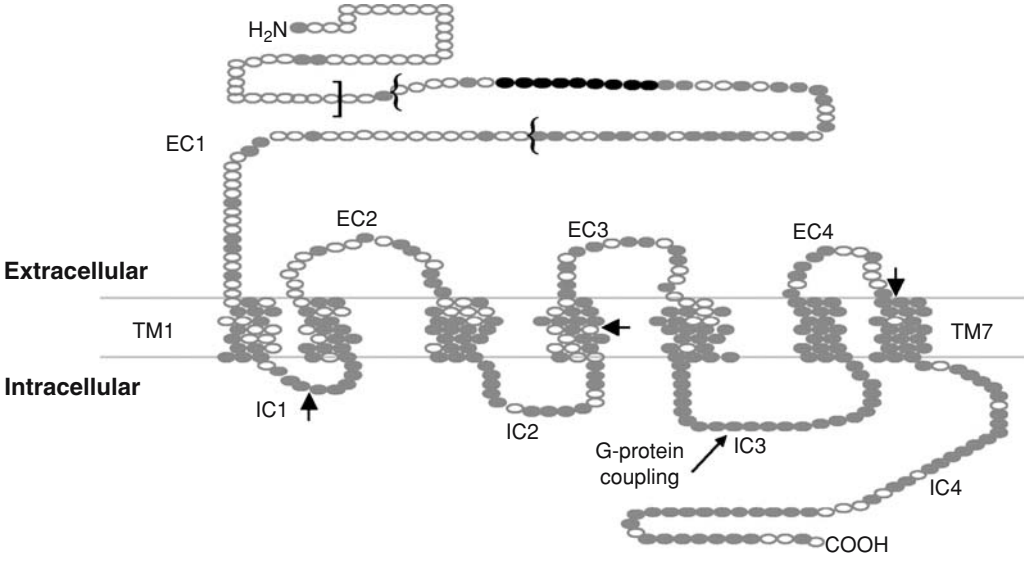
CRF is a 41-amino-acid polypeptide found in brain areas important for stress adaptation, learning, and memory, such as the paraventricular nucleus of the hypothalamus (PVN), central nucleus of the amygdala (CeA), and hindbrain regions in the CNS (Bale and Vale, 2004). It is generated by cleaving the C terminus of the 196-amino-acid precursor called preproCRF (Dautzenberg and Hauger, 2002). The C-terminal region is required for CRF to function properly, and the N-terminal can exist in heterogeneous forms both within and between species (Vale et al., 1981). The primary function of CRF is to activate the HPA axis during stressful conditions. Specifically, it will activate the transcription of the proopiomelanocortin (POMC) gene and stimulate the release of ACTH and β-endorphin from cells in the anterior pituitary gland. The animal study designed to observe the effect of chronic HPA axis activation by overexpressing CRF (CRF-OE) shows that constitutively high level of ACTH and corticosteroid due to high CRF concentration leads to the development of Cushing's syndrome-like symptoms in mice (Stenzel-Poore et al., 1992). In addition, these CRF-OE mice exhibit delayed and attenuated HPA axis hormone responses to stress that may result from desensitization of the HPA axis (Coste et al., 2001). On the other hand, mice deficient in CRF show that corticosteroid levels are blunted in both basal and acute stress conditions (Muglia et al., 1995).

### 2.2 CRF Receptors

There are two known receptors for the CRF ligand family: CRFR1 located at chromosome arm 17q12–q22 and CRFR2 located at chromosome arm 7p15.1 (Horn et al., 2001). Both receptors have seven transmembrane domains (*Figure 26-2*) and are predominantly linked to the activation of adenylate cyclase and protein kinase A (PKA) through $G_s$. They belong to the class B subtype of G-protein-coupled receptors (GPCRs), which can usually be distinguished from the rest of GPCRs by two features: (1) Class B GPCR have a large extracellular N-terminal domain that is critical for ligand binding; (2) They also contain six highly conserved cysteine residues that are likely involved in disulfide bond formation (Rashid et al., 2004).

**Figure 26-2**

**Two-dimensional structure of the human corticotropin-releasing factor 1 (CRF1) and corticotropin-releasing factor 2 (CRF2) receptors. Identical amino acids between both receptors are represented as *filled circles* whereas divergent residues are shown as *open circles*. The *arrows* indicate sites for insertion or deletion of exons in nonfunctional variants of the CRF1 and CRF2 receptor (Chen et al., 1993; Myers et al., 1998; Grammatopoulos et al., 1999; Miyata et al., 1999). The symbols { and } indicate the deletion of a 40-amino-acid exon in a nonfunctional splice variant of the human CRF1 receptor (Ross et al., 1994) whereas the symbol ] represents the common splice site for the three CRF2 variants $CRF2_a$, $CRF2_b$, and $CRF2_c$ (From Pharmacol Rev. 2003, 55:21-26).**




There are several splice variants of CRFR1 and CRFR2 expressed throughout central and peripheral tissues. CRFR1 has α and β isoforms in addition to subtypes designated c–h; however, some of them are not functional (Grammatopoulos and Chrousos, 2002). CRFR2 is expressed in three functional subtypes, α, β, and γ through the use of alternative 5′-exons. These isoforms differ in their N-terminal sequence for ligand selectivity as well as their distribution in both tissues and species (Bale and Vale, 2004). CRFR1 and CRFR2 are very similar (over 80% identical) at the transmembrane domains and the intracellular domains including the third intracellular loop where G-proteins interact with most GPCRs (Perrin and Vale, 1999). Through mutagenesis and chimeric-receptor studies, it is now known that the second and third extracellular domains, the N terminus and its juxtamembrane region are important in determining the specificity of ligand–receptor binding (Perrin et al., 2003). Among the four ligands in the family, CRF has tenfold higher affinity for CRFR1 than CRFR2. UcnI seems to have equal affinities for both receptors, while UcnII and UcnIII appear to have higher affinity for CRFR2, although UcnII may also activate CRFR1 at higher concentrations (Perrin et al., 1995).

The tissue distribution of CRFR variants is related to their endogenous functions. CRFR1 is the dominative receptor in the CNS, and CRFR2 is expressed at several limited locations (Lovenberg et al., 1995). Basically, CRFR1 is distributed throughout the cerebral cortex, cerebellum, olfactory bulb, medial septum, hippocampus, amygdala, and the pituitary, where the HPA axis signaling continues (Potter et al., 1994). Central CRFR2 is predominantly limited to sites in the lateral septum, PVN of the hypothalamus, and choroid plexus. In peripheral tissues, however, CRFR2 is the dominative receptor found to be expressed in the cardiac myocytes, gastrointestinal tract, lung, skeletal muscle, and vasculature (Perrin et al., 1995).

The current consent is that the stress response is mediated by the homeostatic balance of both CRFR1 and CRFR2's functions. Many experiments have shown the importance of CRFR1 in the stress response through the HPA axis. Antagonists of CRFR1 can reduce the release of ACTH following a stress response but not the basal hormone level (Bale and Vale, 2004). Two independent lines of CRFR1 knockout (CRFR1-KO) mice have demonstrated that in the absence of CRFR1, these mice showed a blunted response to restraint stress as revealed by a minimal increase in plasma ACTH and corticosterone, compared with the significant release seen in wild-type littermates (Smith et al., 1998; Timpl et al., 1998).

The role of CRFR2 in the stress response remains unclear. Central administration of the CRFR2 antagonist produces little effect on the ACTH response to or recovery from a restraint stress (Pelleymounter et al., 2002). In addition, CRFR2 knockout (CRFR2-KO) mice developed by three independent laboratories (Bale et al., 2000b; Coste et al., 2000; Kishimoto et al., 2000) all showed normal basal levels of ACTH and corticosterone and a normal circadian rhythm of hormone levels. Two lines of these mice, however, revealed heightened sensitivity to stress (Bale et al., 2000b; Coste et al., 2000). These studies suggest that this receptor may function endogenously to dampen or modulate the stress response associated with CRFR1 activation. Interestingly, no differences were found in basal CRF mRNA or protein levels in the PVN in CRFR2-KO mice (Bale et al., 2000b; Coste et al., 2000). However, it has been found that the expression levels of vasopressin (AVP) in the PVN is increased, which suggests that the CRF response is augmented in these mice, and thus results in the increased sensitivity and hormone levels (Bale et al., 2002).

The response to stress in mice deficient in both CRF receptors (CRFR1/2-KO) has also been examined. In the absence of either known receptor, mice display remarkably little HPA axis response to a restraint stress (Bale et al., 2002). ACTH and corticosterone levels following restraint stress are significantly lower in the CRFR1/2-KO mice compared with CRFR1-KO mice, suggesting a possible role of CRFR2 in mediating HPA-axis sensitivity (Bale et al., 2002). Since CRFR1 is abundantly expressed in anterior pituitary corticotropes, and CRFR2 is not, it suggests that a possible new involvement of CRFR2 in HPA axis activation may occur upstream of corticotropic cell stimulation, possibly in CRF cell bodies within the hypothalamus. Taken together, these data suggest that both CRF receptors participate in the maintenance and regulation of homeostasis in response to stress.

These studies support a hypothetical model in which CRFR1 and CRFR2 play important and opposing roles in regulation of organismal responses to stress and perturbations of homeostasis. This model suggests that following a challenge, CRF activation of CRFR1 stimulates the HPA axis and sympathetic nervous system, in order to maintain physiologic equilibrium under acute and chronic perturbations for energy mobilization and redistribution. CRFR2, however, may function as an inhibitory or modulatory receptor to dampen the actions of CRFR1. Regulation of the relative contribution of the two CRF receptors to brain CRF pathways may be essential in coordinating physiological responses to stress. The development of disorders related to heightened stress sensitivity and dysregulation of stress-coping mechanisms appears to involve regulatory mechanisms of CRF family members (Bale and Vale, 2004).

## 2.3 CRF Binding Protein

The binding protein of CRF (CRF-BP) is a 37 kDa N-linked glycoprotein expressed in several regions in the body, such as cerebral cortex, hippocampus, pituitary, liver, placenta, and in the circulation. Both CRF and UcnI have high affinities for the CRF-BP, but neither UcnII nor UcnIII binds to the CRF-BP.

It has been proposed that CRF-BP can modulate CRF activities by competing for ligand availability with CRF Receptors, and it has also been proposed to prevent inappropriate pituitary–adrenal stimulation during pregnancy (Potter et al., 1991). Consistent with its proposed role, about 40–60% of the human brain CRF is bound with CRF-BP by forming dimer complexes (CRF2/BP2) (Jahn et al., 2002; Bale and Vale, 2004). Recombinant CRF-BP is known to reduce the endocrine activities of CRF, which then leads to the blocking of CRF-induced ACTH secretion from the anterior pituitary (Potter et al., 1992, 1994; Chen et al., 1993). However, the data from CRF-BP overexpression (CRF-BP-OE) and knockout (CRF-BP-KO) experiments show the opposite results. In both cases, although high levels of circulating CRF-BP were detected in these mice, the HPA axis stress response remained unchanged in basal or acute stress conditions (Burrows

et al., 1998; Lovejoy et al., 1998; Karolyi et al., 1999). Increased levels of CRF and AVP were detected in the PVN of these mice, which suggests that a compensation of increased ligand expression to overcome the increased production of CRF-BP was taking place.

As inferred from the data on CRF-BP-KO and CRF-BP-OE mice, CRF-BP is not a critical component in regulation of the stress response. In addition, CRF-BP has been detected in brain regions not associated with CRF activity, suggesting that it may also have CRF-independent actions. It appears that the function of CRF-BP is not very clear and is in need of more data at this stage.

# 3 Physiological Function of CRF

## 3.1 Intracellular Signal Transduction of CRF

### 3.1.1 PKA and PKC Pathways

As mentioned previously, CRFR1 and CRFR2 located in the nervous system are predominately linked to $G_s$, adenylate cyclase, and the PKA pathway (Dautzenberg et al., 2000). However, other G proteins have been observed to link to CRF receptors in various tissues (Grammatopoulos et al., 2001). For example, in Leydig cells and the placenta, CRFR1 might signal exclusively via $G_q$-mediated stimulation of phospholipase C (PLC) and formation of inositol phosphates (Grammatopoulos et al., 2000). In addition, the observation that CRF increases intracellular $Ca^{2+}$ concentrations in astrocytes and melanoma cells suggests that the undefined CRF receptor subtypes in these settings might also signal via the PLC pathway (Fazal et al., 1998). Even within the nervous system, CRFR1 in cerebral cortex can couple to both $G_s$ and $G_q$ proteins, thereby activating cyclic adenosine monophosphate (cAMP) and PLC pathways (Dieterich et al., 1996). Data obtained from physiological experiments on hippocampus slices also show that CRF can increase neuronal activity by activating different intracellular signaling pathways (PKC or PKA) in two mouse inbred strains, BALB/c and C57BL/6N (Blank et al., 2003).

### 3.1.2 MAPK Pathway

The mitogen-activated protein kinase (MAPK) superfamily of signaling cascade includes the extracellular signal-regulated kinase (ERK) 1/2 (which is also known as the p42/p44 MAPKs), the p38 MAPK, and the stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK). The ERK cascade has been linked to many important cellular functions. Originally, it was discovered as a critical regulator of gene expression, cell division, and differentiation. Later, the ERK cascade was found to be heavily involved in the neuronal synaptic plasticity and memory formation, especially during the late phase of long-term potentiation (LTP) mediated through the *N*-methyl-D-aspartic acid (NMDA) receptors (Atkins et al., 1998; Kandel, 2001; Matynia et al., 2001; Sweatt, 2001).

Previous studies have shown that memory can be markedly enhanced by acute stressful experiences (Shors et al., 1992), and modulation of ERK1/2-dependent signaling by hippocampal CRFR2 is selectively involved in the enhancing effects of stress on memory consolidation (Sananbenesi et al., 2003). It has been found that the phosphorylation and activation of p42/p44 MAPK and calcium/cAMP response element-binding protein (CREB) in the CHO cell line transfected with CRFR1 or CRF2$\alpha$ is increased, but not p38MAPK or SAPK (Rossant et al., 1999). However, the pathways from CRF to MAPK and CREB are not simple. Apparently CRF does not activate MAPK through the usual cAMP/PKA pathway. Instead, CRF activation of MAPK is mediated by either $\beta\gamma$-subunits of heterotrimeric G proteins or phosphatidylinositol-3 kinase (PI3K). CREB is activated almost entirely by CRF through the cAMP/PKA pathway (Rossant et al., 1999).

Another very important cellular function of MAPK is the cytoprotective effects against external insults. In organotypic hippocampal cultures, CRF in low physiological concentrations (2 pM) can prevent glutamate-induced neurotoxicity primarily via CRFR1-mediated signaling through the PKA/ERK pathway

(Elliott-Hunt et al., 2002). In cultured rat hippocampal neurons, CRF can increase the resistance of the cells to oxidative and excitotoxic insults through CRFR1. This neuroprotective effect is mediated by multiple protein kinases including PKA, PKC, and the downstream ERK1/2 (Pedersen et al., 2002). This cytoprotective effect, mediated through MAPK, is not limited to the CNS. For example, UcnII and UcnIII can mediate a cardioprotective effect through CRFR2 and ERK1/2 in rat heart (Brar et al., 2004b). Interestingly, the phosphorylation of ERK1/2 in this case is regulated by the PI3K and Ras/Raf-1 pathway, but independent of cAMP/PKA pathway.

Furthermore, CRF has been found to function as a differentiating factor, through cAMP-and ERK-signaling pathways, which triggers neurite outgrowth and morphological changes in a neuronal cell line that expresses CRFR1 (Cibelli et al., 2001). It is clear now that the activation of MAPK by CRF varies according to the cell type, the CRFR subtypes, and the signaling pathways (Brar et al., 2004a). Since CRFR can couple to multiple G proteins, the CRF modulation of MAPK signaling can be very complex (Grammatopoulos et al., 2001).

## 3.2 Extrahypothalamic Functions of CRF in Different Brain Regions

### 3.2.1 CRF in the Dorsal Raphe Nucleus

The dorsal raphe nucleus (DR) contains heterogeneous populations of neurons, with distinct morphologies, projections, and neurochemical characteristics. The majority of the DR neurons are serotonergic, and they have parallel and overlapping ascending projections to many forebrain structures (Hornung, 2003). However, the serotonergic neurons could be subcategorized further according to their morphology and firing patterns. What makes it even more complex is that different subtypes of serotonergic neurons may respond to the same stimuli differently. The primary function of DR includes the regulation of sleep–awake states, behavioral arousal, and stress-related affective conditions (Abrams et al., 2004). The mRNA and protein of both CRFRs have been detected in the DR, but CRFR2 mRNAs exist in a much greater amount than that of CRFR1 (Day et al., 2004). Given the involvement of both serotonin (5-HT) and CRF in stress, it is important to understand the role of CRF in serotonergic DR nucleus.

CRF in the DR regulates the release of serotonin in forebrain terminal regions. By measuring the extracellular levels of serotonin by in vivo microdialysis in the DR neurons, one study has shown that the concentration of extracellular 5-HT is increased during and after stress (e.g., inescapable electric tailshocks) (Maswood et al., 1998). Subsequent experiments show that the inescapable shock increased 5-HT release from the DR, similar to the CRF effect, which further supports the positive regulatory function of CRF at DR (Hammack et al., 2002). Another study has shown that intracerebroventricular administration of CRF produces a bimodal effect on extracellular levels of 5-HT in the lateral septum. Doses of 0.3 and 1.0 μg decreased extracellular 5-HT levels, whereas both a higher (3.0 μg) and a lower (0.1 μg) dose had no effect (Price and Lucki, 2001). Similarly, another study shows that low concentrations of CRF produce primarily an inhibitory effect on DR discharge, and the effect diminishes or becomes excitatory at higher CRF concentration (Kirby et al., 2000). Depending on the targeting area, CRF can have opposite effects as well. For example, the midline raphe neurons are positively regulated by CRF, while the more rostral dorsal raphe serotonergic neurons are negatively regulated by CRF (Lowry et al., 2000). The same study demonstrates that a subpopulation of serotonergic DR neurons responds to CRF by increasing the firing frequency (Lowry et al., 2000). In general, CRF can interfere with the DR serotonergic system by changing the firing rate of neurons and the amount of serotonin released.

The serotonergic neurons in the DR are constantly under inhibitory control of GABAergic neurons located in the same area (Tao and Auerbach, 2000; Varga et al., 2001). Several studies demonstrate that CRF can also directly affect the GABAergic neurons in the dorsolateral DR to indirectly regulate the activities of serotonergic neurons under certain conditions. For example, CRF has been found to increase the expression of the immediate early gene, c-fos, of the GABAergic neurons following a swim stress task through a CRFR1-mediated pathway (Roche et al., 2003). By interacting with GABAergic neurons in the dorsolateral DR, CRF can reduce the amount of 5-HT released at the DR target areas (Roche et al., 2003). Another study

shows that activation of CRFR2 on the DR serotonergic neurons inhibits neuronal activities, whereas activation of CRFR2 on the nonserotonergic neurons indirectly excites the DR serotonergic neurons through disinhibition (Pernar et al., 2004). Both observations described above support earlier observations of Kirby et al. Once again they demonstrate the complex nature of the mechanisms by which CRF regulates neurons in the DR region.

### 3.2.2 CRF in the Hippocampus and Synaptic Plasticity

The hippocampus has a long-established role in certain forms of memory. The ventral hippocampus, a subregion of the hippocampus, is anatomically connected to prefrontal cortex, amygdala, and other subcortical regions associated with the HPA axis (Sananbenesi et al., 2003). As a target of CRF, hippocampus shows many responses to stressful stimuli. For example, repeated stress leads to atrophy of the dendrites in the CA3 region and the suppression of neurogenesis of dentate gyrus granule neurons (McEwen, 1999; Chen et al., 2004). In addition, the AMPA, but not NMDA, portion of synaptic responses of dentate gyrus neurons is significantly increased after corticosterone administration in chronically stressed rats (Karst and Joels, 2003).

Acute inescapable stress can dramatically affect the induction and the direction of plasticity at glutamatergic synapses in the hippocampus (Shors et al., 1997; Xu et al., 1997). The induction of activity-dependent persistent increases in synaptic efficacy, such as long-term potentiation (LTP), is inhibited by behavioral stress (Shors et al., 1989, 1997), while the induction of stable homosynaptic long-term depression (LTD), which is persistent decreases in synaptic efficacy, in the hippocampal CA1 area is facilitated by exposure to stress (Kim et al., 1996; Xu et al., 1997). However, another study shows that CRF application and acute stress facilitate (prime) LTP of population spikes (PS-LTP) in the mouse hippocampus and enhance context-dependent fear conditioning (Blank et al., 2002). Interestingly, the CA1 hippocampal extracellular 5-HT, which is released by serotonergic neurons located in the DR, can mimic the effect of stress by blocking the induction of LTP in nonstressed animals either through an indirect 5-$HT_3$ receptor or a direct 5-$HT_{1A}$-receptor-mediated pathway (Shakesby et al., 2002). With in vivo microdialysis, it has been shown that the stress-induced increase of extracellular 5-HT in hippocampus is mediated by CRF. Both CRFR1 and CRFR2 are thought to be involved in this modulation (Linthorst et al., 2002).

### 3.2.3 CRF in the Prefrontal Cortex

The prefrontal cortex (PFC) is important for its "executive" capacity to process ongoing information and plan future actions. Stress has been recognized to strongly influence cognitive and emotional processes subserved by PFC, including working memory, attention, and inhibition of inappropriate responses (Arnsten, 1998). Elucidation of the functional role of key neuromodulators in PFC, such as serotonin, dopamine, and CRF, is central to understanding why prefrontal cortical deficits are so prominent in many mental illnesses that are exacerbated by stress. In response to stress, PFC shows neurochemical changes and mediates altered behavior (Wellman, 2001). For example, chronic corticosterone treatment can reorganize the apical dendrites by increasing the amount of dendritic material proximally and decreasing the distal dendritic material in the PFC of rats (Wellman, 2001). Another study has shown that endogenous glucocorticoids are essential for maintaining working memory through a $D_1$-receptor-mediated hypodopaminergic mechanism in the PFC (Mizoguchi et al., 2004).

PFC is composed of two major neuronal populations: glutamatergic pyramidal projection neurons and GABAergic interneurons. One of the key roles of GABAergic inhibition in PFC is to shape the temporal flow of information, and thus regulating working memory (Constantinidis et al., 2002). As the primary inhibitory transmitter in the CNS, the downregulated GABA system has been linked to the pathophysiology of several anxiety disorders. This suggests that the GABA system plays a role in homeostasis during stress, opposing the action of CRF on HPA axis (Lydiard, 2003). The GABAergic inhibitory transmission is one of the main targets of the serotonergic system in PFC (Zhou and Hablitz, 1999; Feng et al., 2001; Yan, 2002). For example, by activating 5-$HT_2$ receptors, 5-HT induces a large desensitizing enhancement of the

amplitude and frequency of spontaneous inhibitory postsynaptic currents (sIPSC) mediated by $GABA_A$ receptors (Zhou and Hablitz, 1999). On the other hand, activation of 5-$HT_4$ receptor produces an activity-dependent bidirectional regulation of GABA-evoked currents (Cai et al., 2002). A recent study (Tan et al., 2004) has established a link between CRF and 5-HT in the PFC, both of which are critically involved in the pathophysiology of stress-related mental disorders such as anxiety and depression. The experimental data have demonstrated that in PFC slices pretreated with CRF or from stressed animals, the 5-HT regulation of sIPSC lasts much longer, suggesting that in response to stressful stimuli, CRF could lead to disturbed PFC functions by altering the serotonergic regulation of GABA transmission (Tan et al., 2004). This finding provides a possible mechanism for the stress-induced exacerbation of psychiatric disorders that are associated with aberrant serotonin actions.

### 3.2.4 CRF in the Amygdala and Fear Memory

Many studies have demonstrated that the amygdala, a collection of nuclei buried deep within the temporal lobe, is critical for providing affective salience to sensory information and is involved in the associative processes for both appetitive and aversive emotions (Maren, 2003; Rainnie et al., 2004). Studies have shown that amygdala, especially the basolateral amygdaloid complex (BLA), is an essential component mediating emotional arousal, stress hormone effect on cognitive functions, and the acquisition and expression of pavlovian fear conditioning (Goldstein et al., 1996; Maren, 2003).

The amygdala is a major extrahypothalamic source of CRF-containing neurons and has high expression levels for the two CRF receptors (Palkovits et al., 1983; Van Pett et al., 2000). Under normal conditions, the function of amygdala can be modulated by stress hormones such as CRF. The dysfunction of amygdala can also contribute to the pathology of disorders such as posttraumatic stress disorder (PTSD) (Maren, 2003). During periods of stress, CRF is released into the amygdala (Cratty et al., 1995; Pich et al., 1995), and local CRF receptor activation has been suggested as a substrate for stress-induced alterations in affective behavior (Gray and Bingaman, 1996; Yu and Shinnick-Gallagher, 1998). In addition, acute stress has been found to correlate with the increased gene expression of CRF-BP, but not CRF or CRFRs, in the BLA region (Herringa et al., 2004). It appears that continuous activation of CRFRs of BLA neurons can cause the animal to develop anxiety-like responses in behavioral tests. These behavioral and autonomic responses persist for over one month in the absence of additional CRFR stimulus. Whole-cell patch-clamp recordings from BLA neurons of these hyperreactive animals revealed a pronounced reduction in both spontaneous and stimulation-evoked IPSPs, leading to a hyperexcitability of the BLA network. This stress-induced plasticity appears to be dependent on NMDA receptors and subsequent calcium/calmodulin-dependent protein kinase II (CaMKII) activation (Rainnie et al., 2004).

The BLA projects extensively to several regions of the prefrontal cortex, where the working memory is formed. Exposure to stress or glucocorticoid administration impairs the induction of LTP of the amygdala–prefrontal cortex pathway and the working memory (Maroun and Richter-Levin, 2003; Roozendaal et al., 2004). Lesions or pharmacological inactivation of BLA can block the modulatory effect of glucocorticoids on working memory (Roozendaal et al., 2004), suggesting that BLA activities are essential for the glucocorticoid effect on PFC functions.

The central nucleus of amygdala is thought to be a key extrahypothalamic region in response to CRF and stress conditions. In acutely dissociated CeA neurons, CRF increases the peak of the whole-cell $Ca^{2+}$ current, which may be functionally related to the autonomic, behavioral, and endocrine response to stress (Yu and Shinnick-Gallagher, 1998). CRF and ethanol have both been found to enhance GABA-mediated neurotransmission in CeA (Roberto et al., 2003; Nie et al., 2004). However, the effect of both CRF and ethanol on GABAergic neurotransmission is lost in CRFR1 knockout mice, suggesting that the behavioral and motivational effects of ethanol are at least partially mediated through CRF signaling (Nie et al., 2004). Another limbic nucleus, lateral septum mediolateral nucleus (LSMLN), which is reciprocally innervated with CeA, is also involved in the stress and affective disorders. Experimental data (Liu et al., 2004) suggest that CRF depresses excitatory glutamatergic transmission through CRFR1-mediated postsynaptic pathways in the CeA, while facilitating glutamatergic transmission in the LSMLN. Conversely, UcnI, with higher

affinity to CRFR2 than CRF, facilitates the excitatory postsynaptic current (EPSC) via CRFR2-mediated presynaptic and postsynaptic pathways in the CeA, while depressing EPSCs in the LSMLN through the same signaling pathway. These data demonstrate that CRF receptors in CeA and LSMLN synapses exert and maintain a significant synaptic tone and regulate excitatory glutamatergic transmission differently in these regions (Liu et al., 2004).

### 3.2.5 CRF in the Ventral Tegmental Area

The ventral tegmental area (VTA), which sends dopaminergic inputs to the nucleus accumbens, PFC, and amygdala, plays a central role in both acute and chronic responses to addictive drugs (Spanagel and Weiss, 1999). The state of stress contributes significantly to various aspects of drug addiction, particularly in acute withdrawal, protracted abstinence, and vulnerability to relapse (Koob and Heinrichs, 1999). CRF appears to be a key link between the behavioral and physiological effects of stress and drugs of abuse (Shaham et al., 2000). For example, acute withdrawal is associated with a negative affective state including dysphoria, depression, irritability, and anxiety, which is mostly, if not all, associated with the CRF and the stress response system (Koob and Heinrichs, 1999).

Similar to drugs of abuse, stress increases the dopamine (DA) released to PFC and nucleus accumbens (De Biasi and Dani, 2003). Moreover, the AMPA/NMDA ratio, an indirect measurement of synaptic strength, in VTA dopaminergic neurons is increased in rats treated with drugs of abuse such as cocaine, morphine, amphetamine, and in stressed animals (Saal et al., 2003). Another study has shown that CRF can potentiate NMDAR-mediated EPSCs in a subset of VTA dopaminergic neurons through the CRFR2/PLC/PKC pathway (Ungless et al., 2003). Taken together, these results suggest that the CRF regulation of VTA dopamine neurons may be a key neural adaptation contributing to addiction.

### 3.2.6 CRF in Other Brain Regions

The locus coeruleus–norepinephrine (LC/NE) autonomic systems is another major component of the stress system besides the CRF (Tsigos and Chrousos, 2002). Data have shown that NE neurons in the brain stem can stimulate PVN to release CRF, and therefore activate the HPA axis. On the other hand, the CRF-containing neurons in the PVN, amygdala, and other areas have projection to the LC to modulate the electrophysiological activities of LC/NE neurons (Dunn et al., 2004). In addition, long-term blockade of CRFR1 increases exploratory behavior, possibly by reducing the LC activity (Mallo et al., 2004). CRF in the LC area might even play an important role in stress-induced suppression of the reproductive system, because CRF administered in LC area reduces the luteinizing hormone (LH) pulse frequency in ovariectomized rats. This result shows that CRF can impact and inhibit the hypothalamic–pituitary–gonadal (HPG) axis and is consistent with the well-established regulatory function of LC during stress (Mitchell et al., 2005).

In the cerebellum, a high concentration of CRF is found in the climbing fiber (CF) afferents, which supply excitatory synapse from the inferior olive to the Purkinje cells. One important function of the CF system is to induce LTD at the parallel fiber synapses of Purkinje cells (Ito, 1989). CRF found in CF appears to play a crucial role in cerebellar LTD, because LTD induction is effectively blocked by specific CRF receptor antagonists, and LTD is no longer observed in CF-deprived cerebella but is restored by CRF replenishment (Miyata et al., 1999).

The impact of CRF as a neuropeptide in various brain areas is summarized in ❯ *Table 26-1*.

## 4 CRF and Stress-Related Neuropsychiatric Disorders

### 4.1 Linkages Between CRF and Mental Disorders

Many findings show that the pathophysiology of several mental disorders, such as major depressive disorder (MDD), anxiety, panic disorder, PTSD, and schizophrenia, is related to the stress response system,

**Table 26-1**
**Extrahypothalamic functions of CRF**

| Brain regions | Impact of CRF | |
|---|---|---|
| | Direct effects[a] | Indirect effects |
| Dorsal raphe nucleus | • CRF has opposite effects on different regions of serotonergic neurons in DR | • In stressed animals, CRF in DR regulates the release of serotonin in forebrain terminal regions<br>• In response to stress, CRF interacts with GABAergic neurons in DR to indirectly regulate the activities of serotonergic neurons and serotonin release |
| Hippocampus | — | • Repeated stress leads to atrophy of dendrites in CA3 and the suppression of neurogenesis of dentate gyrus granule neurons<br>• The induction of hippocampal LTP is inhibited by behavioral stress<br>• The induction of hippocampal LTD is facilitated by exposure to stress |
| Prefrontal cortex | • CRF alters the serotonergic regulation of GABA transmission in PFC | • In response to stress, PFC shows neurochemical changes and mediates altered behavior |
| Amygdala | • Continuous activation of CRFRs of BLA neurons induces anxiety-like responses in animal behavioral tests, which is attributable to the reduction of GABAergic inhibition and hyperexcitability of the BLA network<br>• Ethanol enhances GABA-mediated transmission in CeA through CRF signaling<br>• CRFRs CeA and LSMLN synapses regulate excitatory glutamatergic transmission differently in these regions | • Exposure to stress or glucocorticoid administration impairs the induction of LTP of the amygdala–prefrontal cortex pathway and the working memory |
| Ventral tegmental area | • CRF potentiates NMDAR-mediated EPSCs in a subset of VTA neurons through the CRFR2/PLC/PKC pathway | • Stress increases the dopamine released to PFC and to nucleus accumbens from VTA<br>• Stress increases the synaptic strength in VTA dopaminergic neurons |
| Locus coeruleus | • The CRF-containing neurons have projection to LC for modulating the electrophysiological activities of norepinephrine neurons<br>• Long-term blockade of CRFR1 increases exploratory behavior, possibly by reducing the LC activity | — |
| Cerebellum | • High concentration of CRF found in the climbing fiber afferents plays a crucial role in cerebellar LTD at the parallel fiber synapses of Purkinje cells | |

[a]Direct effect of CRF: data obtained from in vitro studies with application of CRF or other CRFR ligands directly on tissues. Indirect effect of CRF: data obtained from in vivo studies with animals exposed to external stressors

to the noncontinuous hypersecretion of cortisol due to increased CRF activities, as well as to systems that regulate the CRF response (Arborelius et al., 1999; Claes, 2004). Many experimental data suggest that alteration of CRF1 and/or CRF2 receptor functioning is involved in the etiology of human stress disorders, particularly anxiety and depression (Arborelius et al., 1999). Severe anxiety and depression has been hypothesized to result from exaggerated neurotransmission in one or more of the following CRF-regulated pathways mediating the stress response: the HPA axis, the LC/NE systems, and the serotonergic DR system (Arborelius et al., 1999; Dautzenberg and Hauger, 2002).

Acute stress, acute experience of loss, chronic stress factors, and early childhood trauma, which depend on a critical time window, the nature of the stressors, presence or absence of supportive environment, and genetic liability, all can induce depression (Arborelius et al., 1999; Rosenblum et al., 2001; Claes, 2004). MDD patients often have clinically significant excessive exposure to glucocorticoids (Gold et al., 2002; Claes, 2004). Glucocorticoids exert negative feedback effects on hypothalamic CRF neurons. In depressed patients, the negative feedback loop fails to bring cortisol levels back to their homeostatic levels, either secondary to loss of glucocorticoid negative feedback or to an overriding stimulus to activation of the HPA axis.

