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Qun-Yong Zhou
Editors

Results and Problems in Cell Differentiation 46

Orphan G Protein-Coupled Receptors and Novel Neuropeptides

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46

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Orphan G Protein-Coupled Receptors and Novel Neuropeptides

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Introduction

Most of the G protein-coupled receptors (GPCRs) started as "orphans": receptors for which natural ligands are unknown. Orphan GPCRs are older than many researchers believe, they have actually been with us since 1987. The first was reported as a putative receptor called G-21, later found to be the serotonin 5-HT-1a receptor. Indeed, G-21 had all of the advantages and disadvantages that characterized the orphan GPCRs that followed. They are exciting for researchers because they offer novelty to basic and potential to applied researches. At the same time they are annoying for the researcher because they cannot be used in pharmacological assays for lack of a natural ligand. In spite of this, orphan GPCRs have gone far, they have been found in large numbers—more than any other gene family—and many have been "deorphanized", i.e., matched to their natural ligands. It is noteworthy that only one year passed between the report of the first orphan GPCR and its deorphanization and that this was paralleled by the concomitant deorphanization of unrelated GPCRs, showing how quickly the field was growing. Furthermore, discussion of orphan GPCRs is not complete without mention that it was the discovery of one family of orphan GPCRs, the olfactory receptors, which set the stage for our better understanding of olfaction.

But foremost, orphan GPCRs are GPCRs. GPCRs, because of their diversity, stand as the major regulators of intercellular communications. They initiate or modulate a wide variety of physiological and behavioral responses. They initiate their actions through activation by specific natural ligands. These ligands are mostly small molecules, such as neurotransmitters, neuropeptides, or lipid mediators but also include the larger glycoprotein hormones. Presently, the number of human GPCRs is estimated to be about 800, of which more than half are sensory receptors. They all exhibit the seven transmembrane domain topology of rhodopsin, the first sequenced GPCR. They form a supergene family implying that they are phylogenetically related and thus that the closest relatives share sequence similarities. Indeed, the vast majority of GPCRs have been found on the basis of expected sequence similarities to already cloned GPCRs. At first they were discovered by applying homology screening approaches and, more recently, using bioinformatics. But the GPCRs found this way suffer from one drawback, they are orphans, they lack the natural ligands that activate them.

Yet the fact that most orphan GPCRs were found to be conserved in higher organisms showed that they carry functional significance, i.e., that they act as G protein-linked receptors. Consequently, most of them should have a natural ligand. In the second part of the 1980s, there existed about 50 transmitters, potential GPCR ligands, which had no cloned cognate receptors. Testing all of these for their binding to new GPCRs was seen as a risky task. Yet hard work, serendipity, and ingenious insight, proved to be successful in matching many orphan GPCRs to known transmitters. To achieve this, the orphan GPCR was expressed by DNA transfection in eukaryotic cells, membranes of these cells were then used as targets to determine the binding of potential transmitters. This strategy has become to be known as reverse pharmacology.

During the first part of the 1990s, however, the overall number of orphan GPCRs was steadily increasing and began to outnumber the known naturally occurring ligands. This led to the conclusion that these receptors must bind ligands that have not been thus far characterized, because inactive receptors should be evolutionarily discarded. This recognition inspired enough confidence in a few researchers (several are contributing to this book) to utilize orphan GPCRs as bait to isolate their natural ligands, which meant identification of novel neurotransmitters or neuropeptides. This approach has led to the discovery of 11 families of novel bioactive peptides, orphanin FQ/nociceptin, the orexins/hypocretins, prolactin-releasing peptide, apelin, ghrelin, kisspeptin/metastin, neuropeptides B and W, prokineticins 1and 2, relaxin 3, neuropeptide S, and neuromedin S. In addition, this approach also led to the discovery of the receptors for melanin-concentrating hormone, urotensin II, and neuromedin U. These discoveries span a period of over ten years.

The discovery of these novel bioactive peptides has opened up new directions for basic as well as applied research. Because most of these bioactive peptides are expressed in the central nervous system, they are viewed as neuropeptides, and deciphering their functional significance has often focused on their implications in brain-directed responses. This book will review 11 of the neuropeptides that were found through or linked to orphan GPCRs. Most of the chapters are written by the researchers who originally deorphanized the receptors. Our aim has been to focus on what has been learned about the roles of these deorphanized systems in the organism. However, we have to remember that many of these discoveries occurred only a few years ago and thus our understanding of their functions is still at an early stage. Yet, these chapters will stand as the state-of-the-art for the field of deorphanization of neuropeptide GPCRs and thus be of interest to biologists interested in new physiological responses and to pharmacologists interested in new therapeutic targets.

Irvine, March 31, 2008

Olivier Civelli
Qun-Yong Zhou

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The Orphanin FQ/Nociceptin (OFQ/N) System

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Abstract Orphanin FQ/nociceptin (OFQ/N) was the first novel neuropeptide discovered as the natural ligand of an orphan G protein-coupled receptor (GPCR). Orphan GPCRs are proteins classified as receptors on the basis of their sequence similarities to known GPCRs but that lack the ligands that activate them in vivo. One such orphan GPCR exhibited sequence similarities with the opioid receptors. OFQ/N was isolated as its natural ligand and shown to also share sequence similarities to the opioid peptides. This led to numerous studies attempting to find functional similarities and differences between the OFQ/N and opioid systems. This chapter will summarize our knowledge of the OFQ/N system and of its roles in the organism.