Increasing amount of data indicate that lifelong susceptibility to anxiety can be determined by both genetic and environmental factors during early development (Gross and Hen, 2004). It has been shown that 5-HT is essential for the establishment of normal anxiety-modulating circuits during postnatal development (Gross et al., 2002; Gross and Hen, 2004). Experimental data also show that application of CRFR antagonists or CRF antisense oligodeoxynucleotide can produce anxiolytic effects in the rats through CRFR1-dependent pathways (Arborelius et al., 1999). In addition, environmental conditions during developmental stages are known to alter the glucocorticoid receptor gene expression in the hippocampus and the HPA axis responses to stress (Weaver et al., 2001).

One of the most consistent findings in the study of PTSD, a subclass of anxiety disorder, is the decrease of the volume of hippocampus, a structure in the medial temporal lobe required for associative memory (Gross and Hen, 2004). The volume and structure of hippocampus, which can be easily damaged by stress hormone and environment factors, might be different from person to person due to genetic variation (McEwen, 1999; Claes, 2004; Gross and Hen, 2004). PTSD is an example of anxiety disorders in which environmental risk factors seem to be modulated by genetic factors. In other words, the hippocampal volume is a preexisting condition that determines the susceptibility to PTSD (Gross and Hen, 2004). Similar to MDD, PTSD is characterized by a central CRF hyperdrive, but unlike in MDD, HPA axis negative feedback is enhanced, resulting in a low cortisol output (Bremner et al., 1997; Heim et al., 1997; Yehuda, 1997; Baker et al., 1999). In panic disorder, another subclass of anxiety disorders, a diminished ACTH response to CRF suggests that the HPA axis is dysfunctional (Roy-Byrne et al., 1986).

Abnormal prefrontal cortical activity and activation of the HPA axis have been extensively reported in patients with affective disorders and schizophrenia. The mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) are two nuclear hormone receptors of primary importance in the control of stress-related HPA activity. A recent study shows that blocking brain MR activity significantly enhances CRF-induced ACTH and cortisol release in humans (Born et al., 1997). The expression of MR is deficient in the prefrontal cortex of patients with schizophrenia and affective disorders (Xing et al., 2004). Moreover, it has been reported that CRF concentrations in the cerebrospinal fluid (CSF) are increased in 18 of 21 male patients with schizophrenia after maintenance haloperidol is replaced by placebo. However, CRF concentrations are not significantly related to severity of psychosis, depression, anxiety or negative symptoms (Forman et al., 1994).

## 4.2 Alteration of the CRF System in Brains of Patients Suffering from Depression and of Suicide Victims

Conflicting results have been obtained regarding the alteration of the CRF system in brains of patients suffering from depression and suicide victims. Some reports show that the CRF level in CSF, the circulating cortisol, or the CRF immunoreactivity in PVN neurons is increased in patients suffering from depression

(Nemeroff et al., 1984; Raddsheer et al., 1995; Austin et al., 2003), whereas others have found a 23% reduction in the number of CRF binding sites in the frontal cortex of the suicide victims (Nemeroff et al., 1988). On the other hand, several reports show that there is no difference between patients suffering from depression and the control groups in CRF immunoreactivity, in receptor binding, or in CRF levels (Pitts et al., 1995; Hucks et al., 1997; Austin et al., 2003). However, postmortem detection of very high concentrations of CRF in the CSF of severely depressed suicide victims has provided additional support for the hypothesis that chronic hypersecretion of CRF plays a leading role in the etiology of major depression (Arborelius et al., 1999; Dautzenberg and Hauger, 2002).

Recent studies show that there are indeed differences between the brains of patients suffering from depression and of control groups. These differences are found at more local regions rather than the whole brain. For example, CRFR1 is increased in the regions related to the NE/5-HT neurotransmission, such as the locus coeruleus, the median raphe, and the caudal dorsal raphe, when compared between suicide victims and control groups. However, no difference exists in dorsal tegmentum or medial parabrachial nucleus. CRFR1 levels are specifically increased in NE- and 5-HT- containing pontine nuclei (Austin et al., 2003). Other differences, such as a shift in the ratio of CRFR1/R2 in the pituitaries of suicide victims, have also been reported (Hiroi et al., 2001). Also, it has been found that in the frontal cortex, mRNA of CRFR1, but not CRFR2, is reduced in brains from suicide victims (Merali et al., 2004).

### 4.3 Mice with Mutant CRF System

The knockout (KO) and overexpression (OE) systems are two valuable tools for investigating how CRF system functions in relation to the stress-related disorders. KO or OE systems have been generated for almost every member in the CRF family. Phenotypes obtained from these animals have greatly expanded or confirmed what we know about the pathophysiological functions of CRF.

CRF-OE mice show increased anxiety-like behaviors and hyperactivity in a novel environment (Stenzel-Poore et al., 1994), which suggests a connection between stress, anxiety, and locomotor activity. Despite strong evidence in support of the involvement of CRF in anxiogenic behaviors, CRF-KO mice display normal behavioral responses to stress (Weninger et al., 1999). A significant increase in anxiety-like behaviors has been found in the UcnI-KO mice (Vetter et al., 2002). CRF-BP-KO mice display an expected increase in anxiety-like behaviors which is presumably the result of increased free CRF or UcnI in the absence of the binding protein (Karolyi et al., 1999). Although these results suggest an important regulatory role for CRF-BP in "dampening" functions in stress-induced behaviors, expression levels and comparisons of unbound CRF ligands have not yet been well characterized in these mice.

Mice deficient for CRFR1 display the predicted phenotype of reduced anxiety and impaired stress response (Smith et al., 1998). These results obtained from global deletion of CRFR1 have confirmed previous studies for an anxiogenic role for this receptor. CRFR2-KO mice show the expected anxiety-like behaviors; supporting the hypothesis that CRFR2 normally functions to reduce the anxiety-generating action of CRFR1 (Bale et al., 2000a). Consistent with the hypothesis, treatment of CRFR2-KO mice with the CRFR1 antagonist decreased the anxiety behaviors in experimental animals. The increased CRF mRNA levels in the central nucleus of the amygdala in CRFR2-KO mice may explain the increased anxiety-like and depression-like behaviors detected in these mice (Bale et al., 2000a), as this nucleus plays a major role in the transduction of stress signals (Liang et al., 1992). Results from testing double CRF-receptor-deficient mice for anxiety-like behaviors have revealed that their responses are sexually dichotomous (Bale et al., 2002). Female CRFR1/2-KO mice display decreased levels of anxiety-like behaviors when compared with wild-type mice, while male CRFR1/2-KO mice display anxiogenic-like behavior.

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## References


Abrams JK, Johnson PL, Hollis JH, Lowry CA. 2004. Anatomic and functional topography of the dorsal raphe nucleus. Ann NY Acad Sci 1018: 46-57.

Arborelius L, Owens M, Plotsky P, Nemeroff C. 1999. The role of corticotropin releasing factor in depression and anxiety disorders. J Endocrinol 160: 1-12.

Arnsten AFT. 1998. The biology of being frazzled. Science 280: 1171-2.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, David SJ. 1998. The MAPK cascade is required for mammalian associative learning. Nat Neurosci 1: 602-609.

Austin M, Janosky J, Murphy H. 2003. Increased corticotropin-releasing hormone immunoreactivity in monoamine-containing pontine nuclei of depressed suicide men. Mol Psychiatry 8: 324-332.

Baker DG, West SA, Nicholson WE, Ekhator NN, Kasckow JW, et al. 1999. Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. Am J Psychiatry 156: 585-588.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, et al. 2000a. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nat Genet 24: 410-414.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF. 2000b. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nat Genet 24: 410-414.

Bale TL, Picetti R, Contarino A, Koob GF, Vale WW, et al. 2002. Mice deficient for both corticotropin-releasing factor receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior. J Neurosci 22: 193-199.

Bale TL, Vale WW. 2004. CRF and CRF receptors: role in stress responsivity and other behaviors. Annu Rev Pharmacol Toxicol 44: 525-557.

Blank T, Nijholt I, Eckart K, Spiess J. 2002. Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor and acute stress: implications for hippocampus-dependent learning. J Neurosci 22: 3788-3794.

Blank T, Nijholt I, Grammatopoulos DK, Randeva HS, Hillhouse EW, et al. 2003. Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. J Neurosci 23: 700-707.

Born J, Steinbach D, Dodt C, Fehm H-L 1997. Blocking of central nervous mineralocorticoid receptors counteracts inhibition of pituitary–adrenal activity in human sleep. J Clin Endocrinol Metab 82: 1106-1110.

Brar BK, Chen A, Perrin MH, Vale W. 2004a. Specificity and regulation of extracellularly regulated kinase1/2 phosphorylation through corticotropin-releasing factor (CRF) receptors 1 and 2{beta} by the CRF/Urocortin family of peptides. Endocrinology 145: 1718-1729.

Brar BK, Jonassen AK, Egorina EM, Chen A, Negro A, et al. 2004b. Urocortin-II and urocortin-III are cardioprotective against ischemia reperfusion injury: an essential endogenous cardioprotective role for corticotropin releasing factor receptor type 2 in the murine heart. Endocrinology 145: 24-35.

Bremner J, Licinio J, Darnell A, Krystal J, Owens M, et al. 1997. Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. Am J Psychiatry 154: 624-629.

Burrows HL, Nakajima M, Lesh JS, Goosens KA, Samuelson LC, et al. 1998. Excess corticotropin releasing hormone-binding protein in the hypothalamic–pituitary–adrenal axis in transgenic mice. J Clin Invest 101: 1439-1447.

Cai X, Flores-Hernandez J, Feng J, Yan Z. 2002. Activity-dependent bidirectional regulation of $GABA_A$ receptor channels by the $5\text{-}HT_4$ receptor-mediated signalling in rat prefrontal cortical pyramidal neurons. J Physiol (Lond) 540: 743-759.

Chen A, Perrin M, Brar B, Li C, Jamieson P, et al. 2004. Mouse corticotropin releasing factor receptor type 2{alpha} gene: isolation, distribution, pharmacological characterization and regulation by stress and glucocorticoids. Mol Endocrinol 19: 441-458.

Chen R, Lewis K, Perrin M, Vale W. 1993. Expression cloning of a human corticotropin-releasing-factor receptor. PNAS 90: 8967-8971.

Cibelli G, Corsi P, Diana G, Vitiello F, Thiel G. 2001. Corticotropin-releasing factor triggers neurite outgrowth of a catecholaminergic immortalized neuron via cAMP and MAP kinase signalling pathways. Eur J Neurosci 13: 1339-1348.

Claes SJ. 2004. Corticotropin-releasing hormone (CRH) in psychiatry: from stress to psychopathology. Ann Med 36: 50-61.

Constantinidis C, Williams GV, Goldman-Rakic PS. 2002. A role for inhibition in shaping the temporal flow of information in prefrontal cortex. Nat Neurosci 5: 175-180.

Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, et al. 2000. Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat Genet 24: 403-409.

Coste SC, Murray SE, Stenzel-Poore MP. 2001. Animal models of CRH excess and CRH receptor deficiency display altered adaptations to stress1. Peptides 22: 733-741.

Cratty MS, Ward HE, Johnson EA, Azzaro AJ, Birkle DL. 1995. Prenatal stress increases corticotropin-releasing factor (CRF) content and release in rat amygdala minces. Brain Res 675: 297-302.

Dautzenberg FM, Hauger RL. 2002. The CRF peptide family and their receptors: yet more partners discovered. Trends Pharmacol Sci 23: 71-77.

Dautzenberg FM, Higelin J, Teichert U. 2000. Functional characterization of corticotropin-releasing factor type 1 receptor endogenously expressed in human embryonic kidney 293 cells. Eur J Pharmacol 390: 51-59.

Day H, Greenwood B, Hammack S, Watkins L, Fleshner M, et al. 2004. Differential expression of 5HT-1A, alpha 1b adrenergic, CRF-R1, and CRF-R2 receptor mRNA in serotonergic, gamma-aminobutyric acidergic, and catecholaminergic cells of the rat dorsal raphe nucleus. J Comp Neurol 474: 364-378.

De Biasi M, Dani JA. 2003. Stress hormone enhances synaptic NMDA response on dopamine neurons. Neuron 39: 387-388.

Dieterich KD, Grigoriadis DE, De Souza EB. 1996. Homologous desensitization of human corticotropin-releasing factor, receptor in stable transfected mouse fibroblast cells. Brain Res 710: 287-292.

Dunn AJ, Swiergiel AH, Palamarchouk V. 2004. Brain circuits involved in corticotropin-releasing factor-norepinephrine interactions during stress. Ann NY Acad Sci 1018: 25-34.

Elliott-Hunt CR, Kazlauskaite J, Wilde GJC, Grammatopoulos DK, Hillhouse EW. 2002. Potential signalling pathways underlying corticotrophin-releasing hormone-mediated neuroprotection from excitotoxicity in rat hippocampus. J Neurochem 80: 416-425.

Fazal N, Slominski A, Choudhry MA, Wei ET, Sayeed MM. 1998. Effect of CRF and related peptides on calcium signaling in human and rodent melanoma cells. FEBS Lett 435: 187-190.

Feng J, Cai X, Zhao J, Yan Z. 2001. Serotonin receptors modulate $GABA_A$ receptor channels through activation of anchored protein kinase C in prefrontal cortical neurons. J Neurosci 21: 6502-6511.

Forman SD, Bissette G, Yao J, Nemeroff CB, van Kammen DP. 1994. Cerebrospinal fluid corticotropin-releasing factor increases following haloperidol withdrawal in chronic schizophrenia. Schizophr Res 12: 43-51.

Gold PW, Drevets WC, Charney DS, Drevets WC. 2002. New insights into the role of cortisol and the glucocorticoid receptor in severe depression. Biol Psychiatry 52: 381-385.

Goldstein LE, Rasmusson AM, Bunney BS, Roth RH. 1996. Role of the amygdala in the coordination of behavioral, neuroendocrine, and prefrontal cortical monoamine responses to psychological stress in the rat. J Neurosci 16: 4787-4798.

Grammatopoulos DK, Chrousos GP. 2002. Functional characteristics of CRH receptors and potential clinical applications of CRH-receptor antagonists. Trends Endocrinol Metab 13: 436-444.

Grammatopoulos DK, Randeva HS, Levine MA, Kanellopoulou KA, Hillhouse EW. 2001. Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. J Neurochem 76: 509-519.

Grammatopoulos DK, Randeva HS, Levine MA, Katsanou ES, Hillhouse EW. 2000. Urocortin, but not corticotropin-releasing hormone (CRH), activates the mitogen-activated protein kinase signal transduction pathway in human pregnant myometrium: an effect mediated via R1{alpha} and R2{beta} CRH receptor subtypes and stimulation of Gq-proteins. Mol Endocrinol 14: 2076-2091.

Gray T, Bingaman E. 1996. The amygdala: corticotropin-releasing factor, steroids, and stress. Crit Rev Neurobiol 10: 155-68.

Gross C, Hen R. 2004. The developmental origins of anxiety. Nat Rev Neurosci 5: 545-552.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, et al. 2002. Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416: 396-400.

Hammack SE, Richey KJ, Schmid MJ, LoPresti ML, Watkins LR, et al. 2002. The role of corticotropin-releasing hormone in the dorsal raphe nucleus in mediating the behavioral consequences of uncontrollable stress. J Neurosci 22: 1020-1026.

Heim C, Owens MJ, Plotsky PM, Nemeroff CB. 1997. The role of early adverse life events in the etiology of depression and posttraumatic stress disorder. Focus on corticotropin-releasing factor. Ann NY Acad Sci 821: 194-207.

Herringa RJ, Nanda SA, Hsu DT, Roseboom PH, Kalin NH. 2004. The effects of acute stress on the regulation of central and basolateral amygdala CRF-binding protein gene expression. Mol Brain Res 131: 17-25.

Hiroi N, Wong ML, Licinio J, Park M, Young PW, et al. 2001. Expression of corticotropin releasing hormone receptors type I and type II mRNA in suicide victims and controls. Mol Psychiatry 6: 540-546.

Horn F, Vriend G, Cohen FE. 2001. Collecting and harvesting biological data: the GPCRDB and NucleaRDB information systems. Nucleic Acids Res 29: 346-349.

Hornung J-P 2003. The human raphe nuclei and the serotonergic system. J Chem Neuroanat 26: 331-343.

Hucks D, Lowther S, Crompton MR, Katona CLE, Horton RW. 1997. Corticotropin-releasing factor binding sites in cortex of depressed suicides. Psychopharmacology 134: 174-178.

Ito M. 1989. Long-term depression. Annu Rev Neurosci 12: 85-102.

Jahn O, Eckart K, Brauns O, Tezval H, Spiess J. 2002. The binding protein of corticotropin-releasing factor: Ligand-binding site and subunit structure. PNAS 99: 12055-12060.

Kandel ER. 2001. The molecular biology of memory storage: A dialogue between genes and synapses. Science 294: 1030-1038.

Karolyi IJ, Burrows HL, Ramesh TM, Nakajima M, Lesh JS, et al. 1999. Altered anxiety and weight gain in corticotropin-releasing hormone-binding protein-deficient mice. PNAS 96: 11595-11600.

Karst H, Joels M. 2003. Effect of chronic stress on synaptic currents in rat hippocampal dentate gyrus neurons. J Neurophysiol 89: 625-633.

Kim JJ, Foy MR, Thompson RF. 1996. Behavioral stress modifies hippocampal plasticity through *N*-methyl-D-aspartate receptor activation. PNAS 93: 4750-4753.

Kirby LG, Rice KC, Valentino RJ. 2000. Effects of Corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. Neuropsychopharmacology 22: 148-162.

Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, et al. 2000. Deletion of Crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat Genet 24: 415-419.

Koob GF, Heinrichs SC. 1999. A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. Brain Res 848: 141-152.

Liang K, Melia K, Campeau S, Falls W, Miserendino M, et al. 1992. Lesions of the central nucleus of the amygdala, but not the paraventricular nucleus of the hypothalamus, block the excitatory effects of corticotropin-releasing factor on the acoustic startle reflex. J Neurosci 12: 2313-2320.

Linthorst ACE, Penalva RG, Flachskamm C, Holsboer F, Reul JMHM. 2002. Forced swim stress activates rat hippocampal serotonergic neurotransmission involving a corticotropin-releasing hormone receptor-dependent mechanism. Eur J Neurosci 16: 2441-2452.

Liu J, Yu B, Neugebauer V, Grigoriadis DE, Rivier J, et al. 2004. Corticotropin-releasing factor and urocortin I modulate excitatory glutamatergic synaptic transmission. J Neurosci 24: 4020-4029.

Lovejoy DA, Aubry JM, Turnbull A, Sutton S, Potter E, et al. 1998. Ectopic expression of the CRF-binding protein: minor impact on HPA axis regulation but induction of sexually dimorphic weight gain. J Neuroendocrinol 10: 483-491.

Lovenberg T, Chalmers D, Liu C, De Souza E. 1995. CRF2 alpha and CRF2 beta receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. Endocrinology 136: 4139-4142.

Lowry CA, Rodda JE, Lightman SL, Ingram CD. 2000. Corticotropin-releasing factor increases in vitro firing rates of serotonergic neurons in the rat dorsal raphe nucleus: evidence for activation of a topographically organized mesolimbocortical serotonergic system. J Neurosci 20: 7728-7736.

Lydiard R. 2003. The role of GABA in anxiety disorders. J Clin Psychiatry 64 (Suppl. 3): 21-27.

Mallo T, Berggard C, Eller M, Damberg M, Oreland L, et al. 2004. Effect of long-term blockade of CRF1 receptors on exploratory behaviour, monoamines and transcription factor AP-2. Pharmacol Biochem Behav 77: 855-865.

Maren S. 2003. What the amygdala does and doesn't do in aversive learning. Learn Mem 10: 306-308.

Maroun M, Richter-Levin G. 2003. Exposure to acute stress blocks the induction of long-term potentiation of the amygdala-prefrontal cortex pathway in vivo. J Neurosci 23: 4406-4409.

Maswood S, Barter JE, Watkins LR, Maier SF. 1998. Exposure to inescapable but not escapable shock increases extracellular levels of 5-HT in the dorsal raphe nucleus of the rat. Brain Res 783: 115-120.

Matynia A, Anagnostaras SG, Silva AJ. 2001. Weaving the molecular and cognitive strands of memory. Neuron 32: 557-559.

McEwen BS. 1999. Stress and hippocampal plasticity. Annu Rev Neurosci 22: 105-122.

Merali Z, Du L, Hrdina P, Palkovits M, Faludi G, et al. 2004. Dysregulation in the suicide brain: mRNA expression of corticotropin-releasing hormone receptors and $GABA_A$ receptor subunits in frontal cortical brain region. J Neurosci 24: 1478-1485.

Mitchell JC, Li XF, Breen L, Thalabard J-C, O'Byrne KT. 2005. The role of the locus coeruleus in corticotropin-releasing hormone and stress-induced suppression of pulsatile luteinizing hormone secretion in the female rat. Endocrinology 146: 323-331.

Miyata M, Okada D, Hashimoto K, Kano M, Ito M. 1999. Corticotropin-releasing factor plays a permissive role in cerebellar long-term depression. Neuron 22: 763-775.

Mizoguchi K, Ishige A, Takeda S, Aburada M, Tabira T. 2004. Endogenous glucocorticoids are essential for maintaining prefrontal cortical cognitive function. J Neurosci 24: 5492-5499.

Muglia L, Jacobson L, Dikkes P, Majzoub JA. 1995. Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 373: 427-432.

Nemeroff C, Widerlov E, Bissette G, Walleus H, Karlsson I, et al. 1984. Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. Science 226: 1342-1344.

Nemeroff CB, Owens MJ, Bissette G, Andorn AC, Stanley M. 1988. Reduced corticotropin releasing factor binding sites

in the frontal cortex of suicide victims. Arch Gen Psychiatry 45: 577-579.

Nie Z, Schweitzer P, Roberts AJ, Madamba SG, Moore SD, et al. 2004. Ethanol augments GABAergic transmission in the central amygdala via CRF1 receptors. Science 303: 1512-1514.

Palkovits M, Brownstein M, Vale W. 1983. Corticotropin releasing factor (CRF) immunoreactivity in hypothalamic and extrahypothalamic nuclei of sheep brain. Neuroendocrinology 37: 302-305.

Pedersen WA, Wan R, Zhang P, Mattson MP. 2002. Urocortin, but not urocortin II, protects cultured hippocampal neurons from oxidative and excitotoxic cell death via corticotropin-releasing hormone receptor type I. J Neurosci 22: 404-412.

Pelleymounter MA, Joppa M, Ling N, Foster AC. 2002. Pharmacological evidence supporting a role for central corticotropin-releasing factor2 receptors in behavioral, but not endocrine, response to environmental stress. J Pharmacol Exp Ther 302: 145-152.

Pernar L, Curtis AL, Vale WW, Rivier JE, Valentino RJ. 2004. Selective activation of corticotropin-releasing factor-2 receptors on neurochemically identified neurons in the rat dorsal raphe nucleus reveals dual actions. J Neurosci 24: 1305-1311.

Perrin M, Donaldson C, Chen R, Blount A, Berggren T, et al. 1995. Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. PNAS 92: 2969-2973.

Perrin MH, DiGruccio MR, Koerber SC, Rivier JE, Kunitake KS, et al. 2003. A soluble form of the first extracellular domain of mouse type 2beta corticotropin-releasing factor receptor reveals differential ligand specificity. J Biol Chem 278: 15595-15600.

Perrin MH, Vale WW. 1999. Corticotropin releasing factor receptors and their ligand family. Ann NY Acad Sci 885: 312-328.

Pich E, Lorang M, Yeganeh M, Fonseca F, Raber J, et al. 1995. Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. J Neurosci 15: 5439-5447.

Pitts AF, Samuelson SD, Meller WH, Bissette G, Nemeroff CB, et al. 1995. Cerebrospinal fluid corticotropin-releasing hormone, vasopressin, and oxytocin concentrations in treated patients with major depression and controls. Biol Psychiatry 38: 330-335.

Potter E, Behan D, Linton E, Lowry P, Sawchenko P, et al. 1992. The central distribution of a corticotropin-releasing factor (CRF)-binding protein predicts multiple sites and modes of interaction with CRF. PNAS 89: 4192-4196.

Potter E, Behan DP, Fischer WH, Linton EA, Lowry PJ, et al. 1991. Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. Nature 349: 423-426.

Potter E, Sutton S, Donaldson C, Chen R, Perrin M, et al. 1994. Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. PNAS 91: 8777-8781.

Price ML, Lucki I. 2001. Regulation of serotonin release in the lateral septum and striatum by corticotropin-releasing factor. J Neurosci 21: 2833-2841.

Raddsheer FC, van Heerikhuize JJ, Lucassen PJ, Hoogendijk WJG, Tilders FJH, et al. 1995. Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. Am J Psychiatry 152: 1372-1376.

Rainnie DG, Bergeron R, Sajdyk TJ, Patil M, Gehlert DR, et al. 2004. Corticotrophin releasing factor-induced synaptic plasticity in the amygdala translates stress into emotional disorders. J Neurosci 24: 3471-3479.

Rashid AJ, O'Dowd BF, George SR. 2004. Minireview: diversity and complexity of signaling through peptidergic G protein-coupled receptors. Endocrinology 145: 2645-2652.

Roberto M, Madamba SG, Moore SD, Tallent MK, Siggins GR. 2003. Ethanol increases GABAergic transmission at both pre- and postsynaptic sites in rat central amygdala neurons. PNAS 100: 2053-2058.

Roche M, Commons KG, Peoples A, Valentino RJ. 2003. Circuitry underlying regulation of the serotonergic system by swim stress. J Neurosci 23: 970-977.

Roozendaal B, McReynolds JR, McGaugh JL. 2004. The basolateral amygdala interacts with the medial prefrontal cortex in regulating glucocorticoid effects on working memory impairment. J Neurosci 24: 1385-1392.

Rosenblum LA, Forger C, Noland S, Trost RC, Coplan JD. 2001. Response of adolescent bonnet macaques to an acute fear stimulus as a function of early rearing conditions. Dev Psychobiol 39: 40-45.

Rossant CJ, Pinnock RD, Hughes J, Hall MD, McNulty S. 1999. Corticotropin-releasing factor type 1 and type 2 {alpha} receptors regulate phosphorylation of calcium/cyclic adenosine 3′,5′-monophosphate response element-binding protein and activation of p42/p44 mitogen-activated protein kinase. Endocrinology 140: 1525-1536.

Roy-Byrne P, Uhde T, Post R, Gallucci W, Chrousos G, Gold PW. 1986. The corticotropin-releasing hormone stimulation test in patients with panic disorder. Am J Psychiatry 143: 896-899.

Saal D, Dong Y, Bonci A, Malenka RC. 2003. Drugs of abuse and stress trigger a common synaptic adaptation in dopamine neurons. Neuron 37: 577-582.

Sananbenesi F, Fischer A, Schrick C, Spiess J, Radulovic J. 2003. Mitogen-activated protein kinase signaling in the hippocampus and its modulation by corticotropin-releasing

factor receptor 2: a possible link between stress and fear memory. J Neurosci 23: 11436-11443.

Shaham Y, Erb S, Stewart J. 2000. Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Res Rev 33: 13-33.

Shakesby AC, Anwyl R, Rowan MJ. 2002. Overcoming the effects of stress on synaptic plasticity in the intact hippocampus: rapid actions of serotonergic and antidepressant agents. J Neurosci 22: 3638-3644.

Shors TJ, Gallegos RA, Breindl A. 1997. Transient and persistent consequences of acute stress on long-term potentiation (LTP), synaptic efficacy, theta rhythms and bursts in area CA1 of the hippocampus. Synapse 26: 209-217.

Shors TJ, Seib TB, Levine S, Thompson RF. 1989. Inescapable versus escapable shock modulates long-term potentiation in the rat hippocampus. Science 244: 224-226.

Shors TJ, Weiss C, Thompson RF. 1992. Stress-induced facilitation of classical conditioning. Science 257: 537-9.

Smith GW, Aubry J-M, Dellu F, Contarino A, Bilezikjian LM, et al. 1998. Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron 20: 1093-1102.

Spanagel R, Weiss F. 1999. The dopamine hypothesis of reward: past and current status. Trends Neurosci 22: 521-527.

Stenzel-Poore M, Cameron V, Vaughan J, Sawchenko P, Vale W. 1992. Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. Endocrinology 130: 3378-3386.

Stenzel-Poore M, Heinrichs S, Rivest S, Koob G, Vale W. 1994. Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. J Neurosci 14: 2579-2584.

Sweatt JD. 2001. The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J Neurochem 76: 1-10.

Tan H, Zhong P, Yan Z. 2004. Corticotropin-releasing factor and acute stress prolongs serotonergic regulation of GABA transmission in prefrontal cortical pyramidal neurons. J Neurosci 24: 5000-5008.

Tao R, Auerbach SB. 2000. Regulation of serotonin release by GABA and excitatory amino acids. J Psychopharmacol 14: 100-113.

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JMHM, et al. 1998. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat Genet 19: 162-166.

Tsigos C, Chrousos GP. 2002. Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress. J Psychosom Res 53: 865-871.

Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, et al. 2003. Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. Neuron 39: 401-407.

Vale W, Spiess J, Rivier C, Rivier J. 1981. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. Science New Series 213: 1394-1397.

Van Pett K, Viau V, Bittencourt JC, Chan RKW, Li H-Y, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE. 2000. Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. J Comp Neurol 428: 191-212.

Varga V, Szekely AD, Csillag A, Sharp T, Hajos M. 2001. Evidence for a role of GABA interneurones in the cortical modulation of midbrain 5-hydroxytryptamine neurones. Neuroscience 106: 783-792.

Vetter DE, Li C, Zhao L, Contarino A, Liberman MC, et al. 2002. Urocortin-deficient mice show hearing impairment and increased anxiety-like behavior. Nat Genet 31: 363-369.

Weaver ICG, La Plante P, Weaver S, Parent A, Sharma S, et al. 2001. Early environmental regulation of hippocampal glucocorticoid receptor gene expression: characterization of intracellular mediators and potential genomic target sites. Mol Cell Endocrinol 185: 205-218.

Wellman CL. 2001. Dendritic reorganization in pyramidal neurons in medial prefrontal cortex after chronic corticosterone administration. J Neurobiol 49: 245-253.

Weninger SC, Dunn AJ, Muglia LJ, Dikkes P, Miczek KA, et al. 1999. Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. PNAS 96: 8283-8288.

Xing G, Russell S, Webster M, Post R. 2004. Decreased expression of mineralocorticoid receptor mRNA in the prefrontal cortex in schizophrenia and bipolar disorder. Int J Neuropsychopharmacol 7: 143-153.

Xu L, Anwyl R, Rowan MJ. 1997. Behavioural stress facilitates the induction of long-term depression in the hippocampus. Nature 387: 497-500.

Yan Z. 2002. Regulation of GABAergic inhibition by serotonin signaling in prefrontal cortex. Mol Neurobiol 26: 203-216.

Yehuda R. 1997. Sensitization of the hypothalamic-pituitary-adrenal axis in posttraumatic stress disorder. Ann NY Acad Sci 821: 57-75.

Yu B, Shinnick-Gallagher P. 1998. Corticotropin-releasing factor increases dihydropyridine- and neurotoxin-resistant calcium currents in neurons of the central amygdala. J Pharmacol Exp Ther 284: 170-179.

Zhou F-M, Hablitz JJ. 1999. Activation of serotonin receptors modulates synaptic transmission in rat cerebral cortex. J Neurophysiol 82: 2989-2999.

# 27 Angiotensins in Brain Function

*J. W. Wright · J. W. Harding*

**Abstract:** This chapter provides a brief historical perspective concerning the discovery of the renin–angiotensin system (RAS), followed by a description of the biochemical pathways that permit synthesis and degradation of active angiotensin peptides, and the three receptor subtypes thus far characterized. This is followed by a review of the physiologies and behaviors mediated by these peptides. These classic physiologies include cardiovascular control, vasopressin release, thirst, and electrolyte balance. More recently angiotensins have been implicated in the mediation of stress, anxiety, depression, learning, and memory consolidation. The chapter concludes with a water shortage scenario that is envisioned to encompass and illustrate the majority of the angiotensin mediated physiologies and behaviors.

**List of Abbreviations:** ACD, acyl-coenzyme A dehydrogenase; ACE, angiotensin-converting enzyme; ACE2, human angiotensin-converting enzyme homologue; ACh, acetylcholine; ACTH, adrenocorticotropic hormone; ADH, antidiuretic hormone; Ang, angiotensin; AngI, angiotensin I; AngII, angiotensin II; AngIII, angiotensin III; AngIV, angiotesnin IV; Ang(1-7), angiotensin II(1-7); Ang(2-7), angiotensin II(2-7); Ang (3-7), angiotensin II(3-7); AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP, area postrema; AP-1, activator protein-1; AP-A, aminopeptidase A; APMA, *p*-aminophenylmercuric acetate; AP-N, aminopeptidase N; AT, angiotensin receptor subtype; Carb-P, carboxypeptidase P; CRH, corticotropin-releasing hormone; CVOs, circumventricular organs; EC27, 2-amino-pentane-1,5-dithiol; EC33, 3-amino-4-thio-butyl-sulfonate; ERK, extracellular signal-regulated kinase; GLUT, glucose transporter molecules; GST, glutathione-s-transferase; GTPγS, guanosine triphosphate γ sulfate; ICV, intracerebroventricular; IRAP, insulin-regulated aminopeptidase; LVV-H7, leucine-valine-valine-hemorphin-7; MAP-K, microtubule-associated protein kinase; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NTS, nucleus of solitary tract; OVLT, organum vasculosum of the lamina terminalis; PAI-1, plasminogen activator inhibitor-1; PO, propyl oligopeptidase; PVN, paraventricular nucleus; RAS, renin–angiotensin system; SFO, subfornical organ; SON, supraoptic nucleus; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator

## 1 Introduction

In 1898 Tiegerstedt and Bergman isolated a kidney extract, later identified as renin, that produced a pressor response when injected into rabbit (Tiegerstedt and Bergman, 1898). Approximately forty years later angiotensin II (AngII) was shown to be the active agent by two independent research groups, and renin was identified as a protease (EC 3.4.23.15) (Munoz et al., 1939; Braun-Menendez et al., 1940; Page and Helmer, 1940). Subsequently the synthesis steps necessary for the formation of the active forms of angiotensins were identified (Elliott and Perart, 1956; Skeggs et al., 1957). By the 1960s and early 1970s research attention moved to the brain facilitated by four complementary observations that provoked interest in determining whether an independent brain renin–angiotensin system (RAS) exists: (1) Bickerton and Buckley (1961) employed cross-circulation experiments to show that AngII, arterially injected into one dog and into the head of a second dog, could produce a pressor response in the lower body of the second dog, even though the only connection between the head and lower body was via nerves. (2) Epstein et al. (1970) discovered that injections of AngII into the brain produced robust drinking in the rat. (3) Ganten et al. (1971a, b) isolated renin in the dog brain, while Fisher-Ferraro et al. (1971) identified renin and AngII in the dog brain. (4) Finally, Sirrett et al. (1977) developed a radio-receptor binding assay to establish the presence of AngII receptors in the rat brain. Taken together these observations suggested an independent brain RAS separate from the blood-borne AngII system. Confirmation of this hypothesis required several more years of work utilizing a variety of techniques including radioimmunoassay, immunohistochemistry, radio-receptor binding assays, and Northern blots of renin and angiotensinogen mRNAs (Phillips et al., 1979; Harding et al., 1981; Ganten et al., 1983; Hermann et al., 1984; Dzau et al., 1986; Lynch et al., 1986).

The most recent events to facilitate our understanding of the RAS occurred during the late 1980s and early 1990s and included two related sets of findings. (1) Two angiotensin receptor subtypes were cloned and sequenced (Chiu et al., 1989; Whitebread et al., 1989; Iwai et al., 1991; Murphy et al., 1991; Kambayashi et al., 1993; Mukoyama et al., 1993). (2) A third angiotensin receptor subtype was discovered (but to date has not been cloned and sequenced) that appears to mediate nonclassical functions including influences

upon blood flow, learning, and memory (Harding et al., 1992; Swanson et al., 1992; Bernier et al., 1994, 1995). These discoveries have rekindled interest in the brain RAS and its potential role in additional physiologies and pathologies.

## 2 Biochemistry of the Renin–Angiotensin System

The RAS mediates several physiologies including blood pressure, sodium and body water balance, cyclicity of reproductive hormones and sexual behaviors, and pituitary gland hormones. These functions appear to be under the control of the $AT_1$ receptor subtype (Allen et al., 2000; deGasparo et al., 2000; Gard, 2002; McKinley et al., 2003; Thomas and Mendelsohn, 2003). A second subtype, the $AT_2$, has also been implicated in the regulation of blood pressure, renal function, and vascular growth (deGasparo and Siragy, 1999; Speth et al., 1995; deGasparo et al., 2000). The octapeptide AngII has traditionally been considered the end product of the RAS, and therefore the active ligand at these receptors subtypes. Accumulating evidence indicates that additional shorter-chain angiotensins also serve as effector peptides in this system. These peptides include the heptapeptide des $Asp^1$-AngII referred to as angiotensin III (AngIII) (Wright and Harding, 1997; Vauquelin et al., 2002), the hexapeptide des $Asp^1$, des $Arg^2$-AngII referred to as AngIV (Wright and Harding, 1994, 1995, 1997; Wright et al., 1995; deGasparo et al., 2000; Albiston et al., 2003; Thomas and Mendelsohn, 2003), and the heptapeptide des $Phe^8$-AngII referred to as Ang(1-7) (Ferrario et al., 1997; Santos et al., 2000; Ferrario, 2003). The proposed functions mediated by AngIV include influences upon blood flow (Kramár et al., 1997; Coleman et al., 1998; Møeller et al., 1999; Slinker et al., 1999), kidney natriuresis (Handa et al., 1998; Hamilton et al., 2001), expression of plasminogen activator inhibitor (PAI-1) in endothelial cells (Kerins et al., 1995; Mehta et al., 2002) and in epithelial cells of the kidney proximal tubule (Gesualdo et al., 1999), and memory facilitation (reviewed in Wright et al., 2002a; Albiston et al., 2003; Bohlen und Halback, 2003). The functions thus far identified for Ang(1-7) include vasopressin, nitric oxide (NO), and prostaglandin release, and facilitation of baroreceptor reflex sensitivity (Santos et al., 2000; Kucharewicz et al., 2002).

### 2.1 Formation of Angiotensin Ligands

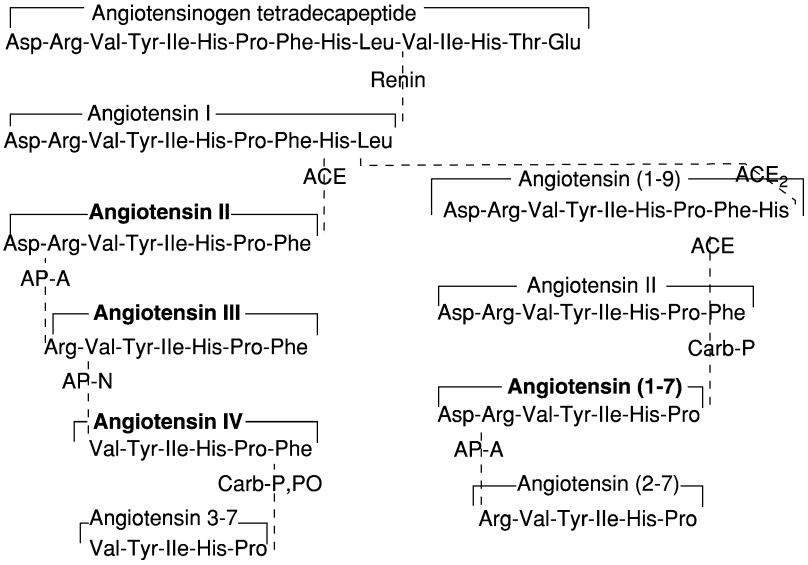
Angiotensinogen serves as a precursor protein to angiotensin peptides (➲ *Figure 27-1*). The decapeptide angiotensin I (AngI) is formed by the protease renin (EC 3.4.23.15) acting upon the amino-terminal of angiotensinogen. AngI is a substrate for angiotensin-converting enzyme (ACE: EC 3.4.15.1), a zinc metalloprotease that hydrolyzes the carboxy-terminal dipeptide His-Leu to form AngII (Johnston, 1990). AngII is converted to AngIII by glutamyl aminopeptidase A (AP-A: EC 3.4.11.7, or A-like activity) that cleaves the Asp residue at the N-terminal (Rich et al., 1984; Wilk and Healy, 1993; Chauvel et al., 1994). Membrane alanyl aminopeptidase N (AP-N: EC 3.4.11.2) cleaves Arg at the N-terminal of AngIII to form AngIV. AngIV can be further converted to Ang(3-7) by carboxypeptidase P (Carb-P) and propyl oligopeptidase (PO) cleavage of the Pro–Phe bond. Endopeptidases such as chymotrypsin are capable of cleaving the Val, Tyr, and Ile residues along with dipeptidyl carboxypeptidase that cleaves the His–Pro bond, reducing AngIV and Ang(3-7) to inactive peptide fragments and amino acid constituents (Unger et al., 1988; Johnston, 1990; Saavedra, 1992; Speth et al., 2003).

AngII can also be converted to Ang(1-7) by Carb-P cleavage of phenylalanine (reviewed in Wright and Harding, 1997) or by ACE cleavage of the dipeptide Phe-His from Ang(1-9) (Vauquelin et al., 2002), and can be further converted to Ang(2-7) by AP-A acting at the Asp-Arg bond (Mentlein and Roos, 1996).

AngI is considered inactive while AngII and AngIII are full agonists at the $AT_1$ and $AT_2$ receptor subtypes (reviewed in deGasparo et al., 2000). AngIV binds with low affinity at the $AT_1$ and $AT_2$ receptor subtypes (Glossman et al., 1974; Bennett and Snyder, 1976; Harding et al., 1992; Swanson et al., 1992), but with high affinity and specificity at the $AT_4$ receptor subtype (Harding et al., 1992; Jarvis et al., 1992; Swanson et al., 1992; Bernier et al., 1994). A specific binding site for Ang(1-7) has been reported (Santos et al., 1994, 2000; Ferrario, 2003; Neves et al., 2003), but not fully elucidated.

**Figure 27-1**

**Summary of the peptide structures and enzymes involved in the conversion of the tetradecapeptide portion of angiotensinogen to angiotensin I (AngI) through shorter fragments. Those forms of angiotensin considered biologically active include AngII, AngIII, AngIV, and Ang(1-7)**




## 2.2 Angiotensin Receptor Subtypes

At present there are three recognized angiotensin receptor subtypes (deGasparo et al., 1995), two that are structurally similar, and a third that appears different. The $AT_1$ and $AT_2$ subtypes are similar given their G protein coupling as compared with the $AT_4$ subtype that is a much larger protein and appears to be insensitive to guanine nucleotides, suggesting that it may not be G-protein-linked.

### 2.2.1 $AT_1$ and $AT_2$ Receptor Subtypes

The $AT_1$ receptor subtype is a G-protein-coupled receptor with signaling via phospholipase-C and calcium. Thus, the angiotensin ligand binds to the $AT_1$ receptor and induces a conformational change in the receptor protein that activates G proteins that, in turn, mediate signal transduction. This transduction involves several plasma membrane mechanisms including phospholipase-C, -$A_2$, -D, and adenylate cyclase, plus L-type and T-type voltage sensitive calcium channels (Sayeski et al., 1998; deGasparo et al., 2000). This $AT_1$ receptor (now designated $AT_{1A}$) is also coupled to intracellular signaling cascades that regulate gene transcription and the expression of proteins that mediate cellular proliferation and growth in many target tissues. Expression cloning was used to isolate the cDNAs encoding this receptor protein (Murphy et al., 1991; Sasaki et al., 1991) and it was found to be a seven transmembrane domain protein consisting of 359-amino-acids with a molecular mass of approximately 41 kDa (Sandberg et al., 1994). Subsequently, a second $AT_1$ subtype was discovered and designated $AT_{1B}$ that was also cloned from the rat (Iwai and Inagami, 1992; Kakar et al., 1992), mouse (Sadamura et al., 1992), and human (Konoshi et al., 1994). This subtype is approximately 92–95% homologous with the amino acid sequence of the $AT_{1A}$ subtype (Guo and Inagami, 1994; Speth et al., 1995). Of these two isoforms the $AT_{1A}$ subtype appears to be responsible for the classic functions associated with the brain angiotensin system (reviewed in Saavedra, 1999; Thomas and Mendelsohn, 2003).

The $AT_2$ receptor subtype has also been cloned using a rat fetus expression library and sequenced (Bottari et al., 1991; Kambayashi et al., 1993). In common with the $AT_1$ subtype, this receptor protein also evidences a seven transmembrane domain characteristic of G-protein-coupled receptors, however it shows only about 32–34% amino acid sequence identity with the rat $AT_1$ receptor. The $AT_2$ receptor protein includes a 363-amino-acid sequence (40 kDa) with 99% sequence agreement between rat and mouse, and 72% homology with human (deGasparo et al., 2000). Even though this $AT_2$ receptor possesses structural features in common with members of the seven transmembrane family of receptors, it displays few if any functional similarities with this group, although it does appear to be G-protein-coupled (Bottari et al., 1991; Kambayashi et al., 1993; Mukoyama et al., 1993; deGasparo et al., 2000). Specific characteristics of the $AT_1$ and $AT_2$ receptor subtypes are summarized in ❯ *Table 27-1*.

**Table 27-1**
**Characteristics of the angiotensin receptor subtypes**

| Characteristic | $AT_1$ | $AT_2$ | $AT_4$ |
|---|---|---|---|
| Affinity | AngII>AngIII>AngI | AngIII>AngII>AngI | AngIV |
| Sensitivity to SH reagents | Inactivation | Enhancement | No effect |
| Selective antagonists | CGP46027, DuP753, DuP532, EXP3174, L158809, GR117289, SK/F108566, SC51316, UP269-6, LR-B/081 | CGP42112A, PD123177, PD121981, PD123319, PD124125 | Divalinal-AngIV, $Nle^1$, $Leual^3$-AngIV |
| Coupling to G protein | Yes | Likely | Unlikely |
| Signal transduction | $\uparrow Ca^{2+}$, ↑P3, ↓adenylyl cyclase, ↑prostaglandins | ↓cGMP/↑cGMP, ↑prostaglandins | Unknown |
| Structure | 359 amino acids, 7 transmembrane domains | 363 amino acids, 7 transmembrane domains | Trimer |
| Molecular size | 41–42 kDa | 40–41 kDa | α 165 kDa, β 50–60 kDa, γ 70–80 kDa |

Adapted from Wright and Harding (1997, 2004), Speth et al. (2003)

### 2.2.2 $AT_4$ Receptor Subtype

Before 1988, angiotensins shorter than AngIII were considered biologically inactive and therefore of little physiological importance. This assumption was based on two facts: (1) AngIV reveals a very poor affinity for the $AT_1$ and $AT_2$ sites (Glossman et al., 1974; Bennett and Snyder, 1976; Harding et al., 1992; Swanson et al., 1992) and (2) AngIV and shorter fragments are considerably less potent than AngII and AngIII in eliciting classic angiotensin-dependent functions (Blair-West et al., 1971; Fitzsimons, 1971; Tonnaer et al., 1982; Unger et al., 1988; Wright et al., 1989). Two findings changed this assumption. First, Jan Braszko et al. (1988) reported that AngIV facilitated acquisition of a conditioned avoidance response in rats. Second, a separate and distinct binding site for AngIV was discovered (Harding et al., 1992; Swanson et al., 1992) and subsequently classified as the $AT_4$ subtype (deGasparo et al., 1995). This subtype was originally identified in bovine adrenal membranes (Harding et al., 1992; Jarvis et al., 1992; Swanson et al., 1992; Bernier et al., 1994). These characterization studies indicated that the $AT_4$ receptor subtype is distinct from the $AT_1$ and $AT_2$ sites given that ligands known to bind to these sites do not bind at the $AT_4$ site (❯ *Table 27-1*) (Harding et al., 1992; Swanson et al., 1992). It was determined that [$^{125}$I]AngIV binds at the $AT_4$ site reversibly, saturably, and with high affinity. This $AT_4$ site has been found in a variety of mammalian tissues including adrenal gland, bladder, colon, heart, kidney, prostate, brain, and spinal cord.

Given that a small peptide is capable of activating the $AT_4$ site, and that the vast majority of small peptide receptors are G-protein-linked, it was logical to predict that the $AT_4$ receptor might be a serpentine G-protein-linked receptor as well. However, this does not appear to be the case since binding to this site was found to be insensitive to guanine nucleotides, suggesting that the $AT_4$ receptor is not G-protein-linked. In addition the $AT_4$ receptor subunit exhibits a molecular weight of between 160 and 190 kDa as determined by reduced SDS-polyacrylamide gel electrophoresis. This receptor appears to be a trimer as suggested by results from a nonreducing gel that indicated two additional subunits. An equivalent molecular weight has been observed for this receptor in other bovine tissues including heart, thymus, kidney, bladder, aorta, and hippocampus (Zhang et al., 1999). Further, Bernier and colleagues (1995, 1998) have reported a similar molecular weight for the binding subunit of the $AT_4$ receptor in bovine aortic endothelial cells. The lack of linkage to G proteins is also supported by the observation that guanosine triphosphate γ sulfate (GTPγS) failed to alter [$^{125}$I]AngIV binding in rabbit heart (Hanesworth et al., 1993), guinea pig brain (Miller-Wing et al., 1993), and rat vascular smooth muscle (Hall et al., 1993). A single report by Dulin et al. (1995) indicates that GTPγS can inhibit $AT_4$ receptor binding in opossum kidney cells. Thus, to date there is little evidence linking the $AT_4$ receptor to G proteins, however, as experienced with the $AT_2$ receptor, a definitive conclusion must await cloning and sequencing of the $AT_4$ receptor.

### 2.2.3 Insulin-Regulated Membrane Aminopeptidase (IRAP)

A potentially important advancement in our understanding of the $AT_4$ receptor system is the recent identification of this receptor as insulin-regulated membrane aminopeptidase (IRAP) (Albiston et al., 2001), a membrane associated aminopeptidase that codistributes with the $GLUT_4$ transporter (Kandror and Pilch, 1994; Keller et al., 1995). The initial identification was based on sequence homology between a tryptic fragment derived from the human brain $AT_4$ receptor and human IRAP, and on the near identical masses of IRAP and the $AT_4$-receptor-binding subunit protein. Subsequent expression of IRAP in HEK293T cells (Lee et al., 2003) yielded an $AT_4$ receptor-like binding site with an affinity for AngIV that was similar to the native receptor. These authors have proposed that the multiple physiological actions of $AT_4$ receptor ligands are due to their ability to competitively inhibit the peptidase activity of IRAP, thus potentiating the actions of endogenous peptides that are normally degraded by IRAP (Lew et al., 2003). This model predicts that the action of all $AT_4$ receptor ligands should be qualitatively equivalent since their action is simply due to their binding to IRAP and competitive interference with IRAP's ability to catabolize endogenous peptides. Clearly this notion does not fit with the existence of both agonists and antagonists that exhibit opposite physiological actions (Kramár et al., 1997, 2001; Wright et al., 1999; Hamilton et al., 2001). Further, this model predicts that the physiological effects of $AT_4$ receptor ligands should be slow since this action requires an accumulation of endogenous ligand. Again this prediction does not agree with the observation that $AT_4$ ligands have amazingly rapid effects on signaling molecules (Chen et al., 2001; Handa, 2001; Li et al., 2002). For example, studies in our laboratory indicate that $AT_4$ receptor activation can lead to a 20-fold increase in ERK activation in C6 glioma cells within 30 s (Harding, Anderson and Meighan, unpublished). Similarly, in vivo studies indicate rapid responses to $AT_4$ receptor ligands. The time course of $AT_4$-dependent changes to blood flow (Kramár et al., 1997), renal oxygen consumption (Handa et al., 1998), and long-term potentiation are rapid (Kramár et al., 2001; Wayner et al., 2001), typically manifesting in less than 1 min. It is difficult to imagine that sufficient peptide would accumulate in such a short period of time to impact physiological responses. More typical time frames for in vivo peptidase inhibitors are hours or days, not seconds. Additionally, the concentrations of $AT_4$ ligands required to effect changes in physiological function can be subpicomolar or subnanomolar (Chen et al., 2001; Handa, 2001; Li et al., 2002), concentrations that are well below that reported for any known enzyme inhibitor. This concern is magnified for IRAP specifically because the recently reported $K_i$ of Norleucine$^1$-Angiotensin IV (Nle$^1$-AngIV) for IRAP of >0.3 μM (Lew et al., 2003) is several orders of magnitude higher than the biologically effective doses of $AT_4$ ligands. Also casting doubt on the hypothesis that $AT_4$ ligands function as competitive substrates is a study by Caron and colleagues (2003), suggesting that AngIV ligands interact allosterically with the IRAP receptor at a site distinct from the active site. The precise characteristics concerning the

structure of AngIV, its analogs, and other angiotensins such as AngIII, that render them nonsubstrates for IRAP, but still retain their ability to bind, are presently unclear (Lew et al., 2003). Finally, this highly unusual situation is in opposition to earlier work by Tsujimoto et al. (1992) who demonstrated that AngIII is an excellent substrate for human placental leucine aminopeptidase (homolog of rat IRAP; Keller et al., 1995).

The discordance between the IRAP inhibitor model and laboratory observations suggests two likely possibilities. First, IRAP may not be the signal transducing $AT_4$ receptor but is instead involved with regulating the extracellular levels of endogenous $AT_4$ receptor ligands. Second, IRAP may be the signal transducing receptor but relies on activities beyond its abilities as an aminopeptidase. If the second possibility is correct then IRAP should possess in its short 109-amino-acid hydrophilic N-terminal segment the information required for signal transduction. Lending credibility to this possibility are previous studies, one indicating that the N terminus of IRAP contains two dileucine motifs and several acidic regions that play important roles in vesicular trafficking (Keller et al., 1995; Waters et al., 1997). A peptide consisting of residues 55–82 of the N terminus, containing one of the dileucine motifs and acidic clusters, was sufficient to cause $GLUT_4$ translocation (Waters et al., 1997). Correspondingly, Ryu et al. (2002) showed in vitro phosphorylation of IRAP Ser80, which is involved in the regulation of insulin-stimulated $GLUT_4$ translocation. The poly (ADP-ribose) polymerase tankyrase was identified in a yeast two-hybrid system and interacted with 96–101 amino acids of IRAP (Chi and Lodish, 2000). Interestingly, acyl-coenzyme A dehydrogenases (ACDs), identified by glutathione-s-transferase (GST) fusion-IRAP (GST-IRAP55-82) is probably involved in retention of $GLUT_4$ vesicles to designated intracellular compartments (Katagiri et al., 2002). Similar mechanisms might exist for IRAP at the plasma membrane resulting in signal transduction, given that several signaling events have been associated with activation of $AT_4$ receptor/IRAP by $AT_4$ ligands (Handa, 2001; Li et al., 2002). Even though the exact role played by IRAP in $AT_4$ ligand signaling is not clear, the high affinity of IRAP for $AT_4$ receptor ligands suggests that its function is substantial.

In addition to AngIV acting as a ligand at the $AT_4$ receptor/IRAP complex, recent studies indicate that a decapeptide, LVV-hemorphin-7 (LVV-H7) isolated from sheep cerebral cortex (Møeller et al., 1997), also acts as an $AT_4$ ligand. Although there is minimal structural overlap between AngIV and LVV-H7, the latter ligand exhibits many of the same effects as the hexapeptide as observed from several in vitro assays including facilitation of cellular proliferation (Mustafa et al., 2001), potassium-evoked acetylcholine release from hippocampal slices (Lee et al., 2001), the inhibition of catalytic activity by IRAP (Albiston et al., 2001), and attenuation of scopolamine-induced interference with acquisition of associative and spatial memory tasks (Albiston et al., 2004). Presumably other putative endogenous ligands will be found in the near future.

### 2.2.4 Angiotensin(1-7)

Ferrario and colleagues (Schiavone et al., 1988) were the first to report biological activity of Ang(1-7) in the form of vasopressin release from the posterior pituitary gland (❯ *Table 27-2*). In the years since that discovery, many investigators have confirmed the biological importance of this peptide (reviewed in Santos et al., 2000; Carey and Siragy, 2003; Kucharewicz et al., 2002). Ang(1-7) opposes several of the actions of AngII and AngIII. Specifically, Ang(1-7) stimulates the release of NO and vasodilator prostaglandins (Meng and Busija, 1993; Osei et al., 1993; Paula et al., 1995; Brosnihan and Ferrario, 1996; Li et al., 1997). Ang(1-7)-stimulated release of NO appears to be primarily from vascular endothelial and smooth muscle cells (Jaiswal et al., 1992; Muthalif et al., 1998) and opposes AngII-induced vasoconstriction (Ueda et al., 2000). It also appears to protect cardiac and endothelium function as well as coronary perfusion, as demonstrated in a heart failure model (Loot et al., 2002). Further, Ang(1-7) has been shown to facilitate baroreceptor reflex sensitivity and modulate circadian rhythm influences on heart rate and blood pressure (Campagnole-Santos et al., 1992; Silva-Barcellos et al., 2001). It is well established that AngII promotes thrombosis primarily via expression of PAI-1 (Feener et al., 1995; Vaughan et al., 1995), although this effect may in fact be via AngIV (Kerins et al., 1995). Kucharewicz and colleagues (2000, 2002) have shown that Ang(1-7) functions as an antithrombotic compound when administered to renal hypertensive rats that served as a venous thrombosis model. A putative binding site with high affinity for Ang(1-7) has been identified but not characterized (Santos et al., 1994). Tallant and colleagues (1997) have reported that

**Table 27-2**
**Summary of angiotensin-mediated physiologies and behaviors**

| Receptor subtype | | | |
|---|---|---|---|
| $AT_1$ | $AT_2$ | $AT_4$ | Ang(1–7) |
| Blood pressure | Blood pressure | Blood flow | Blood pressure |
| Thirst | Thirst | Kidney natriuresis | Vasopressin release |
| Body water balance | Renal function | PAI-1 expression | NO release |
| Cyclicity of reproductive hormones and behaviors | Vascular growth | ACh release | Prostaglandin release |
| Sympathetic activation | | Memory | Baroreceptor reflex |
| ACTH release | | Cognitive effect | Antithrombosis |
| Memory | | | |
| Cognitive effect | | | |

Ang(1-7) binding to this site cannot be inhibited by $AT_1$ or $AT_2$ receptor subtype antagonists, but can be blocked by sarile ($Sar^1$,$Ile^8$-AngII) in bovine aortic endothelial cells. On the other hand, Santos et al. (2000) have noted the action of Ang(1-7) to be inhibited by losartan and $AT_2$ receptor antagonists. The intracellular signaling mechanisms are presently undetermined (reviewed in Santos et al., 2000).

## 3 Classic Brain Angiotensin-Mediated Physiologies and Behaviors

The RAS is critical for the mediation of blood pressure and the maintenance of volume and electrolyte homeostasis. AngII has been assumed to be the primary effector peptide responsible for vasoconstriction, facilitation of sympathetic activation, and control of body fluid balance. However, there is accumulating evidence that AngIII, and perhaps Ang(1-7), also play roles in the central regulation of these physiologies. Angiotensins have also been implicated in the processes of anxiety and depression primarily through influences upon the autonomic nervous system. In addition, both AngII and AngIV are prominently involved in learning and memory. This latter topic has captured considerable attention, especially with the discovery of the $AT_4$ receptor subtype and IRAP as possible binding sites for AngIV and LVV-H7.

### 3.1 Cardiovascular Control

The peripheral RAS contributes to cardiovascular functioning by direct inotropic influences upon the heart and via increased vascular resistance (reviewed in Johnston, 1990; Wright and Harding, 1997). This influence upon vascular resistance occurs due to direct action on vascular smooth muscle and indirect action via the brain resulting in sympathetic nervous system arousal, stimulation of vasopressin release, and inhibition of the baroreceptor reflex (Unger et al., 1988; Phillips and Sumners, 1998; Culman et al., 2002).

Discovery of components of the RAS in the brain led to the notion of a local and independent brain RAS. Considerable evidence now supports the existence of two primary brain angiotensinergic pathways (reviewed in Llorens-Cortes and Mendelsohn, 2002). The first is a forebrain pathway that integrates circumventricular organs (CVOs see below) with the paraventricular nuclei (PVN), supraoptic nuclei (SON), and median preoptic nuclei. A second pathway bridges the hypothalamus and medulla (including area postrema (AP) and nucleus of the solitary tract (NTS)). Since CVOs possess fenestrated capillaries and are heavily distributed with angiotensin receptors, activation of these receptors by blood-borne angiotensins is thought to impact central cardiovascular circuits thus permitting interaction between the peripheral

and central RASs. Given that the $AT_1$ receptor subtype binds AngII and AngIII with approximately the same affinity, and similarly provoke changes in blood pressure, thirst, and vasopressin release, it has been postulated that either AngII and AngIII are equivalently potent at the $AT_1$ subtype, or AngII must be converted to AngIII in order to activate this receptor subtype (reviewed in Wright and Harding, 1997; Llorens-Cortes and Mendelsohn, 2002).

This section summarizes the influences of AngII and AngIII upon blood pressure and vasopressin release. There is continuing debate over the identity of the active ligand at the $AT_1$ receptor subtype.

### 3.1.1 Angiotensin II

Intracerebroventricular (ICV) injections of AngII produce reliable pressor responses via activation of $AT_1$ receptors located in the CVOs (subfornical organ (SFO), organum vasculosum lamina terminalis (OVLT), and AP) that directly or indirectly project to the PVN and SON to induce vasopressin release, a potent vasoconstrictor (reviewed in Wright and Harding, 1992; Phillips and Sumners, 1998). The primary mechanism mediating vasopressin release from these nuclei appears to be norepinephrine activation of α-adrenergic receptors located on PVN and SON neurons (Culman et al., 1995). Microinjections of AngII into the SFO, OVLT, and PVN also elicit elevations in blood pressure (reviewed in Wright and Harding, 1992). The pressor response induced by circulating AngII appears to be mediated primarily by the SFO and AP. The absence of a blood–brain barrier at these CVO sites also permits penetration by other circulating hormones.

AngII also activates $AT_1$ receptors in the medulla in the control of blood pressure. Target structures include NTS, AP, and anterior ventrolateral medulla (Culman et al., 2002). In particular, the AP appears to detect blood-borne AngII while AngII activation of the NTS influences the baroreceptor reflex. Thus, circulating levels of AngII impact the baroreceptor reflex via a pathway from the AP to the NTS (Phillips, 1987; Muratami et al., 1996). Finally, AngII activation of $AT_1$ receptors in the anterior ventrolateral medulla increases blood pressure by activation of the sympathetic nervous system, tachycardia, and catecholamine release from the adrenal medulla (Unger et al., 1985; Dampney et al., 1996; Head, 1996; Muratami et al., 1996; Allen et al., 2001).

### 3.1.2 Angiotensin III and Shorter Fragments

In the 1970s and 1980s, Fitzsimons and colleagues investigated the potency of centrally applied AngIII and found it to possess 50% or less the potency of AngII depending upon the infusion site (reviewed in Wright and Harding, 1992, 1997; Fitzsimons, 1998). Tonnaer et al. (1982) reported the greatest pressor activity for ICV injected AngII followed by AngI and AngIII (pmol range), with less activity induced by AngII(3-8), (4-8), (5-8), and (6-8) (nmol range). The C-terminal dipeptide AngII(7-8) and other dipeptides were inactive. Studies by Fink and Bruner (1985) and Wright et al. (1985) reevaluated the potency of AngIII and corrected potential shortcomings by siliconizing all glassware to discourage adherence of peptides, by reducing the doses in order to minimize the half-life advantage of AngII over AngIII, and by utilizing degradation resistant analogs in an effort to reduce the in vivo conversion of AngII to AngIII and AngIII to AngIV. Under these conditions pressor responses induced by ICV infusion of AngII, AngIII, and successively shortened C-terminal fragments of AngII(5-8) were compared (Wright et al., 1985, 1989). The results indicated that AngII, AngIII, [$Sar^1$]AngII, and [$Sar^1$]AngIII were identical with respect to pressor responses in the alert rat, while AngIV and [$Sar^1$]AngIV revealed 70% of the activity of the above compounds. The activity of the shorter C-terminal fragments dropped to below 35%.

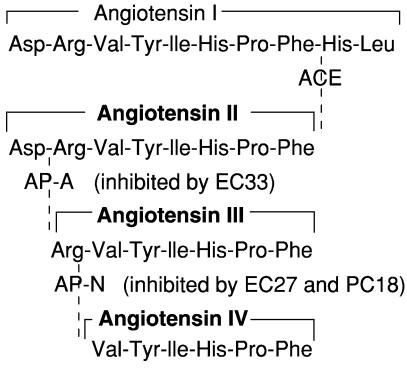
In an effort to determine the active ligand at the $AT_1$ receptor subtype, our laboratory also employed aminopeptidase inhibitors in combination with metabolically resistant angiotensin analogs (reviewed in Wright and Harding, 1992, 1997). Pretreatment with the nonspecific AP-A inhibitor, amastatin, significantly reduced subsequent pressor responses to [D-$Asp^1$]AngII, by inhibiting conversion to AngIII. In contrast, ICV pretreatment with the AP-N and aminopeptidase B inhibitior, bestatin, potentiated

subsequent pressor responses to [D-Arg$^1$]AngIII by inhibiting its conversion to AngIV. Intracerebroventicular pretreatment with the nonspecific $AT_1$ receptor antagonist sarthran greatly diminished subsequent pressor responses to [D-Asp$^1$]AngII, [D-Arg$^1$]AngIII, AngII, and AngIII, suggesting that these ligands act at the same angiotensin receptor site in the brain. These results support the hypothesis that AngIII, and/or AngIII-like ligands, serve as active forms with respect to cardiovascular function.

Zini and colleagues (1996) have developed selective inhibitors of AP-A and AP-N (*Figure 27-2*). The AP-A inhibitor (3-amino-4-thio-butyl-sulfonate: EC33) has been shown to increase the half-life of AngII by

**Figure 27-2**

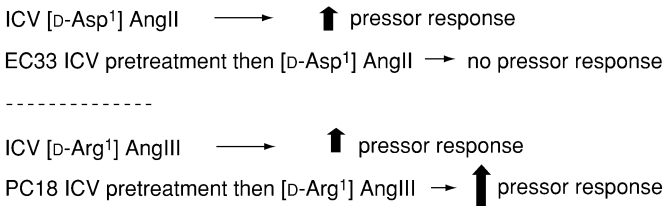
**Location of enzymatic activities of aminopeptidase-A (AP-A) and aminopeptidase-N (AP-N) that are inhibited by 3-amino-4-thio-butyl-sulfonate (EC33) and 2-amino-pentane-1,5-dithiol (EC27), respectively. PC18 has a similar inhibitory action upon AP-N as EC27. EC33's inhibitory potency is approximately 100× greater for AP-A ($K_i = 0.29\ \mu M$) than for AP-N ($K_i = 25\ \mu M$). EC27's inhibitory potency is about 100× greater for AP-N ($K_i = 0.03\ \mu M$) than for AP-A ($K_i = 2.4\ \mu M$). (Modified from Llorens-Cortes and Mendelsohn, 2002; Wright and Harding, 2004)**




2.6-fold as measured in hypothalamic homogenates, and completely blocked the formation of AngIII. An AP-N inhibitor (2-amino-pentane-1,5-dithiol: EC27) increased the half-life of AngIII by 2.3-fold. When AngII was ICV injected into mice, plasma vasopressin levels were increased twofold; however, the coapplication of EC33 inhibited this AngII-induced vasopressin response in a dose-dependent fashion. In contrast, ICV injection of EC27 alone increased plasma vasopressin levels in a dose-dependent fashion. This EC27 stimulation of vasopressin release could be blocked by the accompanying injection of the nonspecific angiotensin receptor antagonist saralasin (Sar$^1$,Ala$^8$-AngII). These results suggest that central angiotensin-induced vasopressin release is dependent upon the conversion of AngII to AngIII, and therefore AngIII may be the main effector peptide in the brain with respect to the mediation of vasopressin release. Consistent with these findings, Song et al. (1997) developed an antiserum with anticatalytic activity against AP-A. When ICV infused, it reduced both drinking and blood pressure responses to the subsequent ICV delivery of AngII by 73% and 59%, respectively. This same antiserum had no effect on ICV-infused AngIII-induced drinking and blood pressure responses.