Keywords Anxiety · Drug dependence · Memory · Neuropeptide · Orphan GPCR · Pain · Stress

Abbreviations

OFQ/N Orphanin FQ/nociceptin
GPCR G protein-coupled receptor
NOP OFQ/N receptor

1 Discovery of OFQ/N

The G protein-coupled receptor (GPCR) ORL-1, also called XOR-1, ROR-C, LC132, Hyp8-1, and C (Bunzow et al. 1994; Chen et al. 1994; Fukuda et al. 1994; Lachowicz et al. 1995; Mollereau et al. 1994; Wang et al. 1994; Wick et al. 1995), had been found through its similarities to the opioid receptors and is known to bind none of the opioid peptides or synthetic opiates (Bunzow et al. 1994; Mollereau et al. 1994; Wang et al. 1994). Its natural ligand was therefore unknown, making it part of the orphan GPCRs.

Purification of the natural ligand of the ORL-1 receptor was achieved simultaneously in two different laboratories starting from either rat or porcine brain extracts (Meunier et al. 1995; Reinscheid et al. 1995). ORL-1 cDNA was transfected into cells that were exposed to the different extracts. Because of its similarities to the opioid receptors, it was assumed that ORL-1 might also bind a peptidergic ligand and share the same coupling mechanism to second

messenger systems as that of the opioid receptors, i.e. inhibition of adenylyl cyclase activity. Extracts were fractionated and active fractions were further purified. Both approaches led to the identification of a 17-residue long peptide with the primary structure FGGFTGARKSARKLANQ. This peptide was named orphanin FQ (OFQ, to mark its origin or nociceptin) (Reinscheid et al. 1995) (N, because of its hyperalgesic activity) (Meunier et al. 1995). Consequently, the ORL1 receptor was classified as the fourth member of the opioid receptor family and renamed OFQ/N peptide (NOP) receptor by NC-IUPHAR.

2 Pharmacology and Cellular Responses Induced by OFQ/N

OFQ/N was first shown to inhibit forskolin-stimulated cAMP accumulation in NOP-transfected cells with a median effective concentration of 1.05 ± 0.21 nM and a maximal effect of ~80% inhibition at 100 nM (Meunier et al. 1995; Reinscheid et al. 1995). To investigate its binding constants, a radioligand was developed (Reinscheid et al. 1995). Because OFQ/N does not contain Tyr residues, a series of Tyr-substituted peptide analogs were synthesized. The Tyr^{14}-substituted OFQ/N was shown to be an agonist with equivalent potency in cAMP assays as the unsubstituted OFQ/N (EC_{50} values of 1.02 ± 0.11 nM). The ^{125}I-labeled Tyr^{14}-substituted peptide displayed saturable, displaceable and reversible binding to membranes of opioid-like orphan receptor transfected cells with a K_d of 0.1 ± 0.02 nM (Reinscheid et al. 1995). Its binding constants are well in the range of affinities observed for other neuropeptides.

Within a short time of its discovery, the OFQ/N receptor was shown to induce a variety of intracellular effects. First, as described above, OFQ/N receptor was shown to induce an inhibition of adenylyl cyclase in CHO cells transfected with the NOP receptor (Meunier et al. 1995; Reinscheid et al. 1995). Next, modulation of cellular excitability was detected when OFQ/N was found to increase inwardly rectifying K-conductance in dorsal raphe nucleus neurons (Vaughan et al. 1996) and in the arcuate nucleus (Wagner et al. 1998); to increase K-conductance in periaqueductal gray neurons (Vaughan et al. 1996) and in locus coeruleus neurons (Connor et al. 1996a); to couple to G protein-activated K channels (Ikeda et al. 1997); to inhibit voltage-gated calcium channels in freshly dissociated CA3 hippocampal neurons (Knoflach et al. 1996); to inhibit T-type Ca channels in sensory neurons (Abdulla et al. 1997); and to inhibit N-type Ca channels in SH-SY5Y cells (Connor et al. 1996b). Also, the OFQ/N receptor appears to couple to K channels in Xenopus oocytes (Matthes et al. 1996). Furthermore, OFQ/N has been shown to inhibit the release of glutamate and GABA from nerve terminals (Faber et al. 1996; Nicol et al. 1996), to block acetylcholine release from retina (Neal et al. 1997) and parasympathetic nerve terminals (Patel et al. 1997), to inhibit synaptic transmission and long-term potentiation in the hippocam-

pus (Yu et al. 1997), to suppress dopamine release in the nucleus accumbens (Murphy et al. 1996), and to inhibit tachykinin and calcitonin gene-related peptide release from sensory nerves (Giuliani et al. 1996; Helyes et al. 1997). OFQ/N was demonstrated to activate mitogen-activated protein kinase in receptor transfected CHO cells (Fukuda et al. 1997). Together, these results show that OFQ/N is able to modulate the biochemical properties of cells, alter the electrophysiological properties of neurons and to affect their transmitter release. In organotypic assays, OFQ/N has been shown to inhibit electrically induced contractions of the vas deferens, ileum, and myenteric plexus preparations (Berzetei-Gurske et al. 1996; Calo et al. 1996; Nicholson et al. 1998; Zhang et al. 1997). Importantly, none of the effects of OFQ/N described were inhibited by opiate antagonists, emphasizing the pharmacological difference between the opioid and the OFQ/N systems (see below).

These data exemplify the breadth of responses that the OFQ/N system may modulate, and merely underscore the fact that the expression of the NOP receptor in a particular neuronal system is sufficient to expect that OFQ/N may modulate this system.

3
Synthesis and Inactivation of OFQ/N

Like all bioactive peptides, OFQ/N is synthesized as part of a larger polypeptide precursor (preproOFQ/N, ppOFQ/N), which has been cloned from rat, human (Mollereau et al. 1996; Nothacker et al. 1996), mice (Saito et al. 1996), and bovine (Okuda-Ashitaka et al. 1998). The primary structure of the precursor protein contains the typical structural elements of a neuropeptide precursor. It starts with an amino terminal signal peptide necessary for its secretion. The OFQ/N sequence is flanked by pairs of Lys-Arg residues, indicating that its maturation requires trypsin-like cleavages (Fig. 1).