Our laboratory has utilized ICV infused EC33 or PC18 (an AP-N inhibitor with similar structure to EC27) followed by the metabolically stable analogs [D-Asp$^1$]AngII or [D-Arg$^1$]AngIII to sort out relative contributions by AngII and AngIII to pressor response in rats (Wright et al., 2002b). Pretreatment by ICV infusion with EC33 blocked the pressor activity induced by the subsequent infusion of [D-Asp$^1$]AngII, while EC33 had no effect on the pressor response to subsequent infusion of [D-Arg$^1$]AngIII (*Figure 27-3*). In contrast, pretreatment infusion with PC18 extended the duration of the [D-Asp$^1$]AngII pressor effect by approximately 2 to 3 times, and the duration of [D-Arg$^1$]AngIII's effect by approximately 10 to 15 times. Pretreatment with the specific $AT_1$ receptor antagonist losartan blocked the pressor responses induced by the subsequent infusion of both analogs, indicating that they act via the $AT_1$ receptor subtype. These results

**Figure 27-3**
**Results from the use of EC33 to block conversion of AngII to AngIII, and PC18 to block conversion of AngIII to AngIV, upon pressor response in the rat. Losartan ICV pretreatment followed by [D-Asp$^1$]AngII or [D-Arg$^1$]AngIII resulted in significant reductions in pressor responses suggesting that both analogs act at the $AT_1$ receptor subtype**




suggest that the brain $AT_1$ receptor may be designed to preferentially bind AngIII in mediating blood pressure maintenance.

## 3.2 Thirst

One of the most dramatic behavioral phenomena associated with the central injection of angiotensin is its ability to induce drinking. Linazasoro et al. (1954) and Nairn et al. (1956) first demonstrated that peripherally infused renal extracts induced drinking in rats and postulated that this response was angiotensin-induced. Since then many investigators have confirmed and extended these initial observations.

### 3.2.1 Angiotensin II

In 1968 Booth discovered that microinjections of AngII into the rostral hypothalamus induced drinking (Booth, 1968). Soon after Epstein et al. (1970) established dose-response drinking to ICV injected AngII. Simpson and Routtenberg (1973) showed that microinjection of AngII into the SFO induced drinking. Buggy et al. (1975; Buggy and Fisher, 1976) found that AngII infused into the third ventricle in the proximity of the OVLT, but not permitted to reach the SFO, also induced drinking. Following additional efforts to sort out the respective contributions of the SFO and OVLT, it was generally agreed that the OVLT detects angiotensins in both the cerebrospinal fluid and blood, while drinking induced by elevations in blood-borne angiotensins is primarily mediated by the highly vascularized SFO (reviewed in Lind, 1988; Wright and Harding, 1992).

### 3.2.2 Angiotensin III and Shorter Fragments

AngIII was originally found to possess about 50% of the dipsogenic activity of AngII when delivered into the diencephalon of the rat, while AngIV, AngII(4-8), and Ang(5-8) produced only slight dipsogenic acivitity (Fitzsimons, 1971, 1980). With the removal of phenylalanine from the C-terminal, i.e., Ang(1-7), a complete loss of activity was noted. Tonnaer et al. (1982) examined AngI, AngII, and several C-terminal fragments for dipsogenic activity when injected ICV into rats. The greatest intakes occurred for AngII, AngI, and AngIII (pmol range) in that order, followed by AngII(4-8), AngIV, AngII(5-8), and AngII(6-8) (nmol range). The C-terminal dipeptide AngII(7-8) and other dipeptide fragments were relatively ineffective. Pretreatment with the ACE inhibitor captopril greatly reduced the drinking induced by AngI suggesting that the conversion of AngI to AngII and/or AngIII is necessary for biological activity in the brain. In addition pretreatment with saralasin blocked drinking to AngI and AngII(4-8); the other angiotensins and fragments were not similarly tested. More recent investigations have established reasonably equivalent drinking

responses in rats to ICV infusions of AngII and AngIII, particularly at low doses (Wright et al., 1985), if precautions are taken to avoid peptide adherence to glass mixing and storage containers, adjustments are made for differences in the purities of the compounds, and the angiotensins are infused rather than bolus injected.

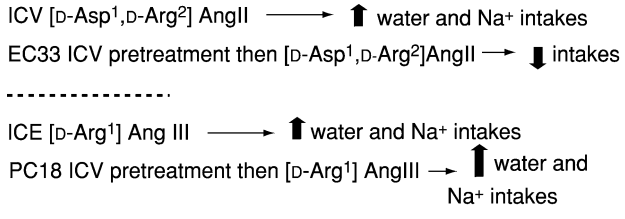
## 3.3 Sodium Appetite

Central application of angiotensins also produces sodium appetite that follows a much slower time course to develop as compared with drinking (reviewed in Fitzsimons, 1998). Body sodium conservation is primarily controlled by the renin–angiotensin–aldosterone system, and these hormones are elevated during sodium deficiency, and in turn, act directly or indirectly on the brain to arouse sodium appetite (Richter, 1936; Epstein, 1982). The salt appetite that develops in a sodium-depleted rat can be suppressed by central application of angiotensin receptor antagonists (Buggy and Jonklaas, 1984; Weiss et al., 1986) or ACE inhibitors (Moe et al., 1984). In fact, sodium appetite induced by adrenalectomy can be suppressed by interruption of the brain RAS (Sakai and Epstein, 1990). AngII-induced sodium intake appears to be a function of activation of forebrain angiotensin receptors, but is not dependent on the SFO. Microinfusion of AngII near the OVLT produced both water and sodium intake in rats, while injections into the SFO elicited only water consumption (Fitts and Masson, 1990).

Peripheral infusions of high doses of AngII are required to provoke a sodium appetite, and at these levels plasma aldosterone is elevated which facilitates central AngII-elicited sodium intake (Summy-Long et al., 1983). Peripheral aldosterone can penetrate the blood–brain barrier and has been shown to elevate brain AngII receptor numbers (Wong et al., 1990). Related to this, there is ongoing discussion concerning whether the appetite for sodium is primary or secondary to an immediate and sustained natriuresis (reviewed in Unger et al., 1988; Fitzsimons, 1998). Intracerebroventricular injections of AngII produced an immediate increase in urinary sodium excretion in alert rats, prepared with a chronic indwelling urethral catheter, which lasted for at least one hour (Unger et al., 1989). Thus, it may be that ICV AngII-induced sodium loss stimulates sodium appetite as a compensatory response.

Little evidence exists concerning AngIII's potential involvement in sodium appetite, and available data are conflicting. Peripheral administration of AngIII has been shown ineffective in eliciting a salt appetite in Fischer 344 and Sprague-Dawley rats, while AngII is capable of producing a sodium appetite (Caputo et al., 1992). Acute AngIII infusions into the preoptic area failed to increase sodium appetite in rats, whereas AngII stimulated the appetite (Avrith and Fitzsimons, 1980). However, ICV infused AngIII is equipotent to AngII in stimulating sodium consumption in baboons (Blair-West et al., 2001). Our laboratory has utilized the AP-A and AP-N inhibitors, EC33 and PC18, respectively, to investigate the roles of AngII and AngIII in salt appetite (Wilson et al., 2005). Rats were sodium depleted with furosemide, followed by endogenous angiotensin blockade with the ACE inhibitor captopril. [D-Asp$^1$,D-Arg$^2$]AngII and [D-Arg$^1$]AngIII were then ICV infused in order to evaluate the relative roles of AngII and AngIII in provoking sodium appetite. Both forms were effective in eliciting water and sodium (0.3 M NaCl) intakes (*Figure 27-4*). AngII

**Figure 27-4**
**Results from the use of EC33 and PC18 upon the subsequent administration of [D-Asp$^1$,D-Arg$^2$]AngII or [D-Arg$^1$] AngIII, respectively, upon water and sodium intakes in the rat**



analog-induced intakes of water and NaCl were decreased following pretreatment with EC33. Use of PC18 produced increased intakes of both fluids following treatment with the AngIII analog. These findings support a role for both peptides in eliciting and mediating sodium appetite.

## 4 Novel Brain Angiotensin-Mediated Physiologies and Behaviors

There is accumulating evidence that angiotensins, by direct action or as modulators, influence the roles of other transmitters important in cognitive processing, depression and mood change, and stress responses. Pharmacological manipulation of brain angiotensins can result in altered mental acuity, antidepressant- and anxiolytic-like effects (reviewed in Gard, 2002). Angiotensins have also been implicated in the enhancement of learning acquisition and memory consolidation. The present section considers available evidence linking brain angiotensins with altered cognitive processing, learning and memory, adaptation to stress, and depression. There is growing recognition that the complexity of the brain RAS has been significantly underestimated. It is now clear that some "metabolic fragments" of AngII are indeed active forms of angiotensin designed, in some cases, to counter-balance and/or modulate the initial actions put into motion by AngII and AngIII via the $AT_1$ receptor subtype.

### 4.1 Angiotensin-Converting Enzyme Inhibitors and Cognition

Accompanying the therapeutic benefits of ACE inhibitors (captopril, enalapril, ramipril) in treating hypertension, congestive heart failure, and following mild cardiac infarction, there appears to be facilitated cognitive functioning and feelings of well-being. Croog et al. (1986) employed 626 mild to moderate hypertensive male patients in randomized double-blind trials over a 24-week study. Patient self-reports indicated improved mental acuity at work, less sexual dysfunction, and increased sense of well-being on captopril. There was no change with propranolol treatment, and a decline in those patients placed on methyldopa. Blood pressure was equivalently controlled in all three treatment groups. Deicken (1986) and Zubenko and Nixon (1984) have reported captopril-induced mood elevating effects in depressed patients. Barnes et al. (1992) posited that elevated brain AngII levels may interfere with acetylcholine (ACh) release that in turn interferes with cognitive processing (Bartus et al., 1982). According to this hypothesis ACE inhibitors may facilitate cognitive functioning by reducing the synthesis of AngII, thus removing an inhibitory influence upon ACh release (Barnes et al., 1990). In support of this hypothesis Costall and colleagues (1989) treated mice with captopril or ceranapril and measured a habituatory response of mice moving from a brightly lit area to the darker area of a light/dark box. The muscarinic ACh receptor antagonist scopolamine impaired habituation, while captopril and ceranapril were both effective at countering this scopolamine effect (Barnes et al., 1992). Scopolamine has also been shown to delay the time required for rats to locate a submerged platform in the Morris water maze task (Morris, 1984). Treatment with ceranapril offset this scopolamine-induced impairment such that escape times were not different from controls. In further support, Barnes et al. (Barnes et al., 1991a, b) reported high binding densities for [$^3$H]-ceranapril in rat striatum and hippocampus, and human caudate, attributed to ACE in the microvasculature and perhaps at extravascular sites. Intravenous pretreatment with captopril reduced subsequent [$^3$H]-ceranapril binding in most areas of the brain, except in the striatum and brain stem, measured 20 min following treatment. Barnes and colleagues (1989) have also reported AngII-induced interference with potassium-mediated release of [$^3$H]-ACh from rat entorhinal cortex slices. This AngII effect could be blocked by sarthran. In line with this, Mondadori and Etienne (1990) found that captopril and enalapril reduced electroshock-induced amnesia in mice. These animals were trained to avoid the dark compartment of a two-chamber passive avoidance apparatus by applying foot shock when in the dark side immediately following an electroconvulsive shock. Recall of the conditioned response was facilitated in those mice given ACE inhibitors one hour prior to the conditioning trial. Flood and Morely (1993) reported similar results using an active avoidance task in mice. Barnes et al. (1990, 1991c) have shown that reasonably

low doses of losartan and the $AT_2$ receptor antagonist P123177 improved scopolamine-impaired performance in the previously described habituation test. Similarly, DeNoble et al. (1991) measured impaired performance on a passive avoidance task in rats ICV treated with renin. This impairment could be offset with ACE inhibitor treatment or by the application of the $AT_1$ receptor antagonists EXP3312 or EXP3880, but not PD123177. The proposal that ACE inhibitors enhance learning has been challenged by Chen and Mendelsohn (1992) who reported that a high oral dose of ceranapril in rats inhibited ACE at the CVOs, but not within blood–brain barrier protected structures. This suggests that ceranapril does not cross the blood–brain barrier.

At present, evidence favoring improved cognitive functioning by ACE inhibitors and $AT_1$ receptor antagonists is stronger in animal tests of habituation and active or passive avoidance tasks than for animals evaluated using spatial learning paradigms. Our laboratory has measured facilitated Morris water maze performance in scopolamine, or mecamylamine, pretreated rats with ICV treatment of AngIV analogs (Pederson et al., 1998; Wright et al., 1999; Olson et al., 2004). This suggests a role for AngIV in the facilitation of cognitive processing noted during treatment with ACE inhibitors. It has also been established that Ang(1-7) and AngI(3-10) levels are elevated during treatment with ACE inhibitors (Lawrence et al., 1990). Both AngII(2-7) and AngI(3-10) bind at the $AT_4$ receptor subtype with affinities nearly comparable to that of native AngIV (Harding et al., unpublished observations; Sardinia et al., 1993). Also, conversion of Ang(1-7) to a ligand that acts at the $AT_4$ receptor is not only possible, but likely.

## 4.2 Learning and Memory

A role for the brain RAS in learning and memory was suggested many years ago, however the assay tools to measure the presumed changes in neuronal plasticity underlying memory consolidation have only recently become available. This section reviews literature supporting the involvement of AngII and AngIV in these processes.

### 4.2.1 Angiotensin II

Memory acquisition can be measured in animals using several protocols including passive and active avoidance conditioning and spatial recognition tasks. Passive and active avoidance conditioning procedures are typically used to assess associative learning. Associative learning is the process of attaching meaning (consequences) to a previously neutral object or event. That consequence is usually a foot shock (a punishment). With passive avoidance conditioning (also called a step-through task), the animal is placed in the lighted side of a two-compartment apparatus and permitted to move to the more preferred dark compartment on several preconditioning trials. Once the animal has habituated to the compartment it typically moves to the dark side within 10–20 s. The final "conditioning trial" consists of placing the animal into the illuminated compartment and allowing it to enter the dark compartment. Once in the dark compartment a guillotine door is dropped to close off the entrance, and a mild foot shock is applied for a short duration, usually 1–2 s. Thus, the conditioning paradigm consists of an association among the cues that denote the preferred dark compartment (conditioned stimuli) and the noxious foot shock (unconditioned stimulus). The conditioned response takes the form of an increased latency (reluctance) to move from the lighted compartment to the dark compartment on subsequent retention trials, i.e. passive avoidance conditioning. These retention trials are usually placed at 24-h intervals following conditioning in order to measure the subsequent strength of the conditioned response.

Circular water and eight-arm radial mazes are used to measure the acquisition of spatial memory by using rodent models. With the circular water maze (Morris water maze) the animal is placed into the water at a different location next to the walls of the tank on each training trial. The goal is to locate a submerged platform (2–3 cm below the surface fixed in position) using extra-maze visual cues placed on the walls surrounding the maze. If the animal is unsuccessful at the end of a trial it is placed on the platform for a short rest period permitting an opportunity to orient using these cues. The number of trials per day can

vary from 1 to as many as 20 or 25. The number of days of training may vary from 1 or 2 days, to a week or longer. The dependent measures may include the latency and distance required to find the platform on each trial, swim speed, and efficiency of search patterns. The eight-arm radial maze protocol requires food reward to motivate the animal. Several arms of the maze are baited with food. The animal is placed at the start point in the middle of the maze (origin of all arms) and must run down each arm to determine whether food is present at the end of the arm. The important measures include the latency required to locate the arms that contain food, and avoid those arms not baited with food, and the number of errors due to reentry into a previously visited arm.

Over thirty years ago Rolls and colleagues (1972) placed rats on a progressive ratio schedule-bar press response for food and water, and found that motivational levels were approximately equal following 24 h of water deprivation and when provided water and prepared with an injection of AngII into the preoptic area of the hypothalamus. Graeff et al. (1973) conditioned water-deprived rats to press a bar for water, and then injected AngII into the septal area and measured equivalent bar pressing when rats were satiated. These results suggest that AngII injections may simulate the motivational characteristics present while water-deprived. At about the same time it was reported that ICV-infused AngII interfered with performance of a variable interval operant task in rabbits (Melo and Graeff, 1975). Similarly, ICV-infused renin 1 min prior to the initiation of acquisition training on a passive avoidance task in rats interfered with recall of that task 1 and 2 days later (Köller et al., 1979). Recall that renin is responsible for the conversion of angiotensinogen to AngI, thus providing additional substrate for ACE-mediated conversion to AngII. Angiotensin II was assumed to be responsible for disruption of recall given that this performance deficit was attenuated by ICV infusion of the ACE inhibitor captopril. It was also reported that AngII injected into the dorsal neostriatum 5 min following passive avoidance conditioning interfered with the recall of the conditioned response 24 h later (Morgan and Routtenberg, 1977). Along these lines, DeNoble et al. (1991) observed that ICV-infused renin disrupted performance of a passive avoidance task in a dose-dependent pattern, i.e. as the renin dose was increased, the level of retention decreased. The coapplication of an $AT_1$ receptor antagonist (EXP3312 or EXP3880) and the ACE inhibitor captopril attenuated this renin-induced deficit. Since coapplication of an $AT_2$ receptor antagonist (PD123177) failed to influence this performance deficit produced by renin, it was concluded that the $AT_1$ receptor subtype mediated this deficit. It follows that compounds that decreased $AT_1$ receptor activation would be expected to facilitate cognitive processing.

In contrast with the above findings, central injections of AngII have been reported by some investigators to improve acquisition and recall. Baranowska et al. (1983) injected AngII (ICV: 1 and 2 μg) 15 min prior to active avoidance conditioning trials in rats. A buzzer served as a conditioned stimulus and foot shock as the unconditioned stimulus. Angiotensin II facilitated acquisition of the response but did not influence extinction. A low ICV dose of AngII (0.5 μg) inhibited the acquisition of this conditioned response. Pretreatment with saralasin or sarile ($Sar^1$,$Ile^8$-AngII) failed to block these AngII effects. These results were interpreted to suggest that AngII exerts a bimodal action upon learning, that is, an inhibitory influence at low doses and a facilitatory effect at higher doses. Subsequent reports from this laboratory indicated that ICV-delivered AngII and AngIV (1 nmol $\approx$ 1 μg), 15 min prior to testing for retention, facilitated recall of a passive avoidance conditioned response (Braszko et al., 1987; Georgiev et al., 1988). These treatments also facilitated the acquisition of a shuttlebox active avoidance task (Braszko et al., 1987, 1988; Georgiev et al., 1988). Further, such treatments facilitated T-maze performance when delivered immediately following acquisition training. However, if AngII and AngIV were administered 15 min prior to testing for recall of T-maze performance, no facilitation of performance was noted (Braszko et al., 1987, 1988).

Along these lines, microinjection of AngII into the CA1 hippocampal field has been shown to facilitate acquisition of an active avoidance (shuttlebox) task in rats (Belcheva et al., 2000). Kulakowska and colleagues (1996) extended this work to an object recognition task in which AngII facilitation could be blocked by pretreatment with losartan. These results suggest that the $AT_1$ receptor mediated this AngII-induced improvement in object recognition. However, Braszko (2002) has recently reported that ICV AngII-induced facilitation of passive avoidance conditioning, conditioned avoidance responding, and open-field locomotor behavior, could be blocked by combined pretreatment with losartan plus an $AT_2$ receptor

antagonist (PD123319), but not by each alone. Further, Braszko and colleagues (2003) have attempted to explain these variable AngII effects upon acquisition by measuring changes in motor and anxiety responses to ICV infusion of AngII. They found significant increases in anxiety as measured using an elevated "plus" maze, and impaired motor coordination as measured with the "chimney" test. Pretreatment with either losartan or PD123319 counteracted the AngII-induced heightened anxiety effects, but only losartan offset the impaired motor coordination effects.

The vast majority of these studies utilized native angiotensins rather than analogs that are resistant to conversion to shorter-chain peptides. Thus, it is very likely that these results are due to a combination of effects resulting from the conversion of AngII to AngIII and perhaps to Ang(1-7), Ang(2-7), Ang(3-7), and AngIV.

### 4.2.2 Angiotensin IV

The frequently noted failure of $AT_1$ and $AT_2$ receptor antagonists to influence performance on cognitive tasks, or block subsequent AngII facilitation of a conditioned response, may indicate that AngII is converted to AngIII, and then to AngIV (or an AngIV-like compound), and it is this ligand that acts at the $AT_4$ receptor subtype to improve performance. Our laboratory has discovered that the ICV infusion of AngIV leads to c-fos expression in the hippocampus and piriform cortices, while similar injection of AngII failed to induce c-fos-like immunoreactivity in these structures, but did activate c-fos expression in circumventricular organs (Roberts et al., 1995) and the hypothalamus (Zhu and Herbert, 1996). Pretreatment with losartan prevented this AngII-induced c-fos immunoreactivity, while pretreatment with the $AT_4$ receptor antagonist, divalinal-AngIV, blocked AngIV-induced c-fos expression (Roberts et al., 1995). There were no crossover effects exhibited by these antagonists. Along these lines, Braszko and colleagues (1988, 1991) were the first to report that ICV injected AngII and AngIV were equivalent at facilitating exploratory behavior in rats tested in an open field, improved recall of passive avoidance conditioning and the acquisition of active avoidance conditioning. Our laboratory confirmed and extended these findings in that ICV-infused AngIV improved the recall of a passive avoidance response in a dose-dependent fashion, with the most prominent facilitation at the highest dose (1 nmol) employed (Wright et al., 1993, 1995). We also found that ICV treatment with divalinal-AngIV, disrupted recall of this response (Wright et al., 1995). Along these lines osmotic pump ICV delivery of divalinal-AngIV during 6 days of training significantly impaired acquisition of the Morris water maze task (Wright et al., 1999). Our laboratory has also determined that ICV injected, metabolically resistant analogs of AngIV can be utilized to facilitate acquisition of successful search patterns in spatial memory tasks as compared with control animals treated with artificial cerebrospinal fluid, or a pentapeptide that does not bind at the $AT_4$ receptor subtype (Stubley-Weatherly et al., 1996; Wright et al., 1999). A similar facilitation of acquisition by AngIV analogs (eg. $Nle^1$-AngIV) has been observed in scopolamine-treated rats (Pederson et al., 1998, 2001) and in perforant path damaged rats (Wright et al., 1999).

Recently our laboratory has reported that ICV treatment with the nicotinic ACh receptor antagonist, mecamylamine, also disrupted acquisition of the Morris water maze task. Once again the ICV application of $Nle^1$-AngIV overcame this deficit in spatial learning (Olson et al., 2004). However, $Nle^1$-AngIV could not compensate for impaired acquisition resulting from the combined application of scopolamine plus mecamylamine. These results suggest that $Nle^1$-AngIV-induced compensation via the $AT_4$ receptor subtype may be dependent upon the brain cholinergic system. This notion is supported by the observation that AngIV and LVV-H7 induced the release of ACh from rat hippocampal slices in a dose-dependent fashion (Lee et al., 2001). This release of ACh could be blocked by divalinal-AngIV. These investigators have also shown AngIV- and LVV-H7-induced facilitation of spatial learning using the Barnes circular maze in which the animal must locate one escape tunnel among eight possible locations (Lee et al., 2004). The rats were tested three trials per day for 10 training days but received only one ICV bolus injection of AngIV or LVV-H7 on day 1, 5 min prior to testing. Taken together, these results suggest an important upstream role for the AngIV/$AT_4$ receptor system in learning and memory processes.

## 4.3 Stress

Considerable evidence supports an important role for the brain RAS in the control of stress-induced physiologies. $AT_1$ receptors are prominent in structures that control stress responses including median eminence, PVN, anterior pituitary gland, adrenal medulla, and zona glomerulosa (reviewed in Wright and Harding, 1992; Armando et al., 2003). Elevations in brain AngII facilitated the release of norepinephrine in vivo and in cell culture (reviewed in Phillips, 1987; Gard, 2002). In turn, elevations in norepinephrine were shown to block AngII release and downregulation of AngII receptors. The application of stressors elevated circulating and brain levels of renin and AngII (Yang et al., 1996; Peng and Phillips, 2001). Stress also upregulates the expression of $AT_1$ receptors within the PVN, where corticotropin-releasing hormone (CRH) cell bodies are located (Castren and Saavedra, 1988; Jezova et al., 1998), and the anterior pituitary (Leong et al., 2002). During stress, locally synthesized AngII within the anterior pituitary facilitates release of adrenocorticotropic hormone (ACTH; Ganong and Murakami, 1987). Thus, stress-induced upregulation of PVN $AT_1$ receptors appears to provoke CRH synthesis that preludes the facilitation of ACTH release and elevated adrenal corticoid secretion. Short periods of isolation stress have been shown to elevate $AT_1$ receptor expression in the PVN, along with correlated elevations in pituitary ACTH, adrenal corticosterone, catecholamines, and aldosterone. Nishimura et al. (2000) have shown that peripheral treatment with the $AT_1$ receptor antagonist, candesartan, prevented $AT_1$ receptor binding following isolation, both in the anterior pituitary and adrenal glands, and in the PVN. This treatment also interfered with the typical elevations in pituitary ACTH and adrenal corticosterone.

A second model that has been examined is gastric ulceration induced by cold restraint. This procedure induces gastric mucosa damage (Overmier and Murison, 2000) due to reduced blood flow and elevated free radical formation (Tuncel et al., 1998). Of particular interest, AngII-mediated constriction of the stomach vasculature via $AT_1$ receptor stimulation appears to be an important mediator of this reduction in blood flow (Heinemann et al., 1999). Bregonzio and colleagues (2003) reported that administration of candesartan significantly decreased the occurrence of gastric ulcerations induced by cold restraint stress. Taken together these results point to an important role for the AngII/$AT_1$ receptor system in the etiology of stress response. Saavedra and colleagues (Armando et al., 2003) have recently recommended that $AT_1$ receptor antagonists be evaluated for clinical efficacy in the treatment of stress-related disease states.

## 4.4 Depression

The first suggestion that the brain RAS may be important in depression came with the observation that captopril induced an antidepressant effect in hypertensive patients who also suffered from depression (Zubenko and Nixon, 1984; Deicken, 1986; Germain and Chouinard, 1988, 1989). There had been previous hints concerning this relationship from animal studies. Specifically, rats treated with antidepressants revealed decreased water intake induced by peripherally or centrally injected isoprenaline either in the presence or absence of a $\alpha_2$-adrenoceptor antagonist (Goldstein et al., 1985; Przegalinski et al., 1988). Further testing indicated that each of the antidepressant drugs fluoxetine, desipramine, and tranylcypromine reduced AngII-induced dipsogenicity in rats (Gard and Mycroft, 1991; Gard et al., 1994).

Captopril treatment has also been shown to protect animals against forced swimming induced learned helplessness/depression. This protocol requires the animal to swim within a small pool of water that has no escape. Eventually the animal stops swimming and becomes immobile. When placed in the pool the next day, it assumes immobility significantly sooner than during the initial trial. On each subsequent test day the latency to evidence immobility decreases, i.e. learned helplessness. Pretreatment with captopril reduced immobility in mice, equivalent to that of treatment with antidepressants, imipramine or mianserine (Giardina and Ebert, 1989). Learned helplessness induced by foot shock in rats could be prevented by pretreatment with captopril to the same degree as imipramine (Martin et al., 1990). Under both protocols the protective effects of captopril were reversed by naloxone, suggesting that the ACE inhibitor was exerting its antidepressant effects, at least in part, via opioid receptors. In addition, this effect is also dependent upon

the brain RAS since pretreatment with losartan also provided protection from immobility in the forced swim test (Gard et al., 1999; Gard, 2002).

These results suggest that antidepressants exert their positive effects in part by inhibiting the brain RAS. The precise mechanism(s) of this inhibition remains to be determined.

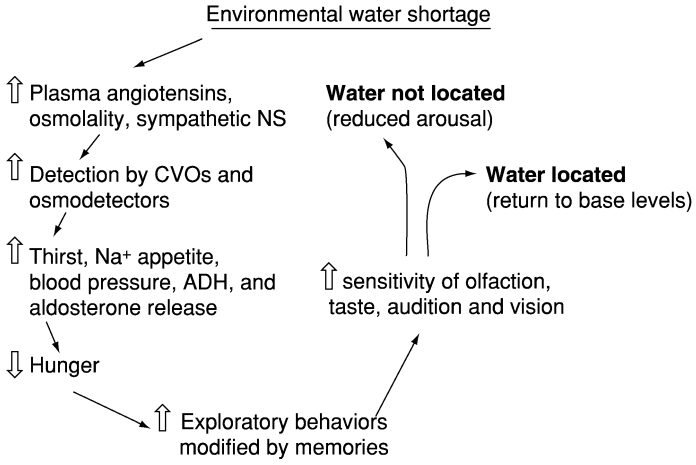
## 5 Conclusions

The existence of a separate and distinct brain RAS is now undisputed. The necessary synthetic precursors and peptidases required for the formation and degradation of the active forms of angiotensins have been identified in brain tissues, and so have three receptor subtypes. There is coordination among the peripheral, pituitary, and central RASs via stimulation of brain angiotensin receptors located within peripheral tissues, the CVOs, and pituitary gland. These CVOs are located in the proximity of brain ventricles, are richly vascularized, and possess a reduced blood–brain barrier permitting access to peptides and other molecules. In this way the brain RAS interacts with many neurotransmitter and neuromodulator systems in the regulation of systemic blood pressure, body fluid homeostasis, vasopressin release, cyclicity of reproductive hormones, and sexual behaviors. These classic functions appear to be primarily mediated by the $AT_1$ receptor subtype. In addition, the brain RAS has been implicated in several novel functions including learning and memory consolidation, cognitive processing, stress, anxiety, and depression. Several of these processes appear to be mediated by a combination of reduced $AT_1$ receptor activation, coupled with increased activation of the $AT_4$ subtype, and/or other binding sites. These observations have significantly expanded our understanding concerning the physiologies and behaviors mediated by the RAS, and facilitated the potential for clinical intervention in the treatment of related disorders.

Some authors have posited that the mammalian CNS is poorly designed to deal with present-day information overload, psychological and sociological stressors, and related problem-solving behaviors, but is better equipped to deal with acute, single, short-duration stressors. On the other hand, many angiotensin researchers have long argued that the RAS is critically important to mammalian ability to deal with multiple, overlapping, environmental challenges. With additional insight concerning the extent and precision of this system's regulatory capacity comes the growing realization of the role of the RAS as a "coping" mechanism designed to address challenges far beyond cardiovascular and body fluid homeostasis.

One can envision the overall activation of this system in the face of a reasonably straightforward environmental challenge such as water shortage (*Figure 27-5*). With time, body water depletion leads to elevated plasma angiotensins and osmolality detected by CVOs and hypothalamic osmodetectors, respectively. These stimuli activate pathways to target structures throughout the brain, including limbic regions, to initiate drinking behavior and sodium appetite, suppress hunger, elevate blood perfusion pressure, and conserve water and sodium via vasopressin and aldosterone release acting at kidney nephrons. Such elevations in angiotensins also stimulate increased exploratory behaviors, modified by the recall of relevant memories, thus increasing the likelihood of discovering water within the animal's temporarily extended environmental range. Accompanying this generalized arousal, the acuity of some of the animal's sensory systems (vision, audition, taste, and olfaction) are facilitated, while the sensitivity of nonessential sensory systems and sexual desire are temporarily reduced. In the event that water is located and ingested, tissue osmolality and circulating levels of angiotensins are reduced, stimulation of CVOs declines, and these systems return to base level functioning. On the other hand, if a long and exhausting search for water is unsuccessful the animal may be forced to adopt a different strategy, one that conserves its remaining energy in favor of longevity. Under these circumstances, reduced arousal (depressive state) in a safe, cool location may be a reasonable option. During this time a reduction in sympathetic arousal would be anticipated, accompanied by decreased blood pressure due to reduced intravascular volume and vasoconstriction, along with reduced plasma angiotensin levels and vasopressin release. Decreased circulating angiotensin levels would be expected to reduce activation of $AT_1$ receptors, and eventually calm the animal. A quiescent state is important given that the animal is forced to patiently await a reversal of bad fortune. This may take the form of a rain shower, moisture from nighttime condensation, or body fluids from prey.

**Figure 27-5**
**Major changes in physiologies and behaviors accompanying environmental water shortage**




As we marvel at how well-adapted the mammal is to the above environmental contingencies, we must also concede that this system is challenged by today's multidimensional stressors and the diseases that may possibly occur should this system fail as in acute or chronic hypertension, atherosclerosis, heart failure, electrolyte imbalance, stress and anxiety disorders, depression, and memory impairment. The development of future treatment strategies must consider the nature of the environmental stressors that shaped the evolution of the RAS, and encourage an understanding of the pathogenesis of this system that includes consideration of both peripheral and brain RASs and the several receptor subtypes involved. Thus, new treatment strategies must employ the medicinal chemistry necessary to develop compounds that gain access to angiotensin receptors located in the periphery, at CVOs, pituitary gland, and especially within structures protected by the blood–brain barrier.

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## References

Albiston AL, McDowall SG, Matsacos D, Sim P, Clune E, et al. 2001. Evidence that the angiotensin IV ($AT_4$) receptor is the enzyme insulin-regulated aminopeptidase. J Biol Chem 276: 48623-48626.

Albiston AL, Mustafa T, McDowall SG, Mendelsohn FA, Lee J, et al. 2003. AT(4) receptor is insulin-regulated membrane aminopeptidase: potential mechanisms of memory enhancement. Trends Endocrinol Metab 14: 72-77.

Albiston AL, Pederson ES, Burns P, Purcell B, Wright JW, et al. 2004. Attenuation of scopolamine-induced learning deficits by LVV-hemorphin-7 in rats in the passive avoidance and water maze paradigms. Behav Brain Res 154: 239-243.

Allen AM, Oldfield BJ, Giles ME, Paxinos G, McKinley MJ, et al. 2000. Localization of angiotensin receptors in the nervous system. Handbook of chemical neuroanatomy.

Quirion R, Bjorklund A, Hodfelt T, editors. Amsterdam: Elsevier; pp. 79-124.

Allen AM, Zhuo J, Mendelsohn FA. 2001. $AT_1$-receptors in the central nervous system. JRAAS 2 (Suppl. 1): S95-S101.

Armando I, Seltzer A, Bregonzio C, Saavedra JM. 2003. Stress and angiotensin II: novel therapeutic opportunities. Curr Drug Targets – CNS Neurol Disord 2: 413-419.

Avrith D, Fitzsimons J. 1980. Increased sodium appetite in the rat induced by intracranial administration components of the renin–angiotensin system. J Physiol 301: 349-364.

Baranowska D, Braszko JJ, Wisniewski K. 1983. Effect of angiotensin II and vasopressin on acquisition and extinction of conditioned avoidance in rats. Psychopharmacology 81: 247-251.

Barnes JM, Barnes NM, Costall B, Coughlan J, Horovitz ZP, et al. 1989. ACE inhibition and cognition. Current advances in ACE inhibition. MacGregor GA, Sever PS, editors. New York: Churchill Livingston Press; pp. 159-171.

Barnes JM, Barnes NM, Costall B, Coughlan J, Kelly ME, et al. 1992. Angiotensin-converting enzyme inhibition, angiotensin, and cognition. J Cardiovasc Pharmacol 19 (Suppl. 6): 563-571.

Barnes NM, Champaneria S, Costall B, Kelly ME, Murphy DA, et al. 1990. Cognitive enhancing actions of DuP 753 detected in a mouse habituation paradigm. Neuroreport 1: 239-242.

Barnes NM, Cheng CH, Costall B, Naylor RJ, Williams TJ, et al. 1991a. Angiotensin converting enzyme density is increased in temporal cortex from patients with Alzheimer's disease. Eur J Pharmacol 20: 289-292.

Barnes NM, Costall B, Egli P, Horovitz ZP, Ironside JW et al. 1991b. Characterization of $^3$H ceranapril recognition sites in rat and human tissue. Neuropharmacology 30: 907-914.

Barnes NM, Costall B, Kelly ME, Murphy DA, Naylor RJ. 1991c. Cognitive enhancing actions of PD123177 detected in a mouse habituation paradigm. Neuroreport 2: 351-353.

Bartus RT, Dean RL, Beer B, Lippa AS. 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science 217: 408-417.

Belcheva I, Ternianov A, Georgiev V. 2000. Lateralized learning and memory effects of angiotensin II microinjected into the rat $CA_1$ hippocampal area. Peptides 21: 407-411.

Bennett JP Jr, Snyder SH. 1976. Angiotensin II binding to mammalian brain membranes. J Biol Chem 251: 7423-7430.

Bernier SG, Bellemare JM, Escher E, Guillemette G. 1998. Characterization of $AT_4$ receptor from bovine aortic endothelium photosensitive analogues of angiotensin IV. Biochemistry 37: 4280-4287.

Bernier SG, Fournier A, Guillemette G. 1994. A specific binding site recognizing a fragment of angiotensin II in bovine adrenal cortex membranes. Eur J Pharmacol 271: 55-63.

Bernier SG, Servant G, Boudreau M, Fournier A, Guillemette G. 1995. Characterization of a binding site for angiotensin IV on bovine endothelial cells. Eur J Pharmacol 291: 191-200.

Bickerton RK, Buckley JP. 1961. Evidence for a central mechanism in angiotensin induced hypertension. Proc Soc Exp Biol Med 106: 834-839.

Blair-West, JR, Carey KD, Denton DA, Madden LJ, Weisingeer RS, et al. 2001. Possible contribution of brain angiotensin III to ingestive behaviors in baboons. Am J Physiol Regul Integr Comp Physiol 281: R1633-R1636.

Blair-West, JR, Coghlan JP, Denton DA, Funder JW, Scoggins BA, et al. 1971. The effect of the heptapeptide (2–8) and hexapeptide (3–8) fragments of angiotensin II on aldosterone secretion. J Clin Endocrinol Metab 32: 575-578.

Bohlen und Halback OV. 2003. Angiotensin IV in the central nervous system. Cell Tissue Res 311: 1-9.

Booth DA. 1968. Mechanism of action of norepinephrine in eliciting an eating response on injection into the rat hypothalamus. J Pharmacol Exp Ther 150: 336-348.

Bottari SP, Raylor V, King IN, Bogdal S, Whitebread S, et al. 1991. Angiotensin II $AT_2$ receptors do not interact with guanine nucleotide binding proteins. Eur J Pharmacol 207: 157-163.

Braszko JJ. 2002. AT(2) but not AT(1) receptor antagonism abolishes angiotensin II increase of the acquisition of conditioned avoidance responses in rats. Behav Brain Res 131: 79-86.

Braszko JJ, Kulakowska A, Winnicka MM. 2003. Effects of angiotensin II and its receptor antagonists on motor activity and anxiety in rats. J Physiol Pharmacol 54: 271-281.

Braszko JJ, Kupryszewski G, Witczuk B, Wisniewski K. 1988. Angiotensin II-(3–8)-hexapeptide affects motor activity, performance of passive avoidance and conditioned avoidance responses in rats. Neuroscience 27: 777-783.

Braszko JJ, Wisniewski K, Kupryszewski G, Witczuk B. 1987. Psychotropic effects of angiotensin II and III in rats: locomotor and exploratory vs. cognitive behavior. Behav Brain Res 25: 195-203.

Braszko JJ, Wlasienko J, Koziolkiewicz W, Janecka A, Wisniewski K. 1991. The 3–7 fragment of angiotensin II is probably responsible for its psychoactive properties. Brain Res 542: 49-54.

Braun-Menendez E, Fasciolo JC, Leloir LF, Munoz JM. 1940. The substance causing renal hypertension. J Physiol (Lond) 98: 283-298.

Bregonzio C, Armando I, Ando H, Jezova M, Baiardi G, et al. 2003. Anti-inflammatory effects of angiotensin II $AT_1$

receptor antagonism prevent stress-induced gastric injury. Am J Physiol 285: G414-G423.

Brosnihan KB, Li P, Ferrario CM. 1996. Angiotensin (1–7) dilates canine coronary arteries through kinins and nitric oxide. Hypertension 27: 523-528.

Buggy J, Fisher AE. 1976. Anteroventral third ventricle site of action for angiotensin induced thirst. Pharmacol Biochem Behav 4: 651-660.

Buggy J, Fisher AE, Hoffman WE, Johnson AK, Phillips MI. 1975. Ventricular obstruction: effect on drinking induced by intracranial injection of angiotensin. Science 233: R44-R52.

Buggy J, Jonklaas J. 1984. Sodium appetite decreased by central angiotensin blockade. Physiol Behav 32: 749-753.

Campagnole-Santos, MJ, Heeringer SB, Batista EN, Khosla MC, Santos RA. 1992. Differential baroreceptor reflex modulation by centrally infused angiotensin peptides. Am J Physiol 263: R89-R94.

Caputo R, Rowland N, Fregly M. 1992. Angiotensin-related intakes of water and NaCl in Fischer 344 and Sprague-Dawley rats. Am J Physiol 262: R382-R388.

Carey RM, Siragy HM. 2003. Newly recognized components of the renin–angiotensin system: potential roles in cardiovascular and renal regulation. Endocr Rev 24: 261-271.

Caron AZ, Arguin G, Guillemette G, 2003. Angiotensin IV interacts with a juxtamembrane site on AT(4)/IRAP suggesting an allosteric mechanism of enzyme modulation. Regul Pept 113: 9-15.

Castren E, Saavedra JM. 1988. Repeated stress increases the density of angiotensin II binding sites in rat paraventricular nucleus and subfornical organ. Endocrinology 122: 370-372.

Chauvel EN, Llorens-Cortes C, Coric P, Wilk S, Roques BP, et al. 1994. Differential inhibition of aminopeptidase A and aminopeptidase N by new beta-amino thiols. J Med Chem 37: 2950-2957.

Chen BZ, Mendelsohn FAO. 1992. Effect of acute and chronic administration of ceranapril on angiotensin converting enzyme in plasma, kidney, lung, brain-regions and cerebrospinal-fluid of rats. Neuropharmacology 31: 929-935.

Chen JK, Zimpelmann J, Harris RC, Burns KD. 2001. Angiotensin IV induces tyrosine phosphorylation of focal adhesion kinase and paxillin in proximal tubule cells. Am J Physiol Renal Physiol 280: F980-F988.

Chi NW, Lodish HF. 2000. Tankyrase is a golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles. J Biol Chem 275: 38437-38444.

Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, et al. 1989. Identification of angiotensin II receptor subtypes. Biochem Biophys Res Commun 165: 196-203.

Coleman JK, Krebs LT, Hamilton TA, Ong B, Lawrence KA, et al. 1998. Autoradiographic identification of kidney angiotensin IV binding sites and angiotensin IV-induced renal blood flow changes in rats. Peptides 19: 269-277.

Costall B, Coughlan J, Horovitz ZP, Kelly ME, Naylor RJ, et al. 1989. The effects of ACE inhibitors captopril and SQ29852 in rodent tests of cognition. Pharmacol Biochem Behav 33: 573-579.

Croog SH, Levine S, Testa MA, Brown B, Bulpitt CJ, et al. 1986. The effects of antihypertensive therapy on the quality of life. N Engl J Med 314: 1657-1664.

Culman J, Blume A, Gohlke P, Unger T. 2002. The renin–angiotensin system in the brain: possible therapeutic implications for $AT_1$-receptor blockers. J Hum Hypertens 16: S64-S70.

Culman J, Hohle S, Qadri F, Edling O, Blume A, et al. 1995. Angiotensin as neuromodulator/neurotransmitter in central control of body fluid and electrolyte homoeostasis. Clin Exp Hypertens 17: 281-293.

Dampney RAL, Hirooka Y, Potts PD, Head GA. 1996. Functions of angiotensin peptides in the rostral ventrolateral medulla. Clin Exp Pharmacol Physiol 3 (Suppl.): S105-S111.

deGasparo M, Catt KJ, Inagami T, Wright JW, Unger T. 2000. International Union of Pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev 52: 415-72.

deGasparo M, Husain A, Alexander W, Catt KJ, Chiu AT, et al. 1995. Proposed update of angiotensin receptor nomenclature. Hypertension 25: 924-39.

deGasparo M, Siragy HM. 1999. The $AT_2$ receptor: fact, fancy and fantasy. Regul Pept 81: 11-24.

Deicken RF. 1986. Captopril treatment of depression. Biol Psychiatry 12: 1425-1428.

DeNoble VJ, De Noble, KF, Spencer KR, Chiu AT, Wong PC, et al. 1991. Non-peptide angiotensin II receptor antagonist and angiotensin-converting enzyme inhibitor: effect on a renin-induced deficit of a passive avoidance response in rats. Brain Res 561: 230-235.

Dulin N, Madhun ZT, Chang CH, Berti-Mattera, L, Dickens D, et al. 1995. Angiotensin IV receptors and signaling in opossum kidney cells. Am J Physiol 269: F644-F652.

Dzau VJ, Ingelfinger J, Pratt RE, Ellison KE. 1986. Identification of renin and angiotensinogen messenger RNA sequences in rat brain. Hypertension 8: 544-548.

Elliott DF, Perart WS. 1956. Amino acid sequence in a hypertensin. Nature 177: 527-528.

Epstein AN. 1982. Mineralocorticoids and cerebral angiotensin may act together to produce sodium appetite. Peptides 3: 493-494.

Epstein AN, Fitzsimons JT, Rolls BJ. 1970. Drinking induced by injection of angiotensin into the brain of the rat. J Physiol (Lond) 210: 457-474.

Feener EP, Northrup JM, Aiello LP, King GL. 1995. Angiotensin II induces plasminogen activator inhibitor-1 and -2 expression in vascular endothelial and smooth muscle cells. J Clin Invest 95: 1353-1362.

Ferrario CM. 2003. Contribution of angiotensin-(1–7) to cardiovascular physiology and pathology. Curr Hypertens Rep 5: 129-134.

Ferrario CM, Chappell MC, Tallant EA, Brosnihan KB, Diz DI. 1997. Counterregulatory actions of angiotensin-(1–7). Hypertension 30: 535-541.

Ferrario CM, Santos RA, Brosnihan KB, Block CH, Schiavone MT, et al. 1988. A hypothesis regarding the function of angiotensin peptides in the brain. Clin Exp Hypertens A 10 (Suppl. 1): 107-121.

Fink GD, Bruner CA. 1985. Hypertension during chronic peripheral and central infusion of angiotensin III. Am J Physiol 249: E201-E208.

Fisher-Ferraro, C, Nahmod VE, Goldstein DJ, Finkielman S. 1971. Angiotensin and renin in the rat and dog brain. J Exp Med 133: 353-361.

Fitts DA, Masson DB. 1990. Preoptic angiotensin and salt appetite. Behav Neurosci 104: 643-650.

Fitzsimons JT. 1971. The effect on drinking of peptide precursors and of shorter chain peptide fragments of angiotensin II injected into the rat's diencephalons. J Physiol (Lond) 214: 295-303.

Fitzsimons JT. 1980. Angiotensin stimulation of the central nervous system. Rev Physiol Biochem Pharmacol 87: 117-167.

Fitzsimons JT. 1998. Angiotensin, thirst, and sodium appetite. Physiol Rev 78: 583-686.

Flood JF, Morley JE. 1993. Dose–response differences in the ability of ramipril to improve retention in diabetic mice. Eur J Pharmacol 240: 311-314.

Ganong WF, Murakami K. 1987. The role of angiotensin II in the regulation of ACTH secretion. Ann N Y Acad Sci 512: 176-186.

Ganten D, Boucher R, Genest J. 1971a. Renin activity in brain tissue of puppies and adult dogs. Brain Res 33: 557-559.

Ganten D, Hermann K, Bayer D, Unger T, Lang RE. 1983. Angiotensin synthesis in the brain and increased turnover in hypertensive rats. Science 221: 869-871.

Ganten D, Marquez-Julio A, Granger P, Hayduk K, Karsunky KP, et al. 1971b. Renin in dog brain. Am J Physiol 221: 1733-1737.

Gard PR. 2002. The role of angiotensin II in cognition and behaviour. Eur J Pharmacol 438: 1-14.

Gard PR, Mandy A, Sutcliffe MA. 1999. Evidence of a possible role of altered angiotensin function in the treatment, but not aetiology, of depression. Biol Psychiatry 45: 1030-1034.

Gard PR, Mandy A, Whiting JM, Nickels DP, Meakin AJ. 1994. Reduction of responses to angiotensin II by antidepressant drugs. Eur J Pharmacol 264: 295-300.

Gard PR, Mycroft N. 1991. Reduction of angiotensin II-induced drinking in rats by 21 hour pretreatment with desipramine. J Pharm Pharmacol 43 (Suppl. 1).

Germain L, Chouinard G. 1988. Treatment of recurrent unipolar major depression with captopril. Biol Psychiatry 23: 637-641.

Germain L, Chouinard G. 1989. Captopril treatment of major depression with serial measurements of blood cortisol concentrations. Biol Psychiatry 25: 489-493.

Georgiev VP, Yonkov DI, Kambourova TS. 1988. Interactions between angiotensin II and baclofen in shuttle-box and passive avoidance performance. Neuropeptides 12: 155-158.

Gesualdo L, Ranieri E, Monno R, Rossiello MR, Colucci M, et al. 1999. Angiotensin IV stimulates plasminogen activator inhibitor-1 expression in proximal tubular epithelial cells. Kidney Int 56: 461-470.

Giardina WJ, Ebert DM. 1989. Positive effects of captopril in the behavioural despair swim test. Biol Psychiatry 25: 697-702.

Glossmann H, Baukal A, Catt KJ. 1974. Angiotensin II receptors in bovine adrenal cortex. Modification of angiotensin II binding by guanyl nucleotides. J Biol Chem 249: 664-666.

Goldstein JM, Knobloch-Litwin, LC, Malick JB. 1985. Behavioural evidence for β-adrenoceptor subsensitivity after subacute antidepressant/$\alpha_2$-adrenoceptor antagonist treatment. Naunyn-Schmiedeberg's Arch Pharmacol 329: 355-358.

Graeff FG, Gentil CG, Peres VL, Covian MR. 1973. Lever-pressing behavior caused by intraseptal angiotensin II in water satiated rats. Pharmacol Biochem Behav 1: 357-359.

Guo DF, Inagami T. 1994. The genomic organization of the rat angiotensin II receptor $AT_{1B}$. Biochim Biophys Acta 1218: 91-94.

Hall KL, Hanesworth JM, Ball AE, Felgenhaner GP, Hosick HL, et al. 1993. Identification and chracterization of a novel angiotensin binding site in cultured vascular smooth muscle cells that is specific for the hexapeptide (3–8) fragment of angiotensin II, angiotensin IV. Regul Pept 44: 225-232.

Hamilton TA, Handa RK, Harding JW, Wright JW. 2001. A role for the $AT_4$/angiotensin IV system in mediating natriuresis in the rat. Peptides 22: 935-944.

Handa RI. 2001. Characterization of signaling of the AT(4) receptor in human proximal tubule epithelial (HK-2) cells. J Am Soc Nephrol 12: 440-449.

Handa RK, Krebs LT, Harding JW, Handa SE. 1998. Angiotensin IV $AT_4$-receptor system in the rat kidney. Am J Physiol 274: F290-F299.

Hanesworth JM, Sardinia JF, Krebs LT, Hall KL, Harding JW. 1993. Elucidation of a specific binding site for angiotensin II(3–8), angiotensin IV, in mammalian heart membranes. J Pharmcol Exp Ther 266: 1036-1042.

Harding JW, Cook VI, Miller-Wing, AV, Hanesworth JM, Sardinia MF, et al. 1992. Identification of an AII (3–8) AIV binding site in guinea pig hippocampus. Brain Res 583: 340-343.

Harding JW, Stone LP, Wright JW. 1981. The distribution of angiotensin II binding sites in rodent brain. Brain Res 205: 265-274.

Head GA. 1996. Role of $AT_1$ receptors in the central control of sympathetic vasomotor function. Clin Exp Pharmacol Physiol 3 (Suppl.): S93-S98.

Heinemann A, Sattler V, Jocic M, Wienen W, Holzer P. 1999. Effect of angiotensin II and telmisartan, an angiotensin 1 receptor antagonist, on rat gastric mucosal blood flow. Aliment Pharmacol Ther 13: 347-355.

Hermann K, Lang RE, Unger T, Bayer C, Ganten D. 1984. Combined high-performance liquid chromatography radioimmunoassay for the characterization and quantitative measurement of neuropeptides. J Chromatogr 312: 273-284.

Iwai N, Inagami T. 1992. Identification of two subtypes in the rat type 1 angiotensin II receptor. FEBS Lett 298: 257-260.

Iwai N, Yamano Y, Chak S, Konishi F, Bardham S, et al. 1991. Rat angiotensin II receptor: cDNA sequence and regulation of gene expression. Biochem Biophys Res Commun 177: 299-304.

Jaiswal N, Diz DI, Chappell MC, Khasla MC, Ferrario CM. 1992. Stimulation of endothelial cell prostaglandin production by angiotensin peptides. Characterization of receptors. Hypertension 21: 900-905.

Jarvis MF, Gessner GW, Ly CG. 1992. The angiotensin hexapeptide 3–8 fragment potently inhibits [$^{125}$I] angiotensin II binding to non-$AT_1$ or -$AT_2$ recognition sites in bovine adrenal cortex. Eur J Pharmacol 219: 319-322.

Jezova D, Ochedalski T, Kiss A, Aguilera GJ. 1998. Brain angiotensin II modulates sympathoadrenal and hypothalamic pituitary adrenocortical activation during stress. Neuroendocrinology 10: 67-72.

Johnston CI. 1990. Biochemistry and pharmacology of the renin–angiotensin system. Drugs 39: 21-31.

Kakar SS, Sellers JC, Devor DC, Musgrove LC, Neill JD. 1992. Angiotensin II type-1 receptor subtype cDNAs: differential tissue expression and hormonal regulation. Biochem Biophys Res Commun 31: 1090-1096.

Kambayashi Y, Bardham S, Takahashi K, Tsuzuki S, Inui H, et al. 1993. Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. J Biol Chem 268: 24543-24546.

Kandror KV, Pilch PF. 1994. gp160, a tissue-specific marker for insulin-activated glucose transport. Proc Natl Acad Sci USA 91 (17): 8017-8021.

Katagiri H, Asano T, Yamada T, Aoyama T, Fukushima Y, et al. 2002. Acyl-coenzyme A dehydrogenases are localized on GLUT4-containing vesicles via association with insulin-regulated aminopeptidase in a manner dependent on its dileucine motif. Mol Endocrinol 16: 1049-1059.

Keller SR, Scott HM, Mastick CC, Aebersold R, Lienhard GE. 1995. Cloning and characterization of a novel insulin-regulated membrane aminopeptidase from Glut4 vesicles. J Biol Chem 270: 23612-23618.

Kerins DM, Hao Q, Vaughan DE. 1995. Angiotensin induction of PAI-1 expression in endothelial cells is mediated by the hexapeptide angiotensin IV. J Clin Invest 96: 2515-2520.

Köller, M, Krause HP, Hoffmeister F, Ganten D. 1979. Endogenous brain angiotensin II disrupts passive avoidance behavior in rats. Neurosci Lett 14: 71-75.

Konoshi H, Kuroda S, Inada Y, Fujisawa Y. 1994. Novel subtype of human angiotensin II type 1 receptor: cDNA cloning and expression. Biochem Biophys Res Commun 199: 467-474.

Kramár, EA, Armstrong DL, Ikeda S, Wayner MJ, Harding JW, et al. 2001. The effects of angiotensin IV on long-term potentiation within the CA1 region of the hippocampus in vitro. Brain Res 897: 114-121.

Kramár, EA, Harding JW, Wright JW. 1997. Angiotensin II-, and IV-induced changes in cerebral blood flow: roles of $AT_1$, $AT_2$, and $AT_4$ receptor subtypes. Regul Pept 68: 131-138.

Kucharewicz I, Chabielska E, Pawlak D, Matys T, Rolkowski R, et al. 2000. The antithrombotic effect of angiotensin (1–7) closely resembles that of losartan. JRAAS 1: 268-272.

Kucharewicz I, Pawlak R, Matys T, Chabielska E, Buczko W. 2002. Angiotensin (1–7): an active member of the renin–angiotensin system. J Physiol Pharmacol 53: 533-540.

Kulakowska A, Karwowska W, Wisniewski K, Braszko JJ. 1996. Losartan influences behavioural effects of angiotensin II in rats. Pharmacol Res 34: 109-115.

Lawrence AC, Evin G, Kladis A, Campbell DJ. 1990. An alternative strategy for the radioimmunoassay of angiotensin peptides using amino-terminal-directed antisera: measurement of eight angiotensin peptides in human plasma. J Hypertens 8: 715-724.

Lee J, Albiston AL, Allen AM, Mendelsohn FAO, Ping SE, et al. 2004. Effect of ICV injection of $AT_4$ receptor ligands, Nle$^1$-angiotensin IV and LVV-hemorphin 7, on spatial learning in rats. Neuroscience 124: 341-349.

Lee J, Chai SY, Mendelsohn FAO, Morris MJ, Allen AM. 2001. Potentiation of cholinergic transmission in the rat hippocampus by angiotensin IV and LVV-hemorphin-7. Neuropharmacology 40: 618-623.

Lee J, Mustafa T, McDowall SG, Mendelsohn FA, Brennan M, et al. 2003. Structure–activity study of LVV-hemorphin-7: angiotensin AT4 receptor ligand and inhibitor of insulin-regulated aminopeptidase. J Pharmacol Exp Ther 305: 205-211.

Leong DS, Terron JA, Falcon-Neri, A, Armando I, Ito T, et al. 2002. Restraint stress modulates brain, pituitary and adrenal expression of angiotensin II AT(1A), AT(1B) and AT(2) receptors. Neuroendocrinology 75: 227-240.

Lew RA, Mustafa T, Ye S, McDowall SG, Chai SY, et al. 2003. Angiotensin $AT_4$ ligands are potent, competitive inhibitors of insulin regulated aminopeptidase (IRAP). J Neurochem 86: 344-350.

Li YD, Block ER, Patel JM. 2002. Activation of multiple signaling modules is critical in angiotensin IV-induced lung endothelial cell proliferation. Am J Physiol Lung Cell Mol Physiol 283: L707-L716.

Li P, Chappell MC, Ferrarion CM, Broshinan KB. 1997. Angiotensin (1–7) augments bradykinin-induced vasodilation by competing with ACE and releasing nitric oxide. Hypertension 29: 394-400.

Linazasoro JM, Diaz CJ, Mendoza HC. 1954. The kidney and thirst regulation. Bull Inst Med Res 7 (2): 53-61.

Lind RW. 1988. Sites of action of angiotensin in the brain. Angiotensin and blood pressure regulation. Harding JW, Wright JW, Speth RC, Barnes CD, editors. San Diego: Academic Press; pp. 135-163.

Llorens-Cortes, C, Mendelsohn FAO. 2002. Organisation and functional role of the brain angiotensin system. JRAAS 3: S39-S48.

Loot AE, Roks AJ, Henning RH, Tio RA, Suurmeijer AM, et al. 2002. Angiotensin-(1–7) attenuates the development of heart failure after myocardial infarction in rats. Circulation 105: 1548-1550.

Lynch KR, Simnad VI, Ben-Ari ET, Garrison JC. 1986. Localization of angiotensinogen messenger RNA sequences in the rat brain. Hypertension 8: 540-543.

Martin P, Massol J, Puech AJ. 1990. Captopril as an antidepressant? Effects on the learned helplessness paradigm in rats. Biol Psychiatry 27: 968-974.

McKinley MJ, Albiston AL, Allen AM, Mathai ML, May CN, et al. 2003. The brain renin–angiotensin system: location and physiological roles. Int J Biochem Cell Biol 35: 901-918.

Mehta JL, Li DY, Yang H, Raizada MK. 2002. Angiotensin II and IV stimulate expression and release of plasminogen activator inhibitor-1 in cultured human coronary artery endothelial cells. J Cardiovasc Pharmacol 39: 789-794.

Melo JC, Graeff FG. 1975. Effect of intracerebroventricular bradykinia and related peptides on rabbit operant behavior. J Pharmacol Exp Ther 193: 1-10.

Meng W, Busija DW. 1993. Comparative effects of angiotensin (1–7) and angiotensin II on piglet pial arterioles. Stroke 24: 2041-2044.

Mentlein R, Roos T. 1996. Proteases involved in the metabolism of angiotensin II, bradykinin, calcitonin gene-related peptide (CGRP), and neuropeptide Y by vascular smooth muscle cells. Peptides 17: 709-720.

Miller-Wing AV, Hanesworth JM, Sardinia MF, Wright JW, Speth RC, et al. 1993. Central angiotensin IV receptors: distribution and specificity in guinea pig brain. J Pharmacol Exp Ther 266: 1718-1726.

Moe KE, Weiss ML, Epstein AN. 1984. Sodium appetite during captopril blockade of endogenous angiotensin II formation. Am J Physiol 247: R356-R365.

Møeller I, Clune EF, Fennessy PA, Bingley JA, Albiston AL, et al. 1999. Up regulation of $AT_4$ receptor levels in carotid arteries following balloon injury. Regul Pept 83: 25-30.

Møeller I, Lew RA, Mendelsohn FA, Smith AI, Brennan ME, et al. 1997. The globin fragment LVV-hemorphin-7 is an endogenous ligand for the $AT_4$ receptor in the brain. J Neurochem 68: 2530-2537.

Mondadori C, Etienne P. 1990. Nootropic effects of ACE inhibitors in mice. Psychopharmacology 100: 301-307.

Morgan TM, Routtenberg A. 1977. Angiotensin injected into the neostriatum after learning disrupts retention performance. Science 196: 87-89.

Morris RG. 1984. Development of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11: 47-60.

Mukoyama M, Nakajiima M, Horiuchi M, Sasamura H, Pratt RE, et al. 1993. Expression cloning of type-2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. J Biol Chem 268: 24539-24542.

Munoz JM, Braun-Menendez E, Fasciolo JC, Leloir LF. 1939. Hypertension: the substance causing renal hypertension. Nature 144: 980.

Muratami H, Teruya H, Sesoko S, Takishita S, Fukiyama K. 1996. Brain angiotensin and circulatory control. Clin Exp Pharmacol Physiol 23: 458-464.

Murphy TJ, Alexander RW, Griendling KK, Runge MS, Berstein KE. 1991. Isolation of a cDNA encoding the vascular type-1 angiotensin receptor. Nature 351: 233-236.

Mustafa T, Chai SY, Mendelsohn FA, Moeller I, Albiston AL, 2001. Characterization of the AT(4) receptor in a human neuroblastoma cell line (SK-N-MC). J Neurochem 76: 1679-1687.

Muthalif MM, Benter IF, Uddin MR, Harper JL, Malik KU. 1998. Signal transduction mechanisms involved in angiotensin (1–7)-stimulated arachidonic acid release and prostanoid synthesis in rabbit aortic smooth muscle cells. J Pharmacol Exp Ther 284: 388-398.

Nairn RC, Mason CM, Corcoran AC. 1956. The production of serous effusions in nephrectomized animals by the administration of renal extracts and renin. J Pathol Bacteriol 71: 155-163.

Neves LA, Averill DB, Ferrario CM, Chappell MC, Aschner JL, et al. 2003. Characterization of angiotensin-(1–7) receptor subtype in mesenteric arteries. Peptides 24: 455-462.

Nishimura Y, Ito T, Hoe KL, Saavedra JM. 2000. Chronic peripheral administration of the angiotensin II AT(1) receptor antagonist candesartan blocks brain AT(1) receptors. Brain Res 871: 29-38.

Olson ML, Olson EA, Qualls JH, Stratton JJ, Harding HW, et al. 2004. Norleucine[1]-angiotensin IV alleviates mecamylamine-induced spatial memory deficits. Peptides 25: 233-241.

Osei SY, Ahima RS, Minkes RK, Weaver JP, Khosla MC, et al. 1993. Differential responses to angiotensin (1–7) in the feline mesenteric and hindquarters vascular beds. Eur J Pharmacol 234: 35-42.

Overmier JB, Murison R. 2000. Anxiety and helplessness in the face of stress predisposes, precipitates, and sustains gastric ulceration. Brain Res 110: 161-174.

Page IH, Helmer OM. 1940. A crystalline pressor substance (angiotonin) resulting from the action between renin and renin-activator. J Exp Med 71: 29-42.

Paula RD, Lima CV, Khosla MC. 1995. Angiotensin (1–7) potentiates hypotensive effect of bradykinin in conscious rats. Hypertension 26: 1154-1159.

Pederson ES, Harding JW, Wright JW. 1998. Attenuation of scopolamine-induced spatial learning impairments by an angiotensin IV analog. Regul Pept 74: 97-103.

Pederson ED, Krishnan R, Harding JW, Wright JW. 2001. A role for the angiotensin $AT_4$ receptor subtype in overcoming scopolamine-induced spatial memory deficits. Regul Pept 102: 147-156.

Peng J, Phillips MI. 2001. Opposite regulation of brain angiotensin type 1 and type 2 receptors in cold-induced hypertension. Regul Pept 97: 91-102.

Phillips MI. 1987. Functions of angiotensin in the central nervous system. Annu Rev Physiol 49: 413-435.

Phillips MI, Sumners C. 1998. Angiotensin II in central nervous system physiology. Regul Pept 78: 1-11.

Phillips MI, Weyhenmeyer JA, Felix D, Ganten D. 1979. Evidence for an endogenous brain renin–angiotensin system. Fed Proc 38 (7): 2260-2266.

Przegalinski E, Siwanowicz J, Baran L. 1988. Effect of repeated administration of antidepressant drugs on the isoprenaline-induced drinking in rats. Pol J Pharmacol 40: 251-258.

Raizada MK, editor. 1995. Current concepts: tissue renin angiotensin systems as local regulators in reproductive and endocrine organs. Plenum Press: New York.

Rich DH, Moon BJ, Harbeson S. 1984. Inhibition of aminopeptidases by amastatin and bestatin derivatives, effect of inhibitor structure on slow-binding processes. J Med Chem 27: 417-422.

Richter CP. 1936. Increased salt appetite in adrenalectomized rats. Am J Physiol 115: 155-161.

Roberts KA, Krebs LT, Kramár, EA, Shaffer MJ, Harding JW, et al. 1995. Autoradiographic identification of brain angiotensin IV binding sites and differential c-Fos expression following intracerebroventricular injection of angiotensin II and IV in rats. Brain Res 682: 13-21.

Rolls BJ, Jones BP, Fallows DJ. 1972. A comparison of the motivational properties of thirst. Physiol Behav 9: 777-782.

Ryu J, Hah JS, Park JS, Lee W, Rampal AL, et al. 2002. Protein kinase C-zeta phosphorylates insulin-responsive aminopeptidase in vitro at Ser-80 and Ser-91. Arch Biochem Biophys 403: 71-82.

Saavedra JM. 1992. Brain and pituitary angiotensin. Endocr Rev 13: 329-380.

Saavedra JM. 1999. Emerging features of brain angiotensin receptors. Regul Pept 85: 31-45.

Sadamura H, Hein L, Krieger JE, Pratt RE, Kobilka BK, et al. 1992. Cloning, characterization, and expression of two angiotensin receptor (AT-1) isoforms from the mouse genome. Biochem Biophys Res Commun 185: 253-259.

Sakai RR, Epstein AN. 1990. Dependence of adrenalectomy-induced sodium appetite on the action of angiotensin II in the brain of the rat. Behav Neurosci 104: 167-176.