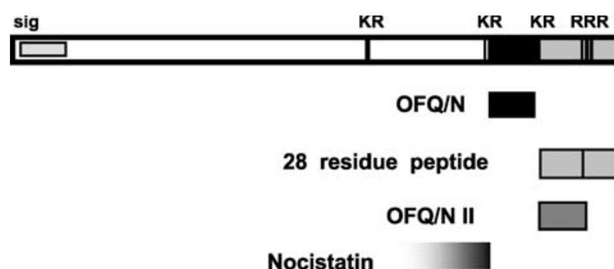

Fig. 1 Synthesis of OFQ/N, showing the structure of the OFQ/N precursor. The bioactive peptides potentially generated by processing are described relative to their corresponding location in the precursor. The nocistatin N-terminus is not described due to its lack of identity in different species. *Sig* signal peptide, *K* Lys, *R* Arg

But, these are not the only putative sites for precursor processing. The C-terminus of the precursor protein downstream of the OFQ/N sequence is conserved among the different species genes and could generate either a 28-residue long peptide or, after cleavage at the Arg triplet, a 17-residue long peptide, whose terminal amino acids are the same as these of OFQ/N (Fig. 1). These two peptides were synthesized but were unable to either bind or activate the NOP receptor. It has been reported that the 17-amino acid peptide (termed NocII or OFQ II) exhibits some effect on locomotion and pain perception (Florin et al. 1997b; Rossi et al. 1998).

The bovine precursor harbors an additional pair of basic amino acids N-terminal to the OFQ/N sequence that could give rise to a 19-amino acid peptide (Okuda-Ashitaka et al. 1998). This putative was isolated from bovine bran and has been reported to possess an anti-OFQ/N activity because it was able to block OFQ/N-induced allodynia and hyperalgesia (Okuda-Ashitaka et al. 1998). This peptide was named nocistatin (Fig. 1) and acts via a receptor different from the NOP receptor. The active part of nocistatin was found to reside in its C-terminal hexapeptide that is also the only conserved structure between all mammalian OFQ/N precursors. The bovine form of nocistatin appears to be species-specific because the human, rat, mouse, and porcine precursor lack the pair of basic amino acids that is used for processing in the bovine precursor protein. The fact that OFQ II or nocistatin require their own receptors but that none have been identified that would exhibit selectivity for these two peptides indicate that these peptides may not be neuropeptides that act through a traditional GPCR system.

Inactivation of OFQ/N has also been studied. Metallopeptidases play a major role. The critical sites of enzymatic cleavage are Phe1–Gly2, Ala7–Arg8, Ala11–Arg12, and Arg12–Lys13 bonds. Aminopeptidase N and endopeptidase 24.15 are the two main enzymes involved in OFQ/N metabolism. Endopeptidase 24.11, which is involved in enkephalin catabolism, does not appear to be critically involved (Montiel et al. 1997; Noble et al. 1997).

4
The OFQ/N and Opioid Systems

There are striking sequence similarities between the OFQ/N and opioid systems. First, the N-terminus of OFQ/N, FGGF, is highly reminiscent of the canonical YGGF of the opioid peptides. Then, the NOP receptor shares more than 60% identity with the three opioid receptors (Bunzow et al. 1994; Mollereau et al. 1994). Furthermore, the OFQ/N precursor protein exhibits several analogous structures as compared to the opioid precursors: the active peptides are located in the C-terminal part and seven Cys residues are found conserved at the N-terminus of ppOFQ/N, prodynorphin, and proenkephalin (Mollereau et al. 1996; Nothacker et al. 1996). Finally, the OFQ/N precursor

gene has retained an intron–exon organization similar to that of the opioid precursor genes (Mollereau et al. 1996)]. The coding sequence is divided over two exons, the smaller one containing the translational start site (AUG), the other encoding the rest of the open reading frame. From this common architecture of the opioid precursor genes, the OFQ/N precursor gene differs in that it contains an additional exon for the 3′-untranslated region of the mRNA. In humans, the OFQ/N gene has been mapped to the chromosomal location 8p21 (Mollereau et al. 1996). Altogether, these data support the view that the receptors, as well as the neuropeptide precursors of both the opioid and the OFQ/N systems, have evolved from common ancestral genes.

While the OFQ/N and opioid systems share significant similarities at the sequence level it has been shown that OFQ/N does not activate opioid receptors nor do the opioid peptides elicit biological activity at the OFQR. The basis for this selectivity is inherent to the primary structures of OFQ/N and the opioid peptides. A series of truncated and/or chimeric peptides led to the conclusion that OFQ/N and dynorphin A, its closest counterpart, contain domains that specifically act to prevent cross-activation of other but their own receptors (Reinscheid et al. 1998). These domains are composed of single residues in key positions together with short stretches of amino acids that do not overlap in both peptides (Fig. 2). It has been further demonstrated that by mutating as few as four amino acids, a receptor can be produced that recognizes dynorphin with very high affinity and yet still binds OFQ/N as well as the wild-type receptor. This indicates that the NOP has evolved features that specifically exclude opioid binding and that these features are distinct from those required for the binding of OFQ/N (Meng et al. 1996) Together these