Sandberg K, Ji H, Catt KJ. 1994. Regulation of angiotensin II receptors in rat brain during dietary sodium changes. Hypertension 23: I-137-I-141.

Santos RA, Campagnole-Santos MJ, Andrade SP. 2000. Angiotensin(1–7): an update. Regul Pept 91: 45-62.

Santos RA, Campagnole-Santos MJ, Baracho NC, Fontes MA, Silva LC, et al. 1994. Characterization of a new angiotensin antagonist selective for angiotensin-(1–7): evidence that the actions of angiotensin-(1–7) are mediated by specific angiotensin receptors. Brain Res Bull 35: 293-298.

Sardinia MF, Hanesworth JM, Krebs LT, Harding JW. 1993. $AT_4$ receptor binding characteristics: D-amino acid- and glycine-substituted peptides. Peptides 14: 949-954.

Sasaki K, Yamano Y, Bardhan S, Iwai N, Murray JJ, et al. 1991. Cloning and expression of a complementary DNA encoding a bovine adrenal angiotensin II type-1 receptor. Nature (Lond) 351: 230-233.

Sayeski PP, Ali MS, Semeniuk DJ, Doan TN, Bernstein KE. 1998. Angiotensin II signal transduction pathways. Regul Pept 78: 19-29.

Schiavone MT, Santos RAS, Brosnihan KB, Khosla MC, Ferrario CM. 1988. Release of vasopressin from the rat hypothalamo-neurohypophysial system by angiotensin (1–7) heptapeptide. Proc Natl Acad Sci USA 85: 4095-4098.

Silva-Barcellos NM, Frezard F, Caligiorne S, Santos RA. 2001. Long-lasting cardiovascular effects of liposome-entrapped angiotensin (1–7) at the rostral ventrolateral medulla. Hypertension 38: 1266-1271.

Simpson JB, Routtenberg A. 1973. Subfornical organ: site of drinking elicitation by angiotensin II. Science 181: 1172-1175.

Sirrett NE, McLean AS, Bray JJ, Hubbard JL. 1977. Distribution of angiotensin II receptors in rat brain. Brain Res 122: 299-312.

Skeggs LT, Kahn JR, Lentz KE, Shumway NP. 1957. The preparation, purification and amino acid sequence of polypeptide renin substrate. J Exp Med 106: 439-453.

Slinker BK, Wu Y, Brennan AJ, Campbell KB, Harding JW. 1999. Angiotensin IV has mixed effects on left ventricle systolic function and speeds relaxation. Cardiovasc Res 42: 660-669.

Song L, Wilk S, Healy DP. 1997. Aminopeptidase A antiserum inhibits intracerebroventricular angiotensin II-induced dipsogenic and pressor responses. Brain Res 744: 1-6.

Speth RC, Brown TE, Barnes RD, Wright JW. 2003. Brain angiotensinergic activity: the state of our current knowledge. Proc West Pharmacol Soc 46: 11-15.

Speth RC, Thompson SM, Johns SJ. 1995. Angiotensin II receptors: structural and functional considerations. Current concepts: tissue rennin angiotensin systems as local regulators in reproductive and endocrine organs. Mukhopadhyay AK, Raizada MK, editors. New York: Plenum Press; pp. 169-192.

Stubley-Weatherly LA, Harding JW, Wright JW. 1996. Effects of discrete kainic acid-induced hippocampal lesions on spatial and contextual learning and memory. Brain Res 716: 29-38.

Summy-Long JY, Keil LC, Sells G, Kirby A, Ohee O, et al. 1983. Cerebroventricular sites for enkephalin inhibition of the central actions of angiotensin. Am J Physiol 244: R522-R529.

Swanson GN, Hanesworth JM, Sardinia MF, Coleman JK, Wright JW, et al. 1992. Discovery of a distinct binding site for angiotensin II (3–8), a putative angiotensin IV receptor. Regul Pept 40: 409-419.

Tallant EA, Lu X, Weiss RB, Chappell MC, Ferrario CM. 1997. Bovine aortic endothelial cells contain an angiotensin-(1–7) receptor. Hypertension 29 (Pt 2): 388-393.

Thomas WG, Mendelsohn FAO. 2003. Molecules in focus: angiotensin receptors: form and function and distribution. Int J Biochem Cell Biol 35: 774-779.

Tiegerstedt R, Bergman PG. 1898. Niere und kreislauf. Skand Arch Physiol 8: 223-231.

Tonnaer JA, Wiegant VM, DeJong W, DeWied, D. 1982. Central effects of angiotensin on drinking and blood pressure; structure–activity relationships. Brain Res 236: 417-428.

Tsujimoto M, Mizutani S, Adachi H, Kimura M, Nakazato H, et al. 1992. Identification of human placental leucine aminopeptidase as oxytocinase. Arch Biochem Biophys 292: 388-392.

Tuncel N, Erkasap N, Sahinturk V, Ak DD, Tuncel M. 1998. The protective effect of vasoactive intestinal peptide (VIP) on stress-induced gastric ulceration in rats. Ann N Y Acad Sci 865: 309-322.

Ueda S, Masumori-Maemoto S, Ashino K, Nagohara T, Gotoh E, et al. 2000. Angiotensin (1–7) attenuates vasoconstriction evoked by angiotensin II but not by noradrenaline in man. Hypertension 35: 998-1001.

Unger T, Badoer E, Ganten D, Lang RE, Rettig R. 1988. Brain angiotensin: pathways and pharmacology. Circulation 77: 140-154.

Unger T, Becker H, Petty M, Demmert G, Schneider B, et al. 1985. Differential effects of central angiotensin II and substance P on sympathetic nerve activity in conscious rats. Implications for cardiovascular adaptation to behavioral responses. Circ Res 56: 563-575.

Unger T, Horst PJ, Bauer M, Demmert G, Rettig R, et al. 1989. Natriuretic action of central angiotensin II in conscious rats. Brain Res 486: 33-38.

Vaughan DE, Lazos SA, Tong K. 1995. Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. A potential link between the renin–angiotensin system and thrombosis. J Clin Invest 95: 995-1001.

Vauquelin G, Michotte Y, Smolders I, Sarre S, Ebinger G, et al. 2002. Cellular targets for angiotensin II fragments: pharmacological and molecular evidence. JRAAS 3: 195-204.

Waters SB, D'Auria M, Martin SS, Nguyen C, Kozma LM, et al. 1997. The amino terminus of insulin-responsive aminopeptidase causes Glut4 translocation in 3T3-L1 adipocytes. J Biol Chem 272: 23323-23327.

Wayner MJ, Armstrong DL, Phelix CF, Wright JW, Harding JW. 2001. Angiotensin IV enhances LTP in rat dentate gyrus in vivo. Peptides 22: 1403-1414.

Weiss MI, Moe KE, Epstein AN. 1986. Interference with central action of angiotensin II suppresses sodium appetite. Am J Physiol 250: R250-R259.

Whitebread S, Mele M, Kamber B, deGasparo M. 1989. Preliminary biochemical characterization of two angiotensin II receptor subtypes. Biochem Biophys Res Commun 163: 284-288.

Wilk S, Healy DP. 1993. Glutamyl aminopeptidase (aminopeptidase A), the BP-1/6C3 antigen. Adv Neuroimmunol 3: 195-207.

Wilson WL, Roques BP Llorens-Coxtes C, Speth RC, Harding JW, et al. 2005. Roles of brain angiotensins II and III in thirst and sodium appetite. Brain Res 1060: 108-117.

Wong PC, Price WA, Chiu AT, Duncia JV, Carini DJ, et al. 1990. Non-peptide angiotensin II receptor antagonists. IX. Antihypertensive activity in rats of DuP 753, an orally active antihypertensive agent. J Pharmacol Exp Ther 252: 726-732.

Wright JW, Harding JW. 1992. Regulatory role of brain angiotensins in the control of physiological and behavioral responses. Brain Res Rev 17: 227-262.

Wright JW, Harding JW. 1994. Brain angiotensin receptor subtypes in the control of physiological and behavioral responses. Neurosci Biobehav Rev 18: 21-53.

Wright JW, Harding JW. 1995. Brain angiotensin receptor subtypes $AT_1$, $AT_2$, and $AT_4$ and their functions. Regul Pept 59: 269-295.

Wright JW, Harding JW. 1997. Important roles for angiotensin III and IV in the brain renin–angiotensin system. Brain Res Rev 25: 96-124.

Wright JW, Harding JW. 2004. The brain angiotensin system and extracellular matrix molecules in neural plasticity, learning, and memory. Prog Neurobiol 72: 263-293.

Wright JW, Jensen LL, Roberts KA, Sardinia MF, Harding JW. 1989. Structure–function analyses of brain angiotensin control of pressor action in rats. Am J Physiol 257: R1551-R1557.

Wright JW, Kramar EA, Meighan SE, Harding JW. 2002a. Extracellular matrix molecules, long-term potentiation, memory consolidation and the brain angiotensin system. Peptides 23: 221-246.

Wright JW, Krebs LT, Stobb JW, Harding JW. 1995. The angiotensin IV system: functional implications. Front Neuroendocrinol 16: 23-52.

Wright JW, Miller-Wing, AV, Shaffer MJ, Higginson C, Wright DE, et al. 1993. Angiotensin II(3-8) [ANG IV] hippocampal binding: potential role in the facilitation of memory. Brain Res Bull 32: 497-502.

Wright JW, Morseth SL, Abhold RH, Harding JW. 1985. Pressor action and dipsogenicity induced by angiotensin II and III in rats. Am J Physiol 249: R514-R521.

Wright JW, Stubley L, Pederson ES, Kramar EA, Hanesworth JM, et al. 1999. Contributions of the brain angiotensin IV-$AT_4$ receptor subtype system to spatial learning. J Neurosci 19: 3952-3961.

Wright JW, Tamura-Myers E, Wilson WL, Roques BP, Llorens-Cortes C, et al. 2002b. Conversion of brain angiotensin II to angiotensin III is critical for pressor response in rats. Am J Physiol Regul Integr Comp Physiol 284: R725-R733.

Yang G, Wan Y, Zhu Y. 1996. Angiotensin II-an important stress hormone. Biol Signals 5: 1-8.

Zhang JH, Hanesworth JM, Sardinia M, Alt JA, Wright JW, et al. 1999. Structural analysis of angiotensin IV receptor ($AT_4$) from selected bovine tissues. J Pharmacol Exp Ther 289: 1075-1083.

Zhu B, Herbert J. 1996. Central antagonism of atrial natriuretic peptides on behavioral and hormonal responses to angiotensin II: mapping with c-Fos. Brain Res 734: 55-60.

Zini S, Fournie-Zaluski MC, Chauvel E, Roques BP, Corvol P, et al. 1996. Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. Proc Natl Acad Sci USA 93: 11968-11973.

Zubenko GS, Nixon RA. 1984. Mood elevating effect of captopril in depressed patients. Am J Psychiatry 141: 110-111.

# 28 Leptin in Brain Function

*J. Harvey*

**Abstract:** In the 21st century obesity affects around 25% of the population and is now one of the main contributors to ill health in Western societies. In recent years major advances have been made in identifying the key hormones that regulate food intake and body weight. In particular the cloning of the ob gene that encodes the endocrine peptide, leptin, has fueled a great deal of research into this area, and has established that this hormone plays a pivotal role in regulating energy balance via its actions in the hypothalamus. This in turn has greatly enhanced our understanding of the highly intricate neural systems within the hypothalamus that regulate energy homeostasis, and the complex interplay that exists between a range of orexigenic and anorexigenic agents (like leptin) to control this process. However, in addition to its fundamental role in regulating food intake and body weight, evidence is emerging that hypothalamic leptin receptors also play a key role in controlling reproductive function and bone formation. Moreover evidence is mounting that in addition to its hypothalamic actions, leptin has widespread actions in the CNS. Indeed in the hippocampus leptin is implicated in associative learning and memory processes as leptin-receptor-deficient rodents (*db/db* mice and *fa/fa* rats) display impairments in both long-term potentiation (LTP) and long-term depression (LTD), and in spatial memory tasks. Furthermore, at the cellular level, leptin has the capacity to convert hippocampal short-term potentiation (STP) into LTP. There is also evidence that leptin, via its ability to modify the activity of specific potassium channels in the hippocampus, can regulate neuronal excitability. More recent studies indicate that the hormone leptin plays a key role in the development of the CNS. Indeed abnormal brain development has been reported in leptin-deficient or insensitive rodents, and recent studies indicate that leptin actively participates in the development of the hypothalamus as specific arcuate nucleus projection pathways are permanently disrupted in *ob/ob* (leptin-deficient) mice. In this chapter the recent advances made in leptin neurobiology are discussed. In particular the key role of leptin in regulating energy balance is addressed, together with the emerging evidence that this hormone play a fundamental role in numerous other CNS functions.

**List of Abbreviations:** α-MSH, α melanocortin-stimulating hormone; ARC, arcuate nucleus; CART, cocaine- and amphetamine-regulated transcript; CIS, cytokine inducible sequence; CNS, central nervous system; *db*, diabetes; IRS, insulin receptor substrate; JAK, janus tyrosine kinase; $K_{ATP}$, ATP-sensitive potassium channel; MAPK, mitogen-activated protein kinase; NPY, neuropeptide Y; *ob*, obese; ObR, leptin receptor; PI-3 kinase, phosphoinositide-3 kinase; POMC, proopiomelanocortin; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription; VMH, ventromedial hypothalamus

## 1 Introduction

It is now well established that a physiological system exists that homeostatically regulates body weight. In 1953, Kennedy was one of the first to propose that the amount of energy stored in adipose mass is a balance between calorie intake and energy expenditure (Kennedy, 1953). In his adipostatic model of body weight regulation he envisaged that adipose mass was maintained at a set point by a homeostatic mechanism, such that changes in food intake and energy expenditure occurred in response to changes in energy stores. In later studies, Hervey (1958) formulated the idea of a circulating satiety factor, as in parabiosis experiments between obese rats (with lesions in to ventral medial hypothalamus; VMH) and control rats, and demonstrated that the lean animals died from starvation. The concept of a circulating satiety factor was further supported by the discovery of natural recessive mutations in the obese (*ob*) and diabetes (*db*) genes, which resulted in hyperphagia, reduced energy expenditure, and obesity in mice (Ingalls et al., 1950). Subsequent parabiosis experiments between wild-type mice and either *ob/ob* or *db/db* mice (Hausberger, 1959; Coleman and Hummel, 1969; Coleman, 1973) suggested that the circulating satiety factor was encoded by the *ob* locus, whereas the *db* locus was necessary for the response to this factor. The cloning of the *ob* and *db* genes (Zhang et al., 1994) confirmed the role for these genetic loci in energy homeostasis proposed by Coleman. The product of the *ob* gene was termed leptin (from the Greek, "leptos" meaning thin) as injection of leptin into leptin-deficient or normal mice resulted in a marked reduction in food intake and body weight (Halaas et al., 1995).

## 2 Leptin

The ob gene, which was first identified from mice by positional cloning techniques, encodes a highly conserved 167-amino-acid protein (Zhang et al., 1994). Leptin is synthesized predominantly, although not exclusively, by white adipose tissue, and it circulates in the plasma at levels proportional to body adiposity (Maffei et al., 1995; Considine et al., 1996). The obese gene product displays a high degree of homology among different species. Leptin is also very similar in structure to other cytokines (Madej et al., 1995), and it contains an intrachain disulphide bond that is required for biological activity (Grasso et al., 1997).

### 2.1 Mutations in the ob Gene

In mice, mutations in the ob gene are known to cause early-onset obesity (Zhang et al., 1994; Campfield et al., 1995). In C57B1/6J *ob/ob* mice, substitution of a cysteine residue with a threonine residue in the ob gene results in the synthesis of a truncated leptin protein that is not secreted (Zhang et al., 1994; Rau et al., 1999). In another leptin-deficient mouse strain ($ob^{2J}/ob^{2J}$), the synthesis of ob mRNA is prevented by the insertion of a transposon in the first intron of the ob gene (Zhang et al., 1994). Both these mutant mice display, deficiencies in leptin, morbid obesity, hypothermia, and hyperphagia.

In humans, mutations in the ob gene are extremely rare. Two children from a Pakistani family were the first reported humans with morbid obesity due to a specific mutation in the ob gene (Montague et al., 1997). In these cases, a truncated form of leptin that is targeted for degradation by proteosomes was synthesized following deletion of a single guanine nucleotide in codon 133 (Rau et al., 1999). Three members of a Turkish family have also been identified with morbid obesity associated with genetic abnormalities in the ob gene. In these individuals a missense mutation in codon 105 had occurred that encodes an abnormal form of leptin that cannot be secreted (Strobel et al., 1998). The rarity of such genetic abnormalities in humans suggests that mutations in the ob gene are unlikely to underlie the resistance to leptin associated with most obese humans.

### 2.2 Sites of Leptin Expression

Initially it was thought that adipose tissue was the only site of leptin expression. However there is now evidence that leptin is widely expressed in numerous extraadipose tissues including skeletal muscle, gastric fundic mucosa, placenta, and mammary epithelium (Casabiell et al., 1997; Masuzaki et al., 1997; Bado et al., 1998; Wang et al., 1998). In these peripheral tissues, the expression of leptin is also influenced by a range of external factors. For instance, in placenta, leptin expression is stimulated by glucocorticoids, insulin, and hypoxia (Mise et al., 1998; Shekhawat et al., 1998). The synthesis of leptin in the gastric fundus is attenuated by feeding, or administration of gastrin, or cholecystokinin (Bado et al., 1998). Infusion of glucose and lipids induces de novo synthesis of leptin in rat skeletal muscle, raising the possibility that leptin has the ability to sense nutrient influx in skeletal muscle and adipose tissue.

In the CNS, leptin mRNA, ob protein, and leptin immunoreactivity are all expressed in various brain regions including hypothalamus, hippocampus, cortex, and cerebellum (Morash et al., 1999; Ur et al., 2002). As well as displaying differential distribution throughout the CNS, the subcellular localization of leptin labeling also varies between neuronal populations. For example in the dentate gyrus region of the hippocampus, leptin labeling is associated with nuclear and perinuclear regions, whereas in the CA2/CA3 region labeling is only evident in the nucleus (Ur et al., 2002). The possibility that leptin may be released and made from specific neuronal populations is demonstrated by localization of leptin labeling to specific subpopulations of neurons. Indeed in the supraoptic nucleus and paraventricular nucleus (PVN), leptin labeling is confined to oxytocin- and vasopressin-containing neurons.

## 2.3 What Regulates Leptin Expression?

### 2.3.1 Status of Energy Stores

Leptin expression is influenced by the status of energy stores as the levels of adipose tissue ob mRNA and serum leptin are elevated in obese animals and humans (Frederich et al., 1995; Hamilton et al., 1995; Maffei et al., 1995). Indeed, fasting in rodents and in humans can attenuate the circulating levels of leptin within hours. In rodents leptin levels also increase within hours after food consumption, whereas in humans alterations in the levels of leptin are only apparent after several days of overeating (Saladin et al., 1995; Kolacznyski et al., 1996). As leptin levels are not enhanced in response to a single meal in humans, leptin is unlikely to act as a satiety signal in a meal-dependent manner. Thus the nutritional regulation of leptin expression is also likely to be mediated, at least in part, by insulin. Indeed, during feeding leptin levels increase after insulin secretion has peaked (Sinha et al., 1996). Insulin is also reported to directly stimulate leptin expression in adipocytes (Rentsch and Chiesi, 1996). In a rodent model of diabetes (streptozotocin-induced) where insulin levels are low, there is also a correlated depression in leptin levels (MacDougald et al., 1995).

### 2.3.2 Hormonal Regulation of Leptin Expression

Leptin expression, in addition to being constitutively regulated, can also be influenced by numerous other hormonal systems. In primary cultures of adipocytes, glucocorticoids directly stimulate synthesis of leptin (De Vos et al., 1995; Murakami et al., 1995; Slieker et al., 1996), whereas in humans, the chronic rise in cortisol levels that occurs in Cushing's syndrome patients is associated with an elevation in leptin expression (Cizza et al., 1997; Leal-Cerro et al., 2001). Interestingly, the plasma levels of leptin and cortisol display an inverse circadian rhythm such that peak glucocorticoid levels occur when leptin levels are low at the start of the light cycle in humans, whereas at night when glucocorticoid levels dip, leptin levels peak (Ahima et al., 1996; Laughlin and Yen, 1997; Licinio et al., 1997).

Leptin levels are higher in prepubertal rodents and prepubertal boys, but the levels of leptin do not appear to be dependent on triglyceride levels or adipose mass (Mantzoros et al., 1997; Ahima et al., 1998). This prepubertal rise in leptin levels occurs prior to increases in testosterone and estradiol, and it has been postulated that this process is involved in the maturation of the gonadal axis (Mantzoros et al., 1997; Ahima et al., 1998). Sex differences also exist in the circulating levels of leptin such that females have higher levels than males when matched for body weight, age, or body fat (Saad et al., 1997). This may be due to differences in the distribution of body fat and the levels of testosterone. Indeed females have higher levels of adipose tissue compared with males, and leptin synthesis is inhibited by testosterone, but is not affected by ovarian sex hormones (Blum et al., 1997; Castracane et al., 1998).

### 2.3.3 Infection and Inflammation

Leptin synthesis can be stimulated by infection, endotoxins, and cytokines, including tumor necrosis factor (TNF) (Sarraf et al., 1997; Bullo et al., 2002), IL-1 (Janik et al., 1997), and leukemia inhibitory factor (Sarraf et al., 1997). The rise in leptin levels in response to these cytokines has been linked to the anorexia and weight loss that develops in inflammatory conditions (Sarraf et al., 1997; Lennie et al., 2001). A number of agents can also influence the leptin levels by means of directly affecting the ob gene. For instance thiazolidinediones, such as troglitazone, and catecholamines reduce the expression of leptin by binding to specific nuclear transcription factor binding sites on the ob gene promoter (Gong et al., 1996; Hwang et al., 1996).

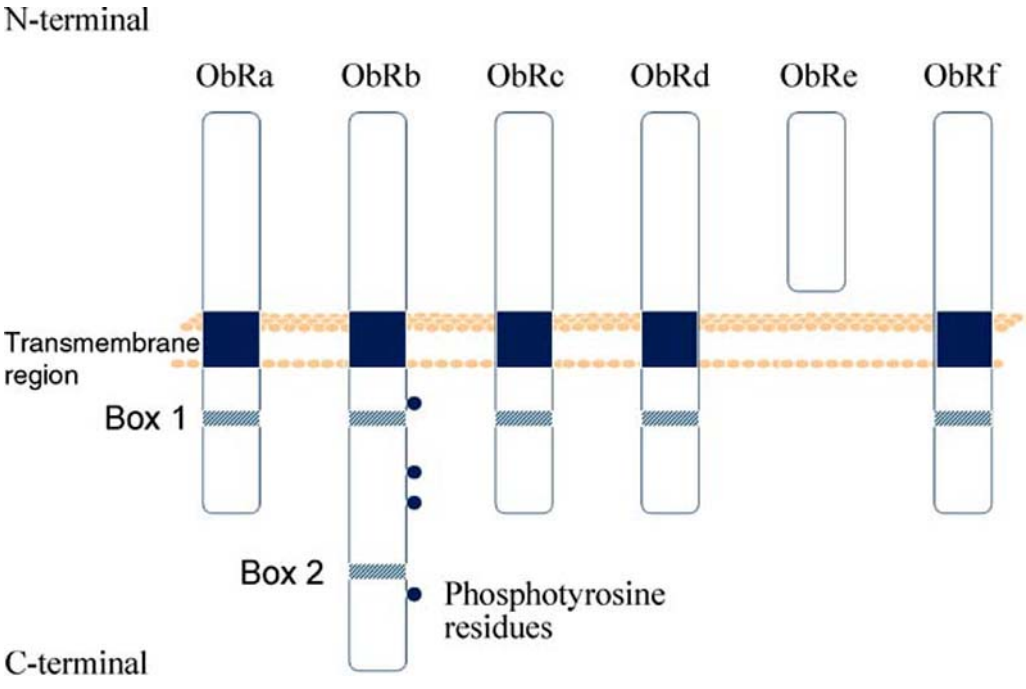
## 3 Leptin Receptor

The leptin receptor (ObR) was first isolated from the choroid plexus by expression cloning strategies (Tartaglia et al., 1995). The diabetes (*db*) gene encodes ObR and at least six receptor isoforms (termed

$ObR_{a–f}$), generated by alternate splicing of the *db* gene, have been identified to date (Lee et al., 1996; Wang et al., 1998). The leptin receptor isoforms have identical N-terminal extracellular ligand-binding domains, but distinct C-terminal intracellular domains. All the isoforms, with the exception of $ObR_e$, are membrane-spanning receptors that have either short- (approximately 30–40 residues) or long- (302 residues) intracellular domains (see ❯ *Figure 28-1*). The extended cytoplasmic domain of the long isoform ($ObR_b$) contains

**Figure 28-1**

**Schematic representation of the structure of the six leptin receptor isoforms (ObR). ObRa,c,d, and f are the short isoforms that have short intracellular domains and limited signaling capacity. ObRb is the long form of the receptor and it has a long intracellular domain that can initiate signaling cascades. ObRe is distinct from the other isoforms as it has no transmembrane domain**




various motifs required for the initiation of signaling cascades following leptin receptor activation. Thus, $ObR_b$ is the main signaling-competent isoform, and it plays a crucial role in the regulation of the obese state as insertion of a premature stop codon in the cytoplasmic domain of the $ObR_b$ mRNA transcript results in an obese phenotype that is comparable to *db/db* mice (Chen et al., 1996). In contrast, the short isoforms ($ObR_{a,c,d,f}$) with their smaller cytoplasmic region, have limited ability to signal. However, there is evidence that in hepatocytes that lack $ObR_b$, leptin antagonizes glucagon-induced cAMP accumulation (Zhao et al., 2000). $ObR_a$ can also stimulate the Ras–MAPK signaling pathway in CHO cells (Yamashita et al., 1998) and HEK293 cells (Bjorbaek et al., 1998). $ObR_e$ is a distinct isoform as it lacks a transmembrane domain (Lee et al., 1997), and it has been proposed to act as a soluble receptor as it is the major site of leptin binding in the plasma.

## 3.1 Leptin Receptor Mutations

In rodents, leptin receptor mutations result in early onset obesity (Chua et al., 1996; Takaya et al., 1996a, b; White et al., 1997). In *db/db* mice (C57B1/K strain) a truncated form of $ObR_b$, that is incapable of

stimulating JAK-STAT signaling, is produced by insertion of a stop codon at the 3′-end of the $ObR_b$ mRNA transcript (Ghilardi et al., 1996; Vaisse et al., 1996). In contrast the other splice variants are expressed normally in *db/db* mice. Mutations in the db gene cause, insensitivity to leptin, hyperphagia, morbid obesity, and various neuroendocrine abnormalities (Campfield et al., 1995; Halaas et al., 1995). In *fa/fa* (fatty) rats, a single point mutation in the extracellular domain of all leptin receptor isoforms occurs, which results in attenuation of the affinity of the receptor for leptin as well as the receptor-driven signal transduction capacity (Da Silva et al., 1998). Obese Koletsky rats have a point mutation at amino acid 763, which results in a stop codon in the extracellular domain and failure of expression of all leptin receptor isoforms (Takaya et al., 1996a, b; Wu-Peng et al., 1997).

In humans, mutations in the leptin receptor have been identified, but these are extremely rare. Three sisters from a Kabilian family were the first reported human cases with mutations in the leptin receptor (Clement et al., 1998). These patients produced truncated leptin receptors lacking both transmembrane and intracellular domains due to a point mutation in the splice donor site of exon 16. Like mutations in the human ob gene, mutations in the db gene cause hyperphagia, early onset obesity, and hypothalamic hypogonadism. However unlike *db/db* mice, humans with this mutation do not develop hyperglycemia, hypercorticism, and hypothermia (Bray and York, 1979; Clement et al., 1998).

## 3.2 Leptin Receptor Expression in the CNS

### 3.2.1 Hypothalamus

In the CNS, the main target for leptin with respect to regulating food intake and body weight is the hypothalamus. Several hypothalamic nuclei including the ventromedial hypothalamus (VMH), arcuate nucleus (ARC), and dorsomedial hypothalamus (DMN) in particular express high levels of leptin receptor mRNA and protein in rodents (Schwartz et al., 1996b; Hakansson et al., 1996, 1998; Elmquist et al., 1998). High levels of leptin receptor mRNA have also been detected in human hypothalamus (Savioz et al., 1997; Burguera et al., 2000). In the hypothalamus leptin receptor expression is influenced by the circulating plasma levels of leptin, as an increase in $ObR_b$ is evident in leptin-deficient rodents (*ob/ob* mice) or fasted rats (Baskin et al., 1998; Lin et al., 2000).

The ARC is particularly enriched with leptin receptors, which correlates well with the role this nucleus plays in converting peripheral signals into neuronal responses (Dawson et al., 1997; Tang-Christensen et al., 1999). Two neuronal populations are critical targets for leptin in the ARC: an orexigenic pathway comprising neuropeptide Y (NPY)- and agouti-related protein (AGRP)-containing neurons and an anorexigenic pathway consisting of proopiomelanocortin (POMC) and cocaine-and amphetamine-regulated transcript (CART)-containing neurons. Leptin-deficient (*ob/ob*) or leptin-insensitive (*db/db*) mice, or fasted rats have elevated levels of NPY/AGRP mRNA and attenuated levels of POMC/CART mRNA (Ahima, 2000). Moreover, administration of leptin to either fasted rats or leptin-deficient mice alleviates these changes (Ahima, 2000). In response to circulating levels of leptin (and other hormones), neurons within the ARC subsequently innervate various second-order neuron centers where further integration of adiposity/satiety signaling occurs. In turn, outputs from these second-order centers descend through hindbrain regions where there is further integration prior to output to spinal neurons and peripheral organs.

### 3.2.2 Extrahypothalamic Brain Regions

In addition to the hypothalamus, high levels of leptin receptor immunoreactivity and $ObR_b$ mRNA have been detected in a number of brain regions that are not generally associated with energy homeostasis, including the hippocampus, thalamus, brain stem, cerebellum, olfactory tract, pyriform cortex, and substantia nigra (Mercer et al., 1996; Elmquist et al., 1998; Hakansson et al., 1998; Baskin et al., 1999). In the hippocampus in particular, the CA1/CA3 regions and the dentate gyrus display widespread expression of leptin receptor mRNA (Huang et al., 1996; Mercer et al., 1996) and leptin receptor immunoreactivity

(Hakansson et al., 1998). Furthermore, as in the hypothalamus, the expression of leptin receptor mRNA is altered in fasted rodents relative to control animals (Lin et al., 1997). In primary neuronal cultures prepared from the CA1/CA3 regions of the hippocampus, leptin receptor immunolabeling is evident on both neurons and glial cells (Shanley et al., 2002b). In dual-labeling studies leptin receptor staining is also associated with somatodendritic regions, axonal processes, and points of synaptic contact (Shanley et al., 2002a, b).

The expression of leptin receptors in many extrahypothalamic brain regions suggests that this hormone, in addition to its role in regulating energy homoeostasis, may play a more fundamental modulatory role in the CNS. In support of this possibility, recent evidence indicates that leptin mRNA, ob protein, and leptin immunoreactivity are all widely expressed in a number of brain regions such as the hippocampus, hypothalamus, and cerebellum (Morash et al., 1999; Ur et al., 2002). This lends support to the possibility that leptin may actually be made and released locally within the CNS. It is not yet known whether centrally derived leptin fulfills the criteria of being either a neurotransmitter or cotransmitter in the brain. However, as circulating leptin can be transported from the plasma, across the blood–brain barrier to all regions of the brain (Banks et al., 2000), leptin originating from peripheral tissues may still be able to function in the brain. Indeed intraperitoneal administration of leptin influences glucocorticoid expression in the hippocampus (Proulx et al., 2000).

## 3.3 Leptin-Receptor-Driven Signal Transduction

ObR shows greatest homology to the class I cytokine receptors (Ihle, 1995); a family of proteins that includes IL-6, leukemia-inhibitory factor, and granulocyte colony- stimulating factor receptors. In a manner similar to other cytokines, binding of leptin to ObR results in the activation of janus tyrosine kinases (JAKs). Numerous studies have demonstrated that JAK2 is preferentially activated during leptin receptor signal transduction (Baumann et al., 1996; Bjorbaek, 1997; Ghilardi, 1997), although there is evidence for signaling via JAK1 (Bjorbaek, 1997). Following leptin binding, JAK2 associates with specific domains within the cytoplasmic (C-terminal) region of the leptin receptor, resulting in *trans*-phosphorylation of JAK2 and subsequent phosphorylation of tyrosine residues on the receptor. These events in turn act as a switch to recruit and activate various downstream signaling pathways, including the STAT (signal transducers and activators of transcription) family of transcription factors, insulin receptor substrate (IRS) proteins, phosphoinositide 3-kinase (PI-3 kinase), and Ras–Raf–MAPK (see ❯ *Figure 28-2*).

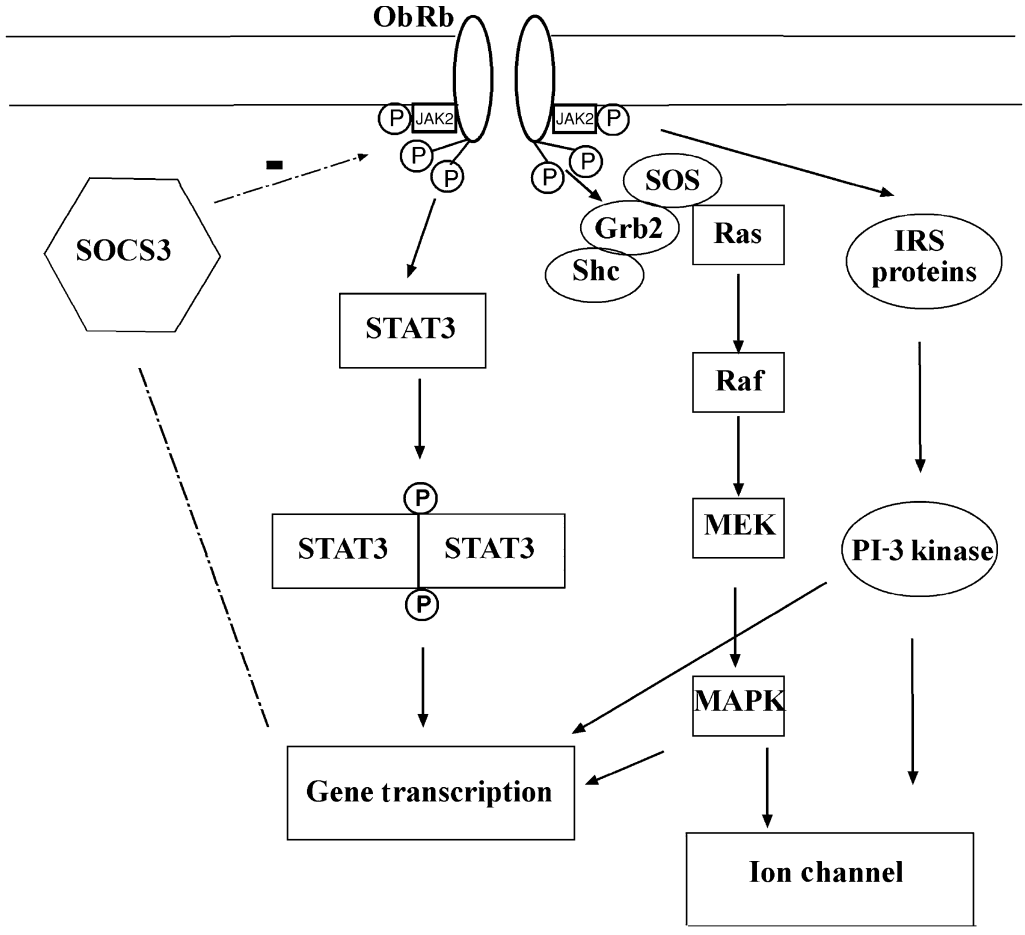
Initially it was thought that the long form of the leptin receptor ($ObR_b$) was the only signaling-competent isoform due to the various motifs required for signaling that are expressed within its long intracellular domain. This suggestion was reinforced by studies performed by Bjorbaek et al. (1998) who demonstrated that the short isoforms are unable to undergo tyrosine phosphorylation. However the short forms of the receptor may have the capacity to signal in some cell types, as activation of recombinant $ObR_a$ can stimulate the MAPK-signaling cascade in CHO cells (Yamashita et al., 1998), whereas in hepatocytes that do not express ObRb, leptin antagonizes the effects of glucagon on cAMP levels (Zhao et al., 2000).

### 3.3.1 The STAT Family of Transcription Factors

Only $ObR_b$ contains the various signaling motifs required for activation of the STAT family of transcription factors. One tyrosine residue (Y1138) on $ObR_b$ enables STAT3 binding, which in turn results in STAT3 dimerization and concomitant translocation to the nucleus. Like other cytokines, $ObR_b$ activation leads to tyrosine phosphorylation of STAT1, 3, and 5 in vitro (Carpenter et al., 1998; Li and Friedman, 1999; Morton et al., 1999). In contrast, in mice intravenous administration of leptin results in specific activation of STAT3 in the hypothalamus. This in turn stimulates activation of the immediate early genes, c-fos and c-jun (Bjorbaek et al., 1998). STAT3 signaling appears to be required for leptin regulation of energy balance as gene-targeted disruption of $ObR_b$–STAT3 signaling in mice results hyperphagia and obesity (Bates et al., 2003; Gao et al., 2004).

**Figure 28-2**

**Schematic representation of leptin-receptor-driven signaling. In a manner similar to other class I cytokines, activation of the long form of the leptin receptor promotes stimulation of a number of signaling molecules including signal transducers and activators of transcription 3 (STAT3), mitogen-activated protein kinase (MAPK), and phosphoinositide-3 (PI-3) kinase**




Recently a new family of cytokine-inducible inhibitors of signaling has been identified, including CIS (cytokine inducible sequence) and SOCS 1–3 (suppressor of cytokine signaling). In the hypothalamus (Bjorbaek et al., 1998) and in COS cells (Bjorbaek et al., 1999), leptin can induce expression of SOCS3 mRNA, which may act as an important regulatory mechanism for controlling leptin-receptor-driven signal transduction at the level of transcription (Bjorbaek et al., 2000). Indeed, recent studies indicate that the level of SOCS3 expression is a critical determinant of leptin sensitivity, and thus susceptibility to obesity (Howard et al., 2004).

### 3.3.2 PI-3 Kinase

In addition to promoting changes in gene transcription via activation of the JAK–STAT signaling pathway, leptin can also evoke more rapid responses (within minutes) by stimulating alternative signaling cascades.

In hypothalamic glucose-responsive ARC neurons, leptin activates ATP-sensitive $K^+$ ($K_{ATP}$) channels via rapid activation of a PI-3 kinase-dependent process (Spanswick et al., 1997). Similarly in peripheral insulin-secreting cells the ability of leptin to stimulate $K_{ATP}$ channel activation involves the activation of PI-3 kinase (Harvey et al., 2000b). A key role for this enzyme has been identified in linking leptin receptor activation to reduced food intake, as intracerebral infusion of selective inhibitors of PI-3 kinase prevent the anorectic effects of leptin (Niswender et al., 2001). PI-3 kinase is also a key element of leptin-receptor-driven signaling in hippocampal neurons (Shanley et al., 2001, 2002a, b).

It is well established that one of the main functions of PI-3 kinase is to phosphorylate phosphoinositides on the 3-position, resulting primarily in phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)$P_3$). Numerous studies also indicate a link between the actin cytoskeleton and the lipid products of PI-3 kinase, as the activity of a number of key cytoskeleton proteins is dependent on the levels of either PtdIns(4,5)$P_2$ or PtdIns(3,4,5)$P_3$ (Janmey, 1998). A functional role for the cytoskeleton in the actions of leptin has also been demonstrated as the ability of leptin to stimulate $K_{ATP}$ channels in peripheral cells has been shown to be due to PI-3-kinase-driven alterations in actin dynamics (Harvey et al., 2000a, b). Indeed leptin-induced activation of $K_{ATP}$ channels in CRI-G1 insulin-secreting cells is prevented by the actin filament stabilizer, phalloidin. Moreover, application of leptin to these cells also evokes rapid disassembly of actin filaments, via stimulation of a PI-3-kinase-dependent process. In addition to direct signaling via its lipid products, PI-3 kinase also possesses serine kinase activity, and this enzyme has been shown to interact with AGC serine kinases, Tec tyrosine kinases, and Rho GTPases. In pancreatic beta cells (Zhao et al., 1998) and hepatocytes (Zhao et al., 2000), there is good evidence that leptin activates cyclic nucleotide phosphodiesterase 3B downstream of PI-3 kinase stimulation. This pathway has also been implicated in the hypothalamic actions of leptin on food intake and body weight as intracerebroventricular (ICV) administration of selective inhibitors of cyclic phosphodiesterase 3B prevent the anorexigenic effects of leptin (Zhao et al., 2002).

#### 3.3.3 Ras–Raf–MAPK Signaling Cascade

In addition to activating the PI-3-kinase signaling cascade, leptin can also rapidly stimulate the Ras–Raf–MAPK pathway. Activation of this pathway involves tyrosine phosphorylation of the adaptor protein, Src homology collagen (Shc), which in turn interacts with Grb2, and this subsequently recruits the Son of sevenless (SOS) exchange protein to the plasma membrane to enable activation by Ras. Once activated, Ras acts like a molecular switch by stimulating a serine kinase cascade through the step-wise activation of Raf, MEK, and ERK. Activation of this pathway downstream of leptin receptor activation has been observed in numerous cell lines including MIN6 insulinoma cells, C3H10T1/2 cells, and HEK293 cells expressing recombinant $ObR_b$ (Tanabe et al., 1997; Takahashi et al., 1997; Banks et al., 2000). Leptin also utilizes this signaling cascade in rat preadipocytes and porcine chromaffin cells (Takekoshi et al., 2001; Machinal-Quelin et al., 2002). In neurons there is also evidence that leptin-receptor-driven activation of the MAPK pathway occurs (Shanley et al., 2001; Morikawa et al., 2004).

## 4 Biological Roles of Leptin

### 4.1 Leptin Transport to the Brain and Sites of Action

Leptin enters the brain via a saturable transport system (Banks et al., 1996), possibly via receptor-mediated transcytosis across the blood–brain barrier. In support of such a mechanism, high levels of the short leptin receptor isoforms are expressed on brain microvessels, and which are capable of binding and internalising leptin (Golden et al., 1997; Bjorbaek et al., 1998). As the key leptin target neurons lie in close proximity to the median eminence, and capillaries in the median eminence lack tight junctions, leptin may reach its hypothalamic targets via diffusion. Leptin may also reach the brain via the cerebrospinal fluid (CSF) (Schwartz et al., 1996a, b). Indeed high levels of $ObR_a$ are expressed in the main site of CSF

production, the choroid plexus, which could mediate blood-to-CSF transport of leptin (Bjorbaek et al., 1998).

## 4.2 Role of Leptin in the Hypothalamus

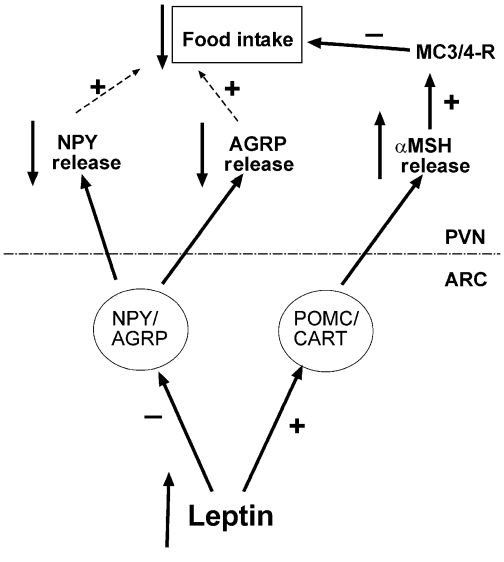
### 4.2.1 Regulation of Food Intake and Body Weight

Within the brain, the hypothalamus is the main site of leptin action with respect to regulating food intake and body weight. Several hypothalamic nuclei are particularly enriched with high levels of leptin receptors including the ARC, VMN, and DMN (Hakansson et al., 1996, 1998; Schwartz et al., 1996a, b; Elmquist et al., 1998). Indeed, intravenous or intraperitoneal injection of leptin results in the activation of all these hypothalamic nuclei (Ahima, 2000). Furthermore, ICV administration of leptin to leptin-deficient *ob/ob* mice and wild-type mice inhibits food intake and attenuates body weight (Elmquist et al., 1999; Ahima and Flier, 2000).

Within the hypothalamus the ARC has been established as the key hypothalamic nucleus that converts peripheral leptin signals into both the neuronal and behavioral responses associated with altering energy homeostasis (Dawson et al., 1997; Tang-Christensen et al., 1999). In the ARC, two neuronal populations are key targets for leptin; an orexigenic pathway consisting of NPY- and AGRP-containing neurons, and an anorexigenic pathway comprising of POMC- and CART- containing neurons. These neurons subsequently innervate various second-order neuronal centres where further integration of satiety/adiposity signaling occurs. These second-order neurons then feed information to hindbrain regions where there is additional integration prior to output to spinal neurons and peripheral organs (see ❯ *Figure 28-3*).

**Figure 28-3**

**Schematic representation of the key leptin-sensitive pathways in the hypothalamus. When leptin levels increase in the arcuate nucleus (ARC), neuropeptide Y and agouti-related protein (NPY/AGRP)-containing neurons are inhibited, which in turn reduces release of these peptides from the paraventricular nucleus (PVN). The resultant reduction in the levels of these orexigenic peptides acts to reduce food intake. An increase in leptin also simultaneously excites proopiomelanocortin and cocaine- and amphetamine-regulated transcript (POMC/CART)-containing neurons in the ARC, which in turn increases release of α-melanocortin-stimulating hormone (αMSH) from the PVN. This stimulates melanocortin receptors (MC3/4-R), which in turn inhibit food intake**



It is now well documented that gene transcriptional changes (mediated by the JAK–STAT signaling pathway) are crucial for the effects of leptin on energy balance. Indeed, targeted disruption of leptin-receptor-driven STAT3 signaling causes obesity in mice (Bates et al., 2003; Gao et al., 2004). More recent studies have also indicated that long lasting alterations in the efficacy of both excitatory and inhibitory synaptic transmission are evident in leptin-deficient rodents, suggesting that the plasticity of specific hypothalamic synapses are also important for the anorexigenic actions of leptin (Pinto et al., 2004). However, it is not clear at this stage whether these leptin-induced changes in the efficacy of synaptic function also involve gene transcriptional changes.

In addition to these long-lasting changes in neuronal function, leptin can evoke acute changes in the properties of ARC neurons, which may also contribute to the anorexigenic actions of leptin. For instance, leptin hyperpolarizes glucose-responsive ARC neurons via activation of $K_{ATP}$ channels (Spanswick et al., 1997), and this effect of leptin is not apparent in Zucker *fa/fa* rats which have dysfunctional leptin receptors. However $K_{ATP}$ channel (Kir $6.2^{-/-}$) knockout mice display no deficits in the ability of leptin to reduce food intake (Miki et al., 2001), indicating that the acute activation of these potassium channels is unlikely to be the only nontranscriptional mechanism underlying leptin-receptor-driven regulation of food intake. Indeed as well as inhibiting glucose-responsive hypothalamic neurons, leptin excites POMC-containing neurons, via a combination of depolarization through activation of a nonselective cation channel and by reducing the release of GABA from NPY-containing neurons (Cowley et al., 2001). Thus at least two distinct populations of hypothalamic neurons are sensitive to leptin: those that are depolarized by leptin with the subsequent release of appetite-reducing hormones such as α-MSH, and those that are hyperpolarized by leptin resulting in a reduction in the release of appetite-stimulating agents, such as NPY and AGRP. Another target for leptin in the regulation of energy homeostasis is AMP-activated protein kinase (AMPK); a kinase that acts like a fuel gauge to monitor the cellular status of energy stores. In the ARC and PVN, leptin inhibits the activity of AMPK, whereas the consitutively active form of AMPK blocks the effects of leptin on food intake and body weight (Minokoshi et al., 2004), suggesting that inhibition of hypothalamic AMPK is necessary for the anorexigenic properties of leptin.

Leptin can also interact with a number of other neurotransmitter systems that are involved in maintaining food intake, including melanocortins (Sahu, 1998), endocannabinoids (Di Marzo et al., 2001), and orexins (Tritos et al., 2001). Thus it is likely that a complex system within the hypothalamus exists whereby a number of effectors are targeted by leptin, and the relative contribution of each of these effector system is crucial for the effects of leptin on energy homeostasis.

### 4.2.2 Role of Leptin Resistance in the Development of Obesity

It is well documented that obesity in humans and many animals is associated with high circulating levels of leptin indicating that a leptin-resistant state rather than leptin deficiency per se is likely to contribute to the development of this disease. One potential cause of leptin resistance is a reduction in the transport of leptin across the blood–brain barrier. In support of this possibility obese humans have lower levels of leptin in the CSF than in the plasma (Caro et al., 1996; Schwartz et al., 1996a, b). Injection of leptin directly into the brain of obese rodents is also more effective at reducing weight than peripheral administration of leptin (Halaas et al., 1997; Van Heek et al., 1997). Moreover the transport rate of leptin across the blood–brain barrier is significantly attenuated in rodent models of obesity compared with lean controls (Banks et al., 1999; Kastin et al., 1999; Burguera et al., 2000; Dube et al., 2000; Banks, 2004), and alterations in the uptake of leptin are evident following obesity-induced lesions of the CNS (Banks et al., 2001), indicating that defective blood-to-brain transport of leptin is likely to be a key factor in producing and reinforcing the leptin-resistant state. Interestingly, this impaired leptin transport can be reversed with even modest weight reduction in rodents (Banks and Farrell, 2003). Recent studies also indicate that triglycerides may be an important cause of leptin resistance as a number of triglycerides, but not their free fatty acid constituents, inhibit transport of leptin across the blood–brain barrier (Banks et al., 2004). It has been postulated that at the cellular level the short isoforms play an important role in leptin transport across the blood–brain barrier as high levels of these isoforms are expressed at the choroid

plexus, and leptin transport is impaired in Koletsky rats that lack the short form of the leptin receptor (Kastin et al., 1999).

Another potential source of leptin resistance is defective or attenuated leptin-receptor-driven signaling in the hypothalamus. Indeed studies on rodents fed a high-fat diet indicate that defects in leptin receptor signaling upstream of STAT3 activation in hypothalamic neurons contributes to leptin resistance (El-Haschimi et al., 2000). Furthermore, in diet-induced obese (DIO) mice, leptin resistance is characterized by a reduction in STAT3, but an increase in SOCS3 levels (Munzberg et al., 2004). The elevations in SOCS3 levels occur specifically in the ARC (Munzberg et al., 2004), indicating that defects in the ARC in particular may play a role in the pathogenesis of leptin-resistant obesity. In support of a key role for SOCS3 in mediating the development of leptin resistance, targeted disruption of SOCS3 in mice increases the leptin sensitivity and reduces diet-induced obesity (Mori et al., 2004; Howard et al., 2004). Other negative regulators of leptin-receptor-driven signaling that may confer resistance to leptin in diet-induced obesity include protein tyrosine phosphatase 1B (Cheng et al., 2002; Zabolotny et al., 2002) and SHP-2 (Carpenter et al., 1998; Li and Friedman, 1999).

### 4.2.3 Role in Reproductive Function

Evidence is accumulating that the reproductive system is also regulated by leptin receptors expressed in the hypothalamus. For instance, animal models that display leptin deficiency and resistance are associated with dysfunctions in the reproductive system (Swerdloff et al., 1976), which can be rescued by leptin treatment (Barash et al., 1996). The onset of puberty in normal (wild type) mice is accelerated by leptin (Ahima et al., 1997), whereas the occurrence of hypothalamic hypogonadism in humans has been linked to mutations in the ob and db genes (Montague et al., 1997; Strobel et al., 1998). Transgenic mice that overexpress leptin also display accelerated puberty (Yura et al., 2000), whereas leptin adminsitration to rodents reverses the delay in sexual maturation induced by fasting. Thus leptin may be the key hormone linking sufficient energy stores and normal reproductive function. In various animal models leptin is also reported to restore the pulsatile release pattern of luteinizing hormone (LH), which is attenuated during fasting (Gonzalez et al., 1999). As there is evidence that leptin directly stimulates gonadotrophin-releasing hormone (GnRH) secretion in the hypothalamus in vivo and the pulsatile nature of LH release is regulated by GnRH, it is feasible that this effect of leptin is a hypothalamic-driven process (Watanobe, 2002). Thus it has been proposed that under conditions where there are adequate nutritional stores, leptin, in conjunction with GnRH and the growth hormone axis, acts to initiate the complex process of puberty.

### 4.2.4 Regulation of Bone Formation

It is also emerging that leptin, via its actions in the hypothalamus is a potent regulator of bone formation. The possibility that bone mass, body weight, and reproduction could all be controlled by the same hormone was suggested by the observations that obesity prevents bone loss whereas gonadal failure results in it (Karsenty, 2001). Leptin- and leptin-receptor-deficient rodents, despite being obese and hypogonadic, exhibit enhanced bone formation, which subsequently leads to high bone mass (HBM) (Ducy et al., 2000). Defects in leptin signaling, as opposed to obesity per se, are thought to contribute to these changes in bone density as the HBM phenotype is evident in leptin-deficient rodents (*ob/ob* mice) prior to the development of obesity. The exact mechanisms underlying the effects of leptin on bone formation are unclear. Although a hypothalamic site for the antiosteogenic actions of leptin has been identified, as ICV infusion of leptin causes bone loss in *ob/ob* and wild-type mice (Ducy et al., 2000), the leptin-receptor-driven hypothalamic networks that regulate bone formation and food intake appear to differ (Takeda et al., 2002). Thus the neuropeptides that mediate the anorexigenic effects of leptin do not affect bone formation. Moreover manipulations that alter the ability of leptin to inhibit bone formation have no effect on body weight (Takeda et al., 2002). Recent studies also indicate that the antiosteogenic and anorexigenic actions of leptin are regulated by similar plasma concentrations of leptin (Elefteriou et al., 2004), indicating that the

circulating levels of leptin are a key determinant of bone formation. Although numerous studies have demonstrated that the hypothalamus plays a pivotal role in the ability of leptin to regulate bone formation, there is also evidence that leptin may act locally to influence bone mass by inhibiting osteoclast generation (Holloway et al., 2002). Thus a complex regulatory mechanism appears to exist that controls bone formation which involves a combination of both central and peripheral actions of leptin.

## 4.3 Role of Leptin in the Hippocampal Formation

### 4.3.1 Is Leptin a Potential Cognitive Enhancer?

It is well documented that the hippocampus is an area of the brain that is critically involved in learning and memory processes. Indeed in this region, the phenomenon of long-term potentiation (LTP), which is a long lasting increase in the efficacy of excitatory synaptic transmission, occurs and this process is thought to be a cellular correlate of certain aspects of learning, memory, and habituation. In particular *N*-methyl-D-aspartate (NMDA) receptor-dependent LTP evoked in the CA1 region of the hippocampus may underlie the formation of spatial memory (Bliss and Collingridge, 1993). A potential role for leptin in hippocampal synaptic plasticity was suggested by recent studies using genetically obese, leptin-receptor-deficient rodents (*db/db* mice and *fa/fa* rats) that displayed impairments in both hippocampal LTP (Li et al., 2002; Gerges et al., 2003) and long-term depression (LTD) (Li et al., 2002). These animals also showed impaired performance in spatial memory tasks in the Morris water maze (Li et al., 2002). Further evidence that implicates leptin in hippocampal synaptic plasticity was obtained by Shanley et al. (2001) and coworkers who demonstrated that leptin has the ability to convert short lasting potentiation (STP) of synaptic transmission (induced by primed burst stimulation of the Schaffer collateral-commissural pathway) into LTP. More recent studies have demonstrated that direct administration of leptin to the dentate region of the rat hippocampus enhances the level of LTP evoked in this region of the brain (Wayner et al., 2004). Leptin adminstration into the CA1 region of the hippocampus also improves memory processing in mice performing T-maze foot shock avoidance and step down passive avoidance tests (Farr et al., 2004).

So what are the potential mechanisms underlying the modulatory effects of leptin on hippocampal synaptic plasticity? It is well established that LTP can be modulated by a number of hormones, and one of the main targets for modulation is the NMDA subtype of glutamate receptor. Indeed leptin can rapidly facilitate NMDA-receptor-mediated synaptic currents and NMDA-evoked $Ca^{2+}$ influx in the hippocampus (Shanley et al., 2001), and this effect is selective for NMDA receptors as leptin fails to alter $Ca^{2+}$ influx via AMPA (α-amino-3-hydroxy-5-methyl-isoxazole) receptors. The ability of leptin to modify NMDA responses involves a PI-3-kinase-driven process as two distinct PI-3 kinase inhibitors, namely LY294002 and wortmannin, attenuate the effects of leptin. Inhibitors of MAPK (PD98059 and U0126) and Src tyrosine kinase (PP1 and lavendustin A) also attenuate leptin-induced facilitation of NMDA responses (Shanley et al., 2001) indicating that MAPK- and Src tyrosine kinase- dependent pathways contribute to the actions of leptin. It is well documented that activation of all these signaling cascades (namley PI-3 kinase; MAPK; Src tyrosine kinases) play a role in hippocampal LTP (English and Sweatt, 1997; Lu et al., 1998; Coogan et al., 1999; Kelly and Lynch, 2000; Huang et al., 2001; Komiyama et al., 2002; Sanna et al., 2002; Man et al., 2003; Opazo et al., 2003; Kelleher et al., 2004). Thus leptin may be released from CA1 synapses during high-frequency stimulation and the induction phase of LTP, and modulate synaptic plasticity by influencing these signaling cascades, and subsequently modifying NMDA receptor function. Alternatively, hormonally released leptin (from adipocytes) may be transported from the periphery to the brain, where it acts to modulate the threshold for the induction of LTP by selective facilitation of NMDA receptors. However as there is no direct evidence that leptin can stimulate these pathways in hippocampal neurons, it is possible that leptin acts in concert with other neurotransmitters/agents capable of stimulating these pathways.

As these findings indicate that leptin is a potential cognitive enhancer, does leptin insensitivity or leptin deficits affect cognitive function in humans? It is known that diabetes is commonly associated with obesity, and cognitive deficits have been found in some diabetic patients. Thus insensitivity to leptin

may be a key contributor to these cognitive deficits. Although there is no direct evidence that human obesity results in cognitive deficits, it is well established that lower cognitive functioning can be associated with patients with obesity and hypertension (Elias et al., 2003), which suggests that obesity may have adverse effects on cognitive performance. In support of such a possibility, functional imaging of cerebral blood flow has demonstrated regional differences in the brain responses of lean and obese subjects, suggesting that the circulating levels of leptin in the brain are altered in obese individuals (Gautier et al., 2000, 2001).

### 4.3.2 Leptin Has Anticonvulsant Properties

Potassium channels are a key cellular target for leptin in both peripheral cells and central neurons. Indeed, in peripheral insulin-secreting cells (Harvey et al., 1997; Kellerer et al., 1997), leptin inhibits insulin secretion via activation of $K_{ATP}$ channels, whereas in hypothalamic glucose-responsive neurons, leptin causes neuronal inhibition via activation of $K_{ATP}$ channels (Spanswick et al., 1997). In contrast, in the hippocampus leptin activates a distinct type of potassium channel, the large conductance $Ca^{2+}$-activated $K^{+}$ (BK) channel. However, like $K_{ATP}$ channel activation by leptin (Harvey et al., 2000a, b), a PI-3 kinase-dependent process connects leptin receptor activation to the stimulation of BK channels (Shanley et al., 2002a).

It is well established that the activity of neuronal BK channels is tightly regulated by both voltage and levels of intracellular $Ca^{2+}$ (Latorre, 1989), and the activity of postsynaptic BK channels is key to regulating the level of neuronal excitability which determines action potential firing rate and burst firing pattern. Thus, BK channels are thought to play a pivotal part in regulating seizure-like activity in the brain (Alger and Williamson, 1988; Shao et al., 1999). Indeed, leptin significantly attenuates the frequency of epileptiform discharges in two distinct models of epilepsy, via a process involving the activation of BK channels (Shanley et al., 2002b). Interestingly, Shanley et al. (2002b) also found that leptin-receptor-deficient rodents (Zucker *fa/fa* rats) displayed an enhanced frequency of seizure-like activity compared with lean control animals, indicating that the degree of neuronal excitability is enhanced in animals with leptin receptor and/or signaling deficits. As unregulated hyperexcitability in the CNS is a hallmark of neurological diseases such as epilepsy, the coupling of leptin receptors to BK channels may provide a novel, and useful therapeutic target in the treatment of this disease.

Interestingly there are numerous reports of an increased frequency of reproductive disorders, in particular polycystic ovary syndrome, in women with epilepsy. The prevalence of such disorders appears to be independent of the antiepileptic medication used or the type of seizure (Bilo et al., 2001). Thus as leptin is a possible pathogenic factor in polycystic ovary syndrome (El Orabi et al., 1999) and as leptin is a potent anticonvulsant, it is tempting to speculate that the occurrence of reproductive disorders in epileptic patients may be linked and possibly may be due to defects in the leptin system.

## 4.4 Leptin and Neuronal Development

The possibility that leptin has a role in developmental processes was originally suggested by the high levels of leptin expression in placenta (Masuzaki et al., 1997). Leptin is also implicated in in utero development as both leptin and leptin receptors are widely expressed in fetal tissues and human umbilical cord (Hoggard et al., 1997; Akerman et al., 2002). Indeed leptin receptor gene expression is evident in a number of embryonic mesoderm-derived tissues including musculoaponeurotic laminae and bone primordia (Camand et al., 2002). Leptin-receptor-driven pathways are also implicated in development of the CNS, as changes in the expression levels and the localization of $ObR_b$ have been detected in rodent brains at different stages of development (Chen et al., 1999, 2000; Matsuda et al., 1999; Morash et al., 2003). For example in mouse embryos (at embryonic day 14.5; E14.5) leptin receptor mRNA is localized to the ventricular zone of the thalamus. However in the ARC and VMN, leptin receptor mRNA was not detected until E18.5 (Udagawa et al., 2000). The expression of the leptin (ob) gene also appears to be developmentally regulated as age-related changes in the expression levels of leptin mRNA levels have been observed in

rodent brains (Morash et al., 2001). For instance in the pituitary, ob mRNA levels peak during postnatal days 7–14, whereas by postnatal day 22 the levels of ob mRNA fall significantly. Interestingly the expression of the leptin gene during the postnatal period is also tissue dependent. For example, in contrast to the pituitary, ob gene levels are low in cerebral cortex in neonatal rats (postnatal days 2–7), but are maximal between postnatal days 14–28 (Morash et al., 2001).

In addition to developmental changes in neuronal ob and $ObR_b$ mRNA levels, specifc changes in the brains of leptin-deficient or insensitive rodents have been observed, which also supports a role for leptin in neuronal development. Indeed reductions in brain weight and protein content have been reported in *ob/ob* and *db/db* mice compared with control mice (Ahima et al., 1999; Steppan and Swick, 1999). The levels of a number of synaptic proteins, including synaptobrevin and syntaxin-1 are also attenuated in hippocampal and neocortical brain regions of *ob/ob* and *db/db* mice, and these deficits are reversed by postnatal administration of leptin (Ahima et al., 1999). Other neuronal deficits that are evident in leptin-deficient (*ob/ob*) rodents include altered dendritic orientation (Bereiter and Jeanrenaud, 1979), reduced neuronal soma size (Bereiter and Jeanrenaud, 1979), as well as reductions in brain myelination (Sena et al., 1985). Alterations in the organization of neuronal connections (Bereiter and Jeanrenaud, 1980) have also been observed in leptin-deficient (*ob/ob*) mice. Moreover recent studies have demonstrated that leptin-deficiency results in profound disruption in the development of ARC projections to the PVN in the hypothalamus (Bouret et al., 2004). Moreover, leptin directly induces neurite growth from neurons within the ARC, and this developmental activity is restricted to a critical period in neonatal development. Thus, it is possible that the neonatal surge in leptin acts as a peripheral signal to direct the development of central circuits in the brain, in particular promoting the formation of specific hypothalamic pathways that convey leptin signals to brain regions involved in regulating energy balance.

### 4.5 Neuroprotective Effects of Leptin

Another possible function of leptin is to act as a neuroprotective agent. Administration of leptin intraperitoneally has been shown to protect (by around 50%) against excitotoxic lesions induced by injection of the glutamatergic analogue ibotenate into the developing mouse brain in vivo (Dicou et al., 2001). In the same study, Dicou et al. (2001) also demonstrated that leptin significantly reduced NMDA-induced cytotoxicity in cultured mouse cortical neurons. Furthermore, in both cases the neuroprotective effects of leptin were inhibited by a selective JAK2 inhibitor, indicating that leptin-receptor-driven activation of a JAK2-dependent process contributes to this process. In human neuroblastoma cells (SH-SY5Y), leptin is also reported to inhibit apoptosis (Russo et al., 2004). The mechanisms underlying leptin-induced suppression of apotosis involves activation of JAK–STAT, PI-3 kinase, and MAPK signaling cascades and subsequent downregulation of the apoptotic factors caspase-10 and tumour necrosis factor (TNF). The antiapoptotic actions of leptin in the CNS parallel its ability to reduce apoptosis in peripheral cells. For instance, in hepatic stellate cells leptin potently abolishes cytcloheximide- and TNF-induced apoptosis, via a process involving activation of ERK and Akt (Saxena et al., 2004).

## 5 Conclusions

It is well documented that the adipocyte-derived hormone leptin plays an important role in controlling feeding behavior and energy expenditure by signaling information regarding the status of energy stores to leptin receptors located on specific neurons within the hypothalamus. However, it is becoming apparent that this hormone has other neuronal functions that are unrelated to its effects on energy homeostasis. Indeed, there is evidence that leptin, via its actions in the hypothalamus, is an important regulator of the reproductive system and of bone formation. However leptin and its receptors are widely expressed in many extrahypothalamic brain regions, including the hippocampus, cerebellum, amygdala and brainstem, suggesting that leptin is a multifaceted hormone in the CNS. Indeed there is good evidence that leptin plays an important role in learning and memory processes as leptin-insensitive rodents display impairments in

hippocampal synaptic plasticity, and a number of studies indicate that leptin can facilitate hippocampal LTP, possibly via enhancement of NMDA receptor function. Alterations in the efficacy of synaptic connections have also been observed in the hypothalamus of leptin-deficient rodents, suggesting that leptin also influences the plasticity of synapses in this region of the brain. Another potential function of leptin is as an anticonvulsant agent, as it can potently regulate hippocampal hyperexcitability by promoting the activation of BK channels. This process may have important implications for diseases, like temporal lobe epilepsy, that are characterized by unregulated excitability in the brain. More recent studies have identified a prominent role for leptin in the development of the CNS as leptin has the ability to promote neurite outgrowth in the hypothalamus, and specific deficits in key hypothalamic feeding pathways have been identified in leptin-deficient rodents.

## References


Ahima RS. 2000. Leptin and the neuroendocrinology of fasting. Front Horm Res 26: 42-56.

Ahima RS, Bjorbaek C, Osei S, Flier JS. 1999. Regulation of neuronal and glial proteins by leptin: implications for brain development. Endocrinology 140: 2755-2762.

Ahima RS, Dushay J, Flier SN, Prabakaran D, Flier JS. 1997. Leptin accelerates the onset of puberty in normal female mice. J Clin Invest 99: 391-395.

Ahima RS, Flier JS. 2000. Leptin. Annu Rev Physiol 62: 413-437.

Ahima RS, Prabakaran D, Flier JS. 1998. Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. J Clin Invest 101: 1020-1027.

Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, et al. 1996. Role of leptin in the neuroendocrine response to fasting. Nature 382 (6588): 250-252.