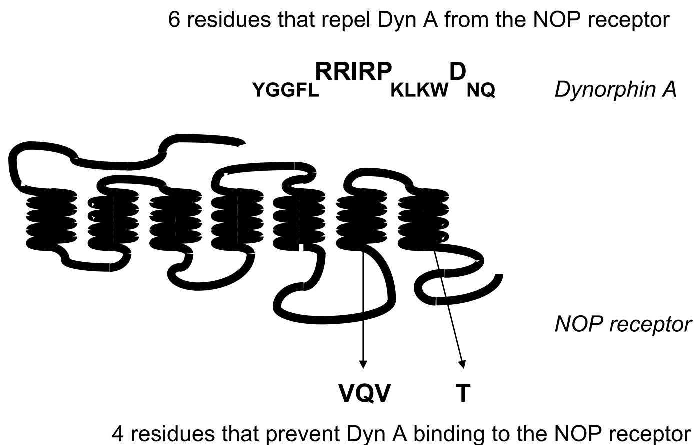

Fig. 2 Configuration of the NOP receptor and of dynorphin A. Dynorphin A's residues *RRIRP* and *D* have been shown to repel dynorphin A from the NOP receptor, while NOP's residues *VQV* and *T* prevent dynorphin A from binding (see text)

data show that while evolutionary related, the OFQ/N and opioid systems have evolve to be pharmacologically distinct.

5
Tissue Distribution of the OFQ/N System

The OFQ/N system is predominantly a CNS system. In situ hybridization, immunhistochemistry, and receptor radiolabeling have been applied to it and have given us a complete understanding of its distribution at the ligand and receptor levels (Anton et al. 1996; Bunzow et al. 1994; Florin et al. 1997a; Houtani et al. 1996; Ikeda et al. 1998; Lachowicz et al. 1995; Lai et al. 1997; Makman et al. 1997; Mollereau et al. 1994; Neal et al. 1999a,b; Nothacker et al. 1996; Pan et al. 1996; Riedl et al. 1996; Schuligoi et al. 1997; Schulz et al. 1996; Sim et al. 1996).

At the receptor level, OFQ/N binding sites have been found to be densest in several cortical regions, the anterior olfactory nucleus, lateral septum, ventral forebrain, several hypothalamic nuclei, hippocampal formation, basolateral and medial amygdala, central gray, pontine nuclei, interpeduncular nucleus, substantia nigra, raphe complex, locus coeruleus, vestibular nuclear complex, and the spinal cord (Florin et al. 1997a; Lai et al. 1997; Makman et al. 1997; Neal et al. 1999a; Riedl et al. 1996; Schuligoi et al. 1997; Schulz et al. 1996; Sim et al. 1996). On the other hand, as determined by in situ hybridization, cells expressing NOP mRNA were most numerous throughout multiple cortical regions, the anterior olfactory nucleus, lateral septum, endopiriform nucleus, ventral forebrain, multiple hypothalamic nuclei, nucleus of the lateral olfactory tract, medial amygdala, hippocampal formation, substantia nigra, ventral tegmental area, central gray, raphe complex, locus coeruleus, multiple brainstem motor nuclei, inferior olive, deep cerebellar nuclei, vestibular nuclear complex, nucleus of the solitary tract, reticular formation, dorsal root ganglia, and spinal cord. Quantitatively, prominent receptor expression was observed in the neocortex, cingulate and piriform cortex, hippocampus, anterior olfactory nucleus, cortical amygdala, claustrum, and endopiriform nucleus. Moderate contents of NOP receptor were found in the central and medial amygdala, dentate gyrus, subiculum, entorhinal cortex, dorsal and ventral pallidum, triangular and medial septum, medial preoptic area, mammilary bodies, and parafascicular and posterior thalamic nuclei, but also at a lesser level in the olfactory system, lateral septum, basal forebrain, thalamus, and hypothalamus (Anton et al. 1996; Mollereau et al. 1994; Neal et al. 1999a) (Fig. 3).

At the neuropeptide level, a good correlation has been found between the distribution of the ppOFQ/N mRNA and that of the OFQ/N peptide measured by immunohistochemistry (Houtani et al. 1996; Mollereau et al. 1996; Neal et al. 1999b; Nothacker et al. 1996; Pan et al. 1996). Both are found in the bed nucleus of the stria terminalis, amygdala, lateral septum, cortex, thalamus,

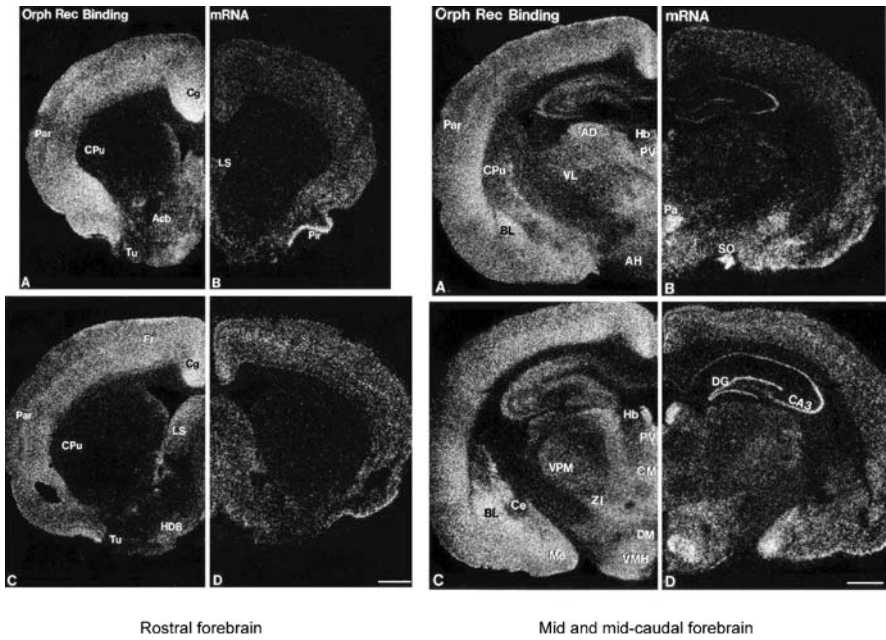

Fig. 3 Darkfield autoradiograms comparing ^{125}I-[Tyr^{14}]-OFQ binding (**a, c**) and orphanin receptor mRNA expression (**b, d**) at representative levels of the rostral forebrain (*left panels*) and of the mid- and mid-caudal forebrain (*right panels*). For abbreviations, see Neal et al. (1999a)

striatum, substantia nigra, hypothalamus, and the trigeminal nucleus. A characteristic scattered distribution of neurons expressing the ppOFQ/N has been observed in the cortex and hippocampus of the rat. Noteworthy, OFQ/N immunoreactivity differs significantly from immunohistochemical localization of the NOP receptor in that it appears much more restricted. Such discrepancies between ligand and receptor distribution have been described in other transmitter systems and may underline the differences between OFQ/N sites of synthesis and sites of action. In the brainstem, a dense network of OFQ/N immunoreactive fibers is observed in many areas (Neal et al. 1999a,b), in particular in the substantia nigra, the locus coeruleus, the tegmental nuclei, several vestibular nuclei, the superior colliculus, and the pons. Intense OFQ/N-positive staining has been detected in the superficial layers of the dorsal horn in the spinal cord, whereas little immunoreactivity was found in the cerebellum. ppOFQ/N mRNA expression was observed in most of the brainstem and spinal cord areas where OFQ/N-immunoreactivity had been detected. Together these data show that OFQ/N is widely expressed and can thus act at numerous sites within the CNS.

The pattern of expression of NOP mRNA in the human, rat, and mouse brains at various developmental stages has also been analyzed (Ikeda et al. 1998;

Neal et al. 2001). In the rat, both NOP and ppOFQ mRNA are detected as early as E12 in the cortical plate, basal forebrain, brainstem, and spinal cord. Expression for both NOP and ppOFQ is strongest during the early postnatal period, remaining strong in the spinal cord, brainstem, ventral forebrain, and neocortex into the adult. In human NOP and ppOFQ expression is observed at 16 weeks gestation, remaining relatively unchanged up to 36 weeks (Neal et al. 2001).

The tissue localization of the OFQ/N system has also been analyzed in peripheral organs. NOP receptor mRNA has been detected in the vas deferens, the intestine, the liver, and the spleen (Wang et al. 1994). The ppOFQ/N mRNA has been found expressed in human spleen and fetal kidney (Nothacker et al. 1996) and at a low level in the ovaries (Mollereau et al. 1996). A functional implication for OFQ/N in the kidney was suggested by a study describing diuretic and antinatriuretic effects after OFQ/N administration (Kapusta et al. 1997). The presence of OFQ/N peptide or expression of the OFQ/N precursor have also been demonstrated in the rat and porcine intestine, respectively, together with a functional role in the regulation of intestinal contractility (Osinski et al. 1999; Yazdani et al. 1999).

The broad distribution of NOP mRNA and OFQ/N binding sites, which mark the sites of action of OFQ/N, supports an extensive role for the OFQ/N system in a multitude of CNS functions. Combined with the data on OFQ/N sites of expression, determined through precursor and peptide analyses, one can infer that the OFQ/N system will have a modulatory effect in motor and balance control, reinforcement and reward, nociception, the stress response, sexual behavior, aggression, and autonomic control of physiologic processes.

6 The Functional Implications of the OFQ/N System

Studies on the role of the OFQ/N system in physiological and behavioral responses began from the time of the discovery of the system and first relied on administration of the neuropeptide. Soon after, NOP- (Manabe et al. 1998) and OFQ/N-deficient (Koster et al. 1999) mice strains were generated by disrupting the NOP and ppOFQ/N genes, respectively, by homologous recombination in embryonic stem cells. Finally, synthetic agonists and antagonists were developed. Together these tools have allowed us to gain a broad knowledge of the function of the OFQ/N system in the organism.

6.1 Locomotion

OFQ/N was first reported to significantly induce a dose-dependent decrease in locomotor activity (i.e., hypolocomotion) when administered centrally

(Reinscheid et al. 1995). This finding was indeed the first to suggest that OFQ/N possesses biological activity in vivo. Then, OFQ/N was found to have a biphasic effect on locomotion, increasing it at low doses while decreasing it at high doses (Florin et al. 1997b). The locomotor-inhibiting effects of high doses of OFQ/N seen in wild-type animals were not seen in the NOP KO mice, demonstrating that they are NOP-mediated. However the NOP KO mice and OFQ/N KO mice do not display any evidence of basal hyperactivity (Nishi et al. 1997), which indicates that the OFQ/N system is not a major player in the regulation of locomotion.

6.2 Pain

The sequence similarities between OFQ/N and the opioid peptides was the impetus to study the role of the OFQ/N system in pain regulation. OFQ/N elicits controversial effects in pain regulation: it is hyperalgesic or analgesic depending on whether administered supraspinally or spinally, respectively (Heinricher 2003; Mogil et al. 2001).

The first report on the central administration of OFQ/N showed its hyperalgesic effects, thus its name nociceptin (Meunier et al. 1995). However, soon after, the hyperalgesic effect of i.c.v. OFQ/N in mice was found to result from an inhibition of stress-induced analgesia (Mogil et al. 1996). Since then, practically all the studies conducting i.c.v. blockade of NOP receptors have obtained antinociceptive responses. Morphine-induced supraspinal analgesia is also enhanced by OFQ/N blockade, which suggests that OFQ/N produces a basal nociceptive tone at the supraspinal level. The tolerance induced by low doses of morphine was reversed by NOP receptor antagonists and was absent in NOP or OFQ/N KO mice. However, the tolerance developed by escalating doses of morphine was not prevented in OFQ/N KO mice (Chung et al. 2006; Kest et al. 2001). Therefore, the supraspinal nociceptive tone of endogenous OFQ/N may contribute to the tolerance development by low doses of morphine, and antagonists may be developed to counter opiate tolerance.

At the spinal level, OFQ/N was found to have an analgesic effect in rodents (Calo et al. 2000; Meunier 1997; Mogil et al. 2001; Zeilhofer et al. 2003) and primates (Ko et al. 2006) yet the role of the OFQ/N system in pain regulation at the spinal level remains controversial. Some studies using different NOP antagonist injections intrathecally suggest that the OFQ/N system plays a protective role in inflammatory but not acute pain (Fu et al. 2006; Rizzi et al. 2006; Yamamoto et al. 2001; Yu et al. 2002). However, opposite results have also been reported for other OFQ/N antagonists in inflammatory (Fu et al. 2006) or neuropathic pain models (Muratani et al. 2002). The OFQ/N system has been also reported to play a role in allodynia (Obara et al. 2005; Tamai et al. 2005).

Plasma and CSF OFQ/N levels have been measured by radioimmunoassay in human subjected to different pain states (Barnes et al. 2004). No significant change was found in the CSF of subjects suffering from labor pain, low back pain, or fibromyalgia. On the other hand, in fibromyalgia and cluster headache attack, and in patients suffering migraine attacks, the OFQ/N plasma levels were significantly lower (Ertsey et al. 2005), while they were elevated in patients in states of either acute or chronic pain.

Nociceptive responses to acute noxious heat in NOP KO, ppOFQ/N KO and double KO mice were indistinguishable from those of wild-type mice. However, NOP KO, ppOFQ/N KO and double KO mice showed markedly stronger nociceptive responses during prolonged nociceptive stimulation. These results indicate that the OFQ/N system contributes significantly to endogenous pain control during prolonged nociceptive stimulation but does not affect acute pain sensitivity (Depner et al. 2003).

These data show that the role of the OFQ/N system in pain regulation is still elusive and that it may differ when acting spinally or supraspinally. Yet, many surrogate antagonist molecules have been developed by the pharmaceutical industry and filed for the management of different pain indications or migraine and should help us understand the function of the OFQ/N system in these therapeutic indications.

6.3 Drug Dependence

In view of its relationships to the opioid system, the OFQ/N system was tested for its role in reward. It was found that neither exogenous OFQ/N on its own, nor the synthetic agonist Ro64-6198, induce conditioned place preference (CPP) (Ciccocioppo et al. 2000; Devine et al. 1996). Instead, OFQ/N was found to have an inhibitory effect on the rewarding system. It reduces morphine withdrawal (Kotlinska et al. 2004; Ueda et al. 2000) and the CPP induced by morphine (Sakoori et al. 2004), cocaine (Kotlinska et al. 2002; Sakoori et al. 2004), amphetamine (Kotlinska et al. 2003), and alcohol (Ciccocioppo et al. 2004b) but not heroin (Murphy et al. 2002). The inhibitory effect of OFQ/N may be explained by inhibition of mesolimbic dopamine release (Koizumi et al. 2004) or may be attributed to inhibition of GABAergic transmission in the central amygdala (Roberto et al. 2006).

The role of the endogenous OFQ/N system in the rewarding response has been analyzed using KO mice and the synthetic antagonist J-113397. Ueda et al. (2000) and Chung et al. (2006) found that NOP KO or OFQ/N KO mice exhibit attenuated development of morphine tolerance as compared to wild-type animals. While the studies by Kest et al. (2001) and Mamiya et al. (2001) contradict these results it should be noted that both of these last studies used doses of morphine that produce OFQ/N-insensitive analgesic tolerance.

6.4 Anxiety and Stress

The observation that OFQ/N is able to reverse stress-induced analgesia (Mogil et al. 1996) and that the NOP receptor is expressed in brain centers known to modulate anxiety prompted investigations into the effects of OFQ/N on stress as it relates to anxiety. Central administration of OFQ/N was found to exert profound anxiolytic effect comparable to classic anxiolytic drugs such as diazepam (Jenck et al. 1997). Low nanomolar doses of OFQ/N decreased behavioral measures of anxiety in rats using the light–dark box and elevated plus maze paradigms. Similar to the biphasic effect of diazepam, higher doses of OFQ/N also showed motor impairment. These findings were later confirmed with mice in the elevated plus maze paradigm (Gavioli et al. 2002) or the hole-board exploration test (Kamei et al. 2004) and in the defense test battery (Griebel et al. 1999), although in the latter paradigm, the effects were observed only after very high stress. Altogether, these experiments provided evidence that the OFQ/N system might be involved in modulation of stress as it relates to anxiety. This conclusion was further supported by two other lines of research.

First, mice devoid of OFQ/N precursor (OFQ/N KO mice) were found to display signs of impaired stress adaptation and increased anxiety-like behavior (Koster et al. 1999), as were antisense-NOP treated rats (Blakley et al. 2004). This effect was, however, not observed in mice devoid of NOP (Mamiya et al. 1998). Interestingly, OFQ/N KO mice failed to habituate to repeated acute stress, such as swim stress, indicating a possible role of OFQ/N in stress adaptation. Also, OFQ/N knockout mice exhibited chronically increased stress-induced analgesia when housed in groups, an environmental condition that may be a source of chronic mild stress. Indeed it was later shown that group housing conditions also increase anxiety-like behavior in knockout animals (Ouagazzal et al. 2003).

Second, a non-peptide NOP receptor agonist (Ro 64-6198) was developed that displays nanomolar affinity and 100-fold selectivity over the opioid receptors. When administered i.p. in rats this agonist produced anxiolytic-like effects in a variety of paradigms such as the elevated plus maze (Jenck et al. 2000), pup isolation-induced ultrasonic vocalization (Varty et al. 2005), fear-potentiated startle (Jenck et al. 2000), Geller–Seifter conflict (Jenck et al. 2000), and conditioned lick suppression (Varty et al. 2005). The anxiolytic-like effects in the elevated plus maze did not show tolerance during a 2 week study (Dautzenberg et al. 2001). In mice, the anxiolytic-like effects of Ro 64-6198 were more difficult to demonstrate because of increased disruptive effects (Jenck et al. 2000). However, it was shown to exhibit selective anxiolytic-like effects in a combined marble burying/locomotor activity test (Nicolas et al. 2006), and in the Geller–Seifter conflict assay (Varty et al. 2005).

The mechanism by which the OFQ/N system modulates stress is not fully understood but implicates regulation of the hypothalamic–pituitary–adrenal (HPA) axis. OFQ/N KO mice exhibit elevated plasma corticosterone levels under both resting and post-stress conditions (Reinscheid et al. 2002), and OFQ/N administrations reverse transient increases in plasma corticosterone precipitated by stressors such as intracerebroventricular injections. Yet glucocorticoid levels were found unchanged in NOP KO mice (Devine et al. 2001; Uezu et al. 2004) while another study reported that central OFQ/N administration increases corticosterone levels (Devine et al. 2001). So, there are indications that the OFQ/N system plays a role in the modulation of the HPA axis, but that this role is rather complex and possibly species-dependent since, in rats, plasma corticosterone levels were found to decrease in antisense-NOP treated rats (Blakley et al. 2004) whereas OFQ/N i.c.v. administrations elevated circulating corticosterone (Fernandez et al. 2004; Vitale et al. 2006).

6.5 Learning and Memory

The first observation that the OFQ/N system may be involved in memory was reported by showing that the performance of NOP KO mice in the water maze (Manabe et al. 1998), passive avoidance (Mamiya et al. 1999), and fear conditioning (Mamiya et al. 2003) tests (but not in the Y-maze test (Mamiya et al. 1999)) were better than those of wild-type mice. OFQ KO mice also show enhanced performances in passive avoidance (Higgins et al. 2002) (but not in the water maze (Koster et al. 1999)). These observations have been corroborated by the studies that showed that central administrations of OFQ/N impair learning and memory performance in mice and rats in water maze (Sandin et al. 2004), fear conditioning (Mamiya et al. 2003), Y-maze, and passive avoidance tests (Hiramatsu et al. 1999; Mamiya et al. 1999). Biphasic effects, enhancement at low doses but impairment at higher doses, were observed in the rat water maze test when OFQ/N was given by intra-hippocampal injection (Sandin et al. 2004) or in the Y-maze and passive avoidance tests (Hiramatsu et al. 2000).

6.6 Depression

The first indication that the OFQ/N system may be involved in modulating depressive states of behavior came from the observation that rat pups who were separated from their mothers exhibit elevated levels of OFQ/N expression in adolescence (Ploj et al. 2001, 2002). That the OFQ/N system may have a role in depression-like symptoms is further indicated by the finding that OFQ/N levels are elevated in postpartum depressive women (Gu et al.

2003) but, more importantly, by studies that showed that OFQ/N antagonists could produce antidepressant effects, such as reduced immobility time in the mouse forced-swim test (Gavioli et al. 2002, 2003; Redrobe et al. 2002), which reflects behavioral despair. Moreover, NOP KO mice also display reduced immobility time in the same assay (Gavioli et al. 2003). The mechanism for the antidepressive effects of OFQ/N antagonists is unknown. OFQ/N is able to attenuate serotonin release from dorsal raphe neurons (Vaughan et al. 1996) and rat cortical serotonergic terminals (Sbrenna et al. 2000), thus acting to reduce serotonin levels. Conversely, OFQ/N antagonists are able to block the OFQ/N-induced inhibition of noradrenaline and serotonin release from cortical synaptosomes (Marti et al. 2003; Mela et al. 2004; Sbrenna et al. 2000).

6.7 Seizures

OFQ/N inhibits glutamate release and blocks T-type Ca channels, which participate in neuronal excitability regulation (Calo et al. 2000). The OFQ/N system may have thus a role in epilepsy. In animals subjected to kainate-induced seizures, OFQ/N release and OFQ/N precursor mRNA levels have been found elevated (Aparicio et al. 2004; Bregola et al. 2002). It is unclear, however, whether these changes may be a cause or an effect of the seizures. OFQ/N itself has been shown to raise the convulsive threshold in seizures induced by pentylenetetrazole, *N*-methyl D-aspartic acid, bicucculine (Rubaj et al. 2002) and penicillin (Feng et al. 2004) but not by electrical shock (Rubaj et al. 2002). Yet, susceptibility to kainate seizures was decreased upon antagonist treatment (J-113397) (Bregola et al. 2002) and in OFQ/N KO mice (Binaschi et al. 2003; Bregola et al. 2002). These observations suggest that the endogenous activation of the OFQ/N system may facilitate the expression of limbic seizures.

6.8 Food Intake

The OFQ/N system has also been shown to modulate food intake. OFQ/N injected i.c.v. increases food consumption in satiated rats and food-deprived rats. This orexigenic effect is thought to result predominantly from inhibition of anorexigenic systems such as the oxytocinergic neurons in the paraventricular nucleus and the alpha-MSH neurons in the arcuate nucleus (Olszewski et al. 2004). OFQ/N has also been found to inhibit stress-induced anorexia at doses that not elicit hyperphagia. In this case, OFQ/N may act as a functional antagonist of the corticotrophin-releasing factor system in the bed nucleus of the stria terminalis (Ciccocioppo et al. 2004a). Two peptidergic antagonists when administered i.c.v. did not affect food intake (Economidou et al. 2006;

Polidori et al. 2000), and one reduced it in food-deprived rats (Polidori et al. 2000). The OFQ/N system may thus display an orexigenic role in response to food deprivation but not in normal feeding.

6.9
Cardiovascular and Renal Systems

OFQ/N administered centrally or peripherally induces bradycardia and hypotension (Kapusta 2000). Centrally, OFQ/N may be acting via the rostral ventrolateral medulla (Chu et al. 1998) while peripherally it may inhibit norepinephrine release from sympathetic nerve terminals in the heart (Giuliani et al. 2000) and blood vessels (Bucher 1998) since NOP receptors are expressed in the endothelial cells of the aorta and in postganglionic sympathetic nerve terminals (Granata et al. 2003). OFQ/N increases water excretion (water diuresis) and decreases sodium excretion (antinatriuresis) (Kapusta et al. 2005a,b) through central and peripheral actions. The NOP receptors in the paraventricular nucleus (PVN) of the hypothalamus are responsible at least partly for the diuretic effect, although a peripheral effect may also play a role. Finally, when OFQ/N is injected into the cavernosal cavity of cats, it produces a strong and long-lasting erectile response (Champion et al. 1997).

OFQ/N inhibits the micturition reflex (Lazzeri et al. 2003). In a clinical trial in patients with neurogenic bladder due to spinal cord injury, intravesical infusion of N/OFQ has been found to increase bladder capacitance and decrease bladder pressure (Lazzeri et al. 2003).

6.10
Cough and Bronchial Constriction

The OFQ/N system has been found to have a powerful effect on cough. OFQ/N or the synthetic agonist Ro 64-6198 inhibit the cough responses provoked by capsaicin in guinea pigs or by mechanical stimulation of intrathoracic airways in cats (Bolser et al. 2001; McLeod et al. 2002, 2004). These antitussive effects may be mediated both centrally and peripherally. OFQ/N inhibits the airway contractions through inhibition of ACh and/or of sensory peptide release (Basso et al. 2005; Peiser et al. 2000). OFQ/N decreases capsaicin-induced calcium influx in nodose ganglia (McLeod et al. 2004), the sensory ganglia involved in cough reflex (Reynolds et al. 2004). Furthermore the medullar nucleus tractus solitarius, which provides polysynaptic inputs to second-order neurons that modulate the respiratory neuron activities (Reynolds et al. 2004), is rich in NOP receptors (Anton et al. 1996). Furthermore, OFQ/N has been reported to inhibit airway microvascular leakage induced by intra-esophageal acid instillation (Rouget et al. 2004).

6.11
Immune System

The NOP receptor mRNA is expressed in a variety of immune cells including mouse lymphocytes (Halford et al. 1995) and human peripheral blood mononuclear cells (PBMCs) (Wick et al. 1995), circulating granulocytes, lymphocytes, and monocytes (Fiset et al. 2003; Peluso et al. 1998). OFQ/N is expressed in neutrophils (Fiset et al. 2003). OFQ/N can function as an immunosuppressant by suppressing antibody production in mouse lymphocytes (Halford et al. 1995), by decreasing proliferation of phytohemagglutinin-stimulated PBMCs (Peluso et al. 2001), and by inhibiting mast cell function (Nemeth et al. 1998). The importance of the OFQ/N system in the immune response, however, remains to be clearly defined.

7
Conclusions

The OFQ/N system is predominantly a neuronal system. The sites of binding of OFQ/N or of expression of the NOP receptor (i.e., the sites of action of OFQ/N) in the CNS are very broadly distributed. This implies that the OFQ/N system will modulate many brain-initiated responses and thus may consequently have many physiological roles. And this is what has been found, as described above. But, ultimately, one may expect that one or a few role(s) may emerge as the prominent function(s) of the OFQ/N system, and that is what one would target for therapeutic intervention. But how to find it among the numerous responses that have been attributed to the OFQ/N system?

Analysis of the responses (Table 1) that have been linked to the activity of the OFQ/N system reveals that the phenotypes of the NOP KO and ppOFQ/N KO mice are mostly the same. They differ in their responses to learning and memory paradigms. In that respect, OFQ/N or agonists confirm the NOP KO mice phenotype. Two responses that are consistent in the two KO mice and confirmed by surrogate molecule treatments are anxiety/stress and morphine tolerance. Activation of the OFQ/N system has an anxiolytic effect, while its inhibition attenuates morphine tolerance. The effect of the OFQ/N system in pain perception has primarily led to the conclusion that activation of the OFQ/N system reverses stress-induced analgesia supraspinally and is analgesic spinally. Blockade of the OFQ/N system tends to decrease depression-like symptoms. Finally, for reasons that are not understood, OFQ/N agonists may act as antitussic agents.

The strong link between the OFQ/N system and anxiety, as well as its, somewhat surprising, effect on memory suggest that post-traumatic stress disorder (PTSD) could be a prominent therapeutic indication for an OFQ/N agonist. Since OFQ/N decreases memory while being anxiolytic it could al-

Table 1 Physiological and behavioral responses modulated by the OFQ/N system

Functional implications of the OFQ/N system					
	ppOFQ/N KO mice	NOP KO