Akerman F, Lei ZM, Rao CV. 2002. Human umbilical cord and fetal membranes co-express leptin and its receptor genes. Gynecol Endocrinol 16: 299-306.

Alger BE, Williamson A. 1988. A transient calcium-dependent potassium component of the epileptiform burst after-hyperpolarization in rat hippocampus. J Physiol 399: 191-205.

Bado A, Levasseur S, Attoub S, Kermorgant S, Laigneau JP, et al. 1998. The stomach is a source of leptin. Nature 394: 790-793.

Banks WA. 2004. The many lives of leptin. Peptides 25: 331-338.

Banks WA, Coon AB, Robinson SM, Moinuddin A, Shultz JM, et al. 2004. Triglycerides induce leptin resistance at the blood-brain barrier. Diabetes 53: 1253-1260.

Banks AS, Davis SM, Bates SH, Myers MG Jr. 2000. Activation of downstream signals by the long form of the leptin receptor. J Biol Chem 275: 14563-14572.

Banks WA, Di Palma CR, Farrell CL. 1999. Impaired transport of leptin across the blood-brain barrier in obesity. Peptides 20: 1341-1345.

Banks WA, Farrell CL. 2003. Impaired transport of leptin across the blood-brain barrier in obesity is acquired and reversible. Am J Physiol Endocrinol Metab 285: E10-E15.

Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM. 1996. Leptin enters the brain by a saturable system independent of insulin. Peptides 17: 305-311.

Banks WA, King BM, Rossiter KN, Olson RD, Olson GA, et al. 2001. Obesity-inducing lesions of the central nervous system alter leptin uptake by the blood-brain barrier. Life Sci 69: 2765-2773.

Barash IA, Cheung CC, Weigle DS, Ren H, Kabigting EB, et al. 1996. Leptin is a metabolic signal to the reproductive system. Endocrinology 137: 3144-3147.

Baskin DG, Seeley RJ, Kuijper JL, Lok S, Weigle DS, Erickson JC, Palmiter RD, Schwartz MW. 1998. Increased expression of mRNA for the long form of the leptin receptor in the hypothalamus is associated with leptin hypersensitivity and fasting. Diabetes 47: 538-543.

Baskin DG, Schwartz MW, Seeley RJ, Woods SC, Porte D Jr, Breininger JF, Jonak Z, Schaefer J, Krouse M, Burghardt C, Campfield LA, Burn P, Kochan JP. 1999. Leptin receptor long-form splice-variant protein expression in neuron cell bodies of the brain and co-localization with neuropeptide Y mRNA in the arcuate nucleus. J Histochem Cytochem 47: 353-362.

Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AW, et al. 2003. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. Nature 421: 856-859.

Baumann H, Morella KK, White DW, Dembski M, Bailon PS, Kim H, Lai CF, Tartaglia LA. 1996. The full-length leptin receptor has signaling capabilities of interleukin 6-type cytokine receptors. Proc Natl Acad Sci USA 93: 8374-8.

Bereiter DA, Jeanrenaud B. 1979. Altered neuroanatomical organization in the central nervous system of the genetically obese (*ob/ob*) mouse. Brain Res 165: 249-260.

Bereiter DA, Jeanrenaud B. 1980. Altered dendritic orientation of hypothalamic neurons from genetically obese (*ob/ob*) mice. Brain Res 202: 201-206.

Bilo L, Meo R, Valentino R, Di Carlo C, Striano S, et al. 2001. Characterization of reproductive endocrine disorders in women with epilepsy. J Clin Endocrinol Metab 86: 2950-2956.

Bjorbaek C, Buchholz RM, Davis SM, Bates SH, Pierroz DD, Gu H, Neel BG, Myers MG Jr, Flier JS. 2000. Divergent roles of SHP-2 in ERK activation by leptin receptors. J Biol Chem 276: 4747-4755.

Bjorbaek C, El-Haschimi K, Frantz JD, Flier JS. 1999. The role of SOCS-3 in leptin signaling and leptin resistance. J Biol Chem 274: 30059-30065.

Bjorbaek C, Elmquist JK, Michl P, Ahima RS, van Bueren A, et al. 1998. Expression of leptin receptor isoforms in rat brain microvessels. Endocrinology 139: 3485-3491.

Bjorbaek C, Uotani S, da Silva B, Flier JS. 1997. Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem 272: 32686-32695.

Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361: 31-39.

Blum WF, Englaro P, Hanitsch S, Juul A, Hertel NT, et al. 1997. Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. J Clin Endocrinol Metab 82: 2904-2910.

Bouret SG, Draper SJ, Simerly RB. 2004. Trophic action of leptin on hypothalamic neurons that regulate feeding. Science 304: 108-110.

Bray GA, York DA. 1979. Hypothalamic and genetic obesity in experimental animals: an autonomic and endocrine hypothesis. Physiol Rev 59: 719-809.

Bullo M, Garcia-Lorda P, Peinado-Onsurbe J, Hernandez M, Del Castillo D, et al. 2002. TNFalpha expression of subcutaneous adipose tissue in obese and morbid obese females: relationship to adipocyte LPL activity and leptin synthesis. Int J Obes Relat Metab Disord 26 (5): 652-658.

Burguera B, Couce ME, Curran GL, Jensen MD, Lloyd RV, et al. 2000. Obesity is associated with a decreased leptin transport across the blood-brain barrier in rats. Diabetes 49: 1219-1223.

Camand O, Turban S, Abitbol M, Guerre-Millo M. 2002. Embryonic expression of the leptin receptor gene in mesoderm-derived tissues. C R Biol 325: 77-87.

Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. 1995. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269 (5223): 546-549.

Caro JF, Kolaczynski JW, Nyce MR, Ohannesian JP, Opentanova I, et al. 1996. Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. Lancet 348: 159-161.

Carpenter LR, Farruggella TJ, Symes A, Karow ML, Yancopoulos GD, et al. 1998. Enhancing leptin response by preventing SH2-containing phosphatase 2 interaction with Ob receptor. Proc Natl Acad Sci USA 95: 6061-6066.

Casabiell X, Pineiro V, Tome MA, Peino R, Dieguez C, et al. 1997. Presence of leptin in colostrum and/or breast milk from lactating mothers: a potential role in the regulation of neonatal food intake. J Clin Endocrinol Metab 82: 4270-4273.

Castracane VD, Kraemer RR, Franken MA, Kraemer GR, Gimpel T. 1998. Serum leptin concentration in women: effect of age, obesity, and estrogen administration. Fertil Steril 70: 472-477.

Chen H, Charlat O, Tartaglia L, Woolf EA, Weng X, et al. 1996. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in *db/db* mice. Cell 84 (3): 491-495.

Chen SC, Cunningham JJ, Smeyne RJ. 2000. Expression of OB receptor splice variants during prenatal development of the mouse. J Recept Signal Transduct Res 20: 87-103.

Chen SC, Kochan JP, Campfield LA, Burn P, Smeyne RJ. 1999. Splice variants of the OB receptor gene are differentially expressed in brain and peripheral tissues of mice. J Recept Signal Transduct Res 19: 245-266.

Cheng A, Uetani N, Simoncic PD, Chaubey VP, Lee-Loy A, McGlade CJ, Kennedy BP, Tremblay ML. 2002. Attenuation of leptin action and regulation of obesity by protein tyrosine phosphatase 1B. Dev Cell 2: 497-503.

Chua SC Jr, White DW, Wu-Peng XS, Liu SM, Okada N, et al. 1996. Phenotype of fatty due to Gln269Pro mutation in the leptin receptor (Lepr). Diabetes 45: 1141-1143.

Cizza G, Lotsikas AJ, Licinio J, Gold PW, Chrousos GP. 1997. Plasma leptin levels do not change in patients with Cushing's disease shortly after correction of hypercortisolism. J Clin Endocrinol Metab 82 (8): 2747-2750.

Clement K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, et al. 1998. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392: 398-401.

Coleman DL. 1973. Effects of parabiosis of obese with diabetes and normal mice. Diabetologia 9 (4): 294-298.

Coleman DL, Hummel KP. 1969. Effects of parabiosis of normal with genetically diabetic mice. Am J Physiol 217 (5): 1298-1304.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, et al. 1996. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334 (5): 292-295.

Coogan AN, O'Leary DM, O'Connor JJ. 1999. P42/44 MAP kinase inhibitor PD98059 attenuates multiple forms of synaptic plasticity in rat dentate gyrus in vitro. J Neurophysiol 81: 103-110.

Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, et al. 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411: 480-484.

da Silva BA, Bjorbaek C, Uotani S, Flier JS. 1998. Functional properties of leptin receptor isoforms containing the gln→pro extracellular domain mutation of the fatty rat. Endocrinology 139: 3681-3690.

Dawson R, Pelleymounter MA, Millard WJ, Liu S, Eppler B. 1997. Attenuation of leptin-mediated effects by monosodium glutamate-induced arcuate nucleus damage. Am J Physiol 273: E202-E206.

De Vos P, Saladin R, Auwerx J, Staels B. 1995. Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. J Biol Chem 270 (27): 15958-15961.

Dicou E, Attoub S, Gressens P. 2001. Neuroprotective effects of leptin in vivo and in vitro. Neuroreport 12: 3947-3951.

Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, et al. 2001. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature 410: 822-825.

Dube MG, Kalra SP, Kalra PS. 2000. Hypothalamic galanin is up-regulated during hyperphagia and increased body weight gain induced by disruption of signaling in the ventromedial nucleus. Peptides 21: 519-526.

Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, et al. 2000. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. Cell 100: 197-207.

Elefteriou F, Takeda S, Ebihara K, Magre J, Patano N, et al. 2004. Serum leptin level is a regulator of bone mass. Proc Natl Acad Sci USA 101: 3258-3263.

El-Haschimi K, Pierroz DD, Hileman SM, Bjorbaek C, Flier JS. 2000. Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. J Clin Invest 105: 1827-1832.

Elias MF, Elias PK, Sullivan LM, Wolf PA, D'Agostino RB. 2003. Lower cognitive function in the presence of obesity and hypertension: the Framingham heart study. Int J Obes Relat Metab Disord 27: 260-268.

Elmquist JK, Bjorbaek C, Ahima RS, Flier JS, Saper CB. 1998. Distributions of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol 395: 535-547.

Elmquist JK, Elias CF, Saper CB. 1999. From lesions to leptin: hypothalamic control of food intake and body weight. Neuron 22: 221-232.

El Orabi H, Ghalia AA, Khalifa A, Mahfouz H, El Shalkani A, et al. 1999. Serum leptin as an additional possible pathogenic factor in polycystic ovary syndrome. Clin Biochem 32: 71-75.

English JD, Sweatt JD. 1997. A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J Biol Chem 272: 19103-19106.

Farr SA, Banks WA, Miller NE, Morley JE. 2004. Leptin administration into the hippocampus improves memory processing in mice. Soc Neurosci Abstr 24: 210.11.

Frederich RC, Lollmann B, Hamann A, Napolitano-Rosen A, Kahn BB, et al. 1995. Expression of ob mRNA and its encoded protein in rodents. Impact of nutrition and obesity. J Clin Invest 96 (3): 1658-1663.

Gao Q, Wolfgang MJ, Neschen S, Morino K, Horvath TL, et al. 2004. Disruption of neural signal transducer and activator of transcription 3 causes obesity, diabetes, infertility, and thermal dysregulation. Proc Natl Acad Sci USA 101: 4661-4666.

Gautier JF, Chen K, Salbe AD, Bandy D, Pratley RE, et al. 2000. Differential brain responses to satiation in obese and lean men. Diabetes 49: 838-846.

Gautier JF, Del Parigi A, Chen K, Salbe AD, Bandy D, et al. 2001. Effect of satiation on brain activity in obese and lean women. Obes Res 9: 676-684.

Gerges NZ, Aleisa AM, Alkadhi KA. 2003. Impaired long-term potentiation in obese zucker rats: possible involvement of presynaptic mechanism. Neuroscience 120: 535-539.

Ghilardi N, Skoda RC. 1997. The leptin receptor activates janus kinase 2 and signals for proliferation in a factor-dependent cell line. Mol Endocrinol 11: 393-399.

Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, et al. 1996. Defective STAT signaling by the leptin receptor in diabetic mice. Proc Natl Acad Sci USA 93: 6231-6235.

Golden PL, Maccagnan TJ, Pardridge WM. 1997. Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. J Clin Invest 99: 14-18.

Gong DW, Bi S, Pratley RE, Weintraub BD. 1996. Genomic structure and promoter analysis of the human obese gene. J Biol Chem 271: 3971-3974.

Gonzalez LC, Pinilla L, Tena-Sempere M, Aguilar E. 1999. Leptin(116-130) stimulates prolactin and luteinizing hormone secretion in fasted adult male rats. Neuroendocrinology 70: 213-220.

Grasso P, Leinung MC, Ingher SP, Lee DW. 1997. In vivo effects of leptin-related synthetic peptides on body weight and food intake in female *ob/ob* mice: localization of leptin activity to domains between amino acid residues 106–140. Endocrinology 138 (4): 1413-1418.

Hakansson ML, Brown H, Ghilardi N, Skoda RC, Meister B. 1998. Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci 18: 559-572.

Hakansson ML, Hulting AL, Meister B. 1996. Expression of leptin receptor mRNA in the hypothalamic arcuate nucleus – relationship with NPY neurones. Neuroreport 7: 3087-3092.

Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, et al. 1997. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. Proc Natl Acad Sci USA 94: 8878-8883.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, et al. 1995. Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269 (5223): 543-546.

Hamilton BS, Paglia D, Kwan AY, Deitel M. 1995. Increased obese mRNA expression in omental fat cells from massively obese humans. Nat Med 1 (9): 953-956.

Harvey J, Hardy SC, Irving AJ, Ashford ML. 2000a. Leptin activation of ATP-sensitive $K^+$ ($K_{ATP}$) channels in rat CRI-G1 insulinoma cells involves disruption of the actin cytoskeleton. J Physiol 527: 95-107.

Harvey J, McKay NG, Walker KS, Van der Kaay J, Downes CP, et al. 2000b. Essential role of phosphoinositide 3-kinase in leptin-induced $K_{ATP}$ channel activation in the rat CRI-G1 insulinoma cell line. J Biol Chem 275: 4660-4669.

Harvey J, McKenna F, Herson PS, Spanswick D, Ashford ML. 1997. Leptin activates ATP-sensitive potassium channels in the rat insulin-secreting cell line, CRI-G1. J Physiol 504: 527-535.

Hausberger FX. 1959. Behavior of transplanted adipose tissue of hereditarily obese mice. Anat Rec 135: 109-113.

Hervey GR. 1958. The effects of lesions in the hypothalamus in parabiotic rats. J Physiol 145: 336-352.

Hoggard N, Hunter L, Duncan JS, Williams LM, Trayhurn P, et al. 1997. Leptin and leptin receptor mRNA and protein expression in the murine fetus and placenta. Proc Natl Acad Sci USA 94: 11073-11078.

Holloway WR, Collier FM, Aitken CJ, Myers DE, Hodge JM, et al. 2002. Leptin inhibits osteoclast generation. J Bone Miner Res 17: 200-209.

Howard JK, Cave BJ, Oksanen LJ, Tzameli I, Bjorbaek C, et al. 2004. Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. Nat Med 10: 734-738.

Huang XF, Koutcherov I, Lin S, Wang HQ, Storlien L. 1996. Localization of leptin receptor mRNA expression in mouse brain. Neuroreport 7: 2635-2638.

Huang Y, Lu W, Ali DW, Pelkey KA, Pitcher GM, et al. 2001. CAKbeta/Pyk2 kinase is a signaling link for induction of long-term potentiation in CA1 hippocampus. Neuron 29: 485-496.

Hwang CS, Mandrup S, MacDougald OA, Geiman DE, Lane MD. 1996. Transcriptional activation of the mouse obese (ob) gene by CCAAT/enhancer binding protein alpha. Proc Natl Acad Sci USA 93: 873-877.

Ihle JN. 1995. Cytokine receptor signalling. Nature 377(6550): 591-594.

Ingalls AM, Dickie MM, Snell GD. 1950. Obese, a new mutation in the house mouse. J Hered 41 (12): 317-318.

Janik JE, Curti BD, Considine RV, Rager HC, Powers GC, et al. 1997. Interleukin 1 alpha increases serum leptin concentrations in humans. J Clin Endocrinol Metab 82 (9): 3084-3086.

Janmey PA. 1998. The cytoskeleton and cell signaling: component localization and mechanical coupling. Physiol Rev 78: 763-781.

Karsenty G. 2001. Leptin controls bone formation through a hypothalamic relay. Recent Prog Horm Res 56: 401-415.

Kastin AJ, Pan W, Maness LM, Koletsky RJ, Ernsberger P. 1999. Decreased transport of leptin across the blood-brain barrier in rats lacking the short form of the leptin receptor. Peptides 20: 1449-1453.

Kelleher RJ III, Govindarajan A, Jung HY, Kang H, Tonegawa S. 2004. Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell 116: 467-479.

Kellerer M, Koch M, Metzinger E, Mushack J, Capp E, Haring HU. 1997. Leptin activates PI-3 kinase in C2C12 myotubes via janus kinase-2 (JAK-2) and insulin receptor substrate-2 (IRS-2) dependent pathways. Diabetologia 40: 1358-1362.

Kelly A, Lynch MA. 2000. Long-term potentiation in dentate gyrus of the rat is inhibited by the phosphoinositide 3-kinase inhibitor, wortmannin. Neuropharmacology 39: 643-651.

Kennedy GC. 1953. The role of depot fat in the hypothalamic control of food intake in the rat. Proc R Soc Lond B Biol Sci 140 (901): 578-596.

Kolacznyski JW, Considine RV, Ohannesian J, Marco C, Opentanova I, et al. 1996. Responses of leptin to short-term fasting and refeeding in humans: a link with ketogenesis but not ketones themselves. Diabetes 45 (11): 1511-1515.

Komiyama NH, Watabe AM, Carlisle HJ, Porter K, Charlesworth P, et al. 2002. SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor. J Neurosci 22: 9721-9732.

Latorre R. 1989. Ion channel modulation by divalent cations. Acta Physiol Scand Suppl 582: 13.

Laughlin GA, Yen SS. 1997. Hypoleptinemia in women athletes: absence of a diurnal rhythm with amenorrhea. J Clin Endocrinol Metab 82 (1): 318-321.

Leal-Cerro A, Soto A, Martinez MA, Dieguez C, Casanueva FF. 2001. Influence of cortisol status on leptin secretion. Pituitary 4 (1–2): 111-116.

Lee G, Li C, Montez J, Halaas J, Darvishzadeh J, Friedman JM. 1997. Leptin receptor mutations in 129 db3J/db3J mice and NIH facp/facp rats. Mamm Genome 8: 445-447.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, et al. 1996. Abnormal splicing of the leptin receptor in diabetic mice. Nature 379 (6566): 632-635.

Lennie TA, Wortman MD, Seeley RJ. 2001. Activity of body energy regulatory pathways in inflammation-induced anorexia. Physiol Behav 73: 517-523.

Li XL, Aou S, Oomura Y, Hori N, Fukunaga K, et al. 2002. Impairment of long-term potentiation and spatial memory in leptin receptor-deficient rodents. Neuroscience 113: 607-615.

Li C, Friedman JM. 1999. Leptin receptor activation of SH2 domain containing protein tyrosine phosphatase 2 modulates Ob receptor signal transduction. Proc Natl Acad Sci USA 96: 9677-9682.

Licinio J, Mantzoros C, Negrao AB, Cizza G, Wong ML, et al. 1997. Human leptin levels are pulsatile and inversely related to pituitary–adrenal function. Nat Med 3 (5): 575-579.

Lin S, Huang XF. 1997. Fasting increases leptin receptor mRNA expression in lean but not obese (ob/ob) mouse brain. Neuroreport 8: 3625-3629.

Lin S, Thomas TC, Storlien LH, Huang XF. 2000. Development of high fat diet-induced obesity and leptin resistance in C57B1/6J mice. Int J Obes Relat Metab Disord 24: 639-646.

Lu YM, Roder JC, Davidow J, Salter MW. 1998. Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. Science 279: 1363-1367.

MacDougald OA, Hwang CS, Fan H, Lane MD. 1995. Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. Proc Natl Acad Sci USA 92 (20): 9034-9037.

Machinal-Quelin F, Dieudonne MN, Leneveu MC, Pecquery R, Giudicelli Y. 2002. Proadipogenic effect of leptin on rat preadipocytes in vitro: activation of MAPK and STAT3 signaling pathways. Am J Physiol Cell Physiol 282: C853-C863.

Madej T, Boguski MS, Bryant SH. 1995. Threading analysis suggests that the obese gene product may be a helical cytokine. FEBS Lett 373 (1): 13-18.

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, et al. 1995. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1 (11): 1155-1161.

Man HY, Wang Q, Lu WY, Ju W, Ahmadian G, et al. 2003. Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. Neuron 38: 611-624.

Mantzoros CS, Flier JS, Rogol AD. 1997. A longitudinal assessment of hormonal and physical alterations during normal puberty in boys. V. Rising leptin levels may signal the onset of puberty. J Clin Endocrinol Metab 82: 1066-1070.

Masuzaki H, Ogawa Y, Sagawa N, Hosoda K, Matsumoto T, et al. 1997. Nonadipose tissue production of leptin: leptin as a novel placenta-derived hormone in humans. Nat Med 3: 1029-1033.

Matsuda J, Yokota I, Tsuruo Y, Murakami T, Ishimura K, et al. 1999. Development changes in long-form leptin receptor expression and localization in rat brain. Endocrinology 140: 5233-5238.

Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Trayhurn P. 1996. Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization. FEBS Lett 387: 113-116.

Miki T, Liss B, Minami K, Shiuchi T, Saraya A, et al. 2001. ATP-sensitive $K^+$ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. Nat Neurosci 4: 507-512.

Minokoshi Y, Alquier T, Furukawa N, Kim YB, Lee A, et al. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428: 569-574.

Mise H, Sagawa N, Matsumoto T, Yura S, Nanno H, et al. 1998. Augmented placental production of leptin in preeclampsia: possible involvement of placental hypoxia. J Clin Endocrinol Metab 83: 3225-3229.

Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, et al. 1997. Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387 (6636): 903-908.

Morash BA, Imran A, Wilkinson D, Ur E, Wilkinson M. 2003. Leptin receptors are developmentally regulated in rat pituitary and hypothalamus. Mol Cell Endocrinol 210: 1-8.

Morash B, Li A, Murphy PR, Wilkinson M, Ur E. 1999. Leptin gene expression in the brain and pituitary gland. Endocrinology 140: 5995-5998.

Morash B, Wilkinson D, Murphy P, Ur E, Wilkinson M. 2001. Developmental regulation of leptin gene expression in rat brain and pituitary. Mol Cell Endocrinol 185: 151-159.

Mori H, Hanada R, Hanada T, Aki D, Mashima R, et al. 2004. Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. Nat Med 10: 739-743.

Morikawa Y, Ueyama E, Senba E. 2004. Fasting-induced activation of mitogen-activated protein kinases (ERK/p38) in the mouse hypothalamus. J Neuroendocrinol 16: 105-112.

Morton NM, Emilsson V, de Groot P, Pallett AL, Cawthorne MA. 1999. Leptin signalling in pancreatic islets and clonal insulin-secreting cells. J Mol Endocrinol 22: 173-184.

Munzberg H, Flier JS, Bjorbaek C. 2004. Region-specific leptin resistance within the hypothalamus of diet-induced-obese mice. Endocrinology 145: 4880-4889.

Murakami T, Iida M, Shima K. 1995. Dexamethasone regulates obese expression in isolated rat adipocytes. Biochem Biophys Res Commun 214 (3): 1260-1267.

Niswender KD, Morton GJ, Stearns WH, Rhodes CJ, Myers MG Jr, et al. 2001. Intracellular signalling. Key enzyme in leptin-induced anorexia. Nature 413: 794-795.

Opazo P, Watabe AM, Grant SG, O'Dell TJ. 2003. Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. J Neurosci 23: 3679-3688.

Pinto S, Roseberry AG, Liu H, Diano S, Shanabrough M, et al. 2004. Rapid rewiring of arcuate nucleus feeding circuits by leptin. Science 304: 110-115.

Proulx K, Clavel S, Nault G, Richard D, Walker CD. 2001. High neonatal leptin exposure enhances brain GR expression and feedback efficacy on the adrenocortical axis of developing rats. Endocrinology 142: 4607-4616.

Rau H, Reaves BJ, O'Rahilly S, Whitehead JP. 1999. Truncated human leptin (delta133) associated with extreme obesity undergoes proteasomal degradation after defective intracellular transport. Endocrinology 140 (4): 1718-1723.

Rentsch J, Chiesi M. 1996. Regulation of ob gene mRNA levels in cultured adipocytes. FEBS Lett 379 (1): 55-59.

Russo VC, Metaxas S, Kobayashi K, Harris M, Werther GA. 2004. Antiapoptotic effects of leptin in human neuroblastoma cells. Endocrinology 145: 4103-4112.

Saad MF, Damani S, Gingerich RL, Riad-Gabriel MG, Khan A, et al. 1997. Sexual dimorphism in plasma leptin concentration. J Clin Endocrinol Metab 82: 579-584.

Sahu A. 1998. Leptin decreases food intake induced by melanin-concentrating hormone (MCH), galanin (GAL) and neuropeptide Y (NPY) in the rat. Endocrinology 139: 4739-4742.

Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, et al. 1995. Transient increase in obese gene expression after food intake or insulin administration. Nature 377 (6549): 527-529.

Sanna PP, Cammalleri M, Berton F, Simpson C, Lutjens R, et al. 2002. Phosphatidylinositol 3-kinase is required for the expression but not for the induction or the maintenance of long-term potentiation in the hippocampal CA1 region. J Neurosci 22: 3359-3365.

Sarraf P, Frederich RC, Turner EM, Ma G, Jaskowiak NT, et al. 1997. Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. J Exp Med 185 (1): 171-175.

Savioz A, Charnay Y, Huguenin C, Graviou C, Greggio B, Bouras C. 1997. Expression of leptin receptor mRNA (long form splice variant) in the human cerebellum. Neuroreport 8: 3123-3126.

Saxena NK, Titus MA, Ding X, Floyd J, Srinivasan S, et al. 2004. Leptin as a novel profibrogenic cytokine in hepatic stellate cells: mitogenesis and inhibition of apoptosis mediated by extracellular regulated kinase (Erk) and Akt phosphorylation. FASEB J 18: 1612-1614.

Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte D Jr. 1996a. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. Nat Med 2: 589-593.

Schwartz MW, Seeley RJ, Campfield LA, Burn P, Baskin DG. 1996b. Identification of targets of leptin action in rat hypothalamus. J Clin Invest 98: 1101-1106.

Sena A, Sarlieve LL, Rebel G. 1985. Brain myelin of genetically obese mice. J Neurol Sci 68: 233-243.

Shanley LJ, Irving AJ, Harvey J. 2001. Leptin enhances NMDA receptor function and modulates hippocampal synaptic plasticity. J Neurosci 21: RC186.

Shanley LJ, Irving AJ, Rae MG, Ashford ML, Harvey J. 2002a. Leptin inhibits rat hippocampal neurons via activation of large conductance calcium-activated $K^+$ channels. Nat Neurosci 5: 299-300.

Shanley LJ, O'Malley D, Irving AJ, Ashford ML, Harvey J. 2002b. Leptin inhibits epileptiform-like activity in rat hippocampal neurones via PI 3-kinase-driven activation of BK channels. J Physiol 545: 933-944.

Shao LR, Halvorsrud R, Borg-Graham L, Storm JF. 1999. The role of BK-type $Ca^{2+}$-dependent $K^+$ channels in spike broadening during repetitive firing in rat hippocampal pyramidal cells. J Physiol 521: 135-146.

Shekhawat PS, Garland JS, Shivpuri C, Mick GJ, Sasidharan P, et al. 1998. Neonatal cord blood leptin: its relationship to birth weight, body mass index, maternal diabetes, and steroids. Pediatr Res 43: 338-343.

Sinha MK, Ohannesian JP, Heiman ML, Kriauciunas A, Stephens TW, et al. 1996. Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. J Clin Invest 97 (5): 1344-1347.

Slieker LJ, Sloop KW, Surface PL, Kriauciunas A, LaQuier F, et al. 1996. Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. J Biol Chem 271 (10): 5301-5304.

Spanswick D, Smith MA, Groppi VE, Logan SD, Ashford ML. 1997. Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. Nature 390: 521-525.

Steppan CM, Swick AG. 1999. A role for leptin in brain development. Biochem Biophys Res Commun 256: 600-602.

Strobel A, Issad T, Camoin L, Ozata M, Strosberg AD. 1998. A leptin missense mutation associated with hypogonadism and morbid obesity. Nat Genet 18 (3): 213-215.

Swerdloff RS, Batt RA, Bray GA. 1976. Reproductive hormonal function in the genetically obese (ob/ob) mouse. Endocrinology 98: 1359-1364.

Takahashi Y, Okimura Y, Mizuno I, Iida K, Takahashi T, et al. 1997. Leptin induces mitogen-activated protein kinase-dependent proliferation of C3H10T1/2 cells. J Biol Chem 272: 12897-12900.

Takaya K, Ogawa Y, Hiraoka J, Hosoda K, Yamori Y, et al. 1996a. Nonsense mutation of leptin receptor in the obese spontaneously hypertensive Koletsky rat. Nat Genet 14: 130-131.

Takaya K, Ogawa Y, Isse N, Okazaki T, Satoh N, et al. 1996b. Molecular cloning of rat leptin receptor isoform complementary DNAs – identification of a missense mutation in Zucker fatty (*fa/fa*) rats. Biochem Biophys Res Commun 225: 75-83.

Takeda S, Elefteriou F, Levasseur R, Liu X, Zhao L, et al. 2002. Leptin regulates bone formation via the sympathetic nervous system. Cell 111: 305-317.

Takekoshi K, Ishii K, Nanmoku T, Shibuya S, Kawakami Y, et al. 2001. Leptin stimulates catecholamine synthesis in a PKC-dependent manner in cultured porcine adrenal medullary chromaffin cells. Endocrinology 142: 4861-4871.

Tanabe K, Okuya S, Tanizawa Y, Matsutani A, Oka Y. 1997. Leptin induces proliferation of pancreatic beta cell line MIN6 through activation of mitogen-activated protein kinase. Biochem Biophys Res Commun 241: 765-768.

Tang-Christensen M, Holst JJ, Hartmann B, Vrang N. 1999. The arcuate nucleus is pivotal in mediating the anorectic effects of centrally administered leptin. Neuroreport 10: 1183-1187.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, et al. 1995. Identification and expression cloning of a leptin receptor, OB-R. Cell 83: 1263-1271.

Tritos NA, Mastaitis JW, Kokkotou E, Maratos-Flier E. 2001. Characterization of melanin concentrating hormone and preproorexin expression in the murine hypothalamus. Brain Res 895 (1–2): 160-166.

Udagawa J, Hatta T, Naora H, Otani H. 2000. Expression of the long form of leptin receptor (Ob-Rb) mRNA in the brain of mouse embryos and newborn mice. Brain Res 868: 251-258.

Ur E, Wilkinson DA, Morash BA, Wilkinson M. 2002. Leptin immunoreactivity is localized to neurons in rat brain. Neuroendocrinology 75: 264-272.

Vaisse C, Halaas JL, Horvath CM, Darnell JE Jr, Stoffel M, et al. 1996. Leptin activation of Stat3 in the hypothalamus of wild-type and *ob/ob* mice but not *db/db* mice. Nat Genet 14: 95-97.

Van Heek M, Compton DS, France CF, Tedesco RP, Fawzi AB, et al. 1997. Diet-induced obese mice develop peripheral, but not central, resistance to leptin. J Clin Invest 99: 385-390.

Watanobe H. 2002. Leptin directly acts within the hypothalamus to stimulate gonadotropin-releasing hormone secretion in vivo in rats. J Physiol 545: 255-268.

Wang J, Liu R, Hawkins M, Barzilai N, Rossetti L. 1998. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. Nature 393: 684-688.

Wayner MJ, Armstrong DL, Phelix CF, Oomura Y. 2004. Orexin-A (Hypocretin-1) and leptin enhance LTP in the dentate gyrus of rats in vivo. Peptides 25: 991-996.

White DW, Wang DW, Chua SC Jr, Morgenstern JP, Leibel RL, et al. 1997. Constitutive and impaired signaling of leptin receptors containing the Gln → Pro extracellular domain fatty mutation. Proc Natl Acad Sci USA 94: 10657-10662.

Wu-Peng XS, Chua SC Jr, Okada N, Liu SM, Nicolson M, et al. 1997. Phenotype of the obese Koletsky (f) rat due to Tyr763Stop mutation in the extracellular domain of the leptin receptor (Lepr): evidence for deficient plasma-to-CSF transport of leptin in both the Zucker and Koletsky obese rat. Diabetes 46: 513-518.

Yamashita T, Murakami T, Otani S, Kuwajima M, Shima K. 1998. Leptin receptor signal transduction: OBRa and OBRb of fa type. Biochem Biophys Res Commun 246: 752-759.

Yura S, Ogawa Y, Sagawa N, Masuzaki H, Itoh H, et al. 2000. Accelerated puberty and late-onset hypothalamic hypogonadism in female transgenic skinny mice overexpressing leptin. J Clin Invest 105: 749-755.

Zabolotny JM, Bence- Hanulec KK, Stricker-Krongrad A, Haj F, Wang Y, Minokoshi Y, Kim YB, Elmquist JK, Tartaglia LA, Kahn BB, Neel BG. 2002. PTP1B regulates leptin signal transduction in vivo. Dev Cell 2: 489-495.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, et al. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372 (6505): 425-432.

Zhao AZ, Bornfeldt KE, Beavo JA. 1998. Leptin inhibits insulin secretion by activation of phosphodiesterase 3B. J Clin Invest 102: 869-873.

Zhao AZ, Huan JN, Gupta S, Pal R, Sahu A. 2002. A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. Nat Neurosci 5: 727-728.

Zhao AZ, Shinohara MM, Huang D, Shimizu M, Eldar-Finkelman H, et al. 2000. Leptin induces insulin-like signaling that antagonizes cAMP elevation by glucagon in hepatocytes. J Biol Chem 275: 11348-11354.

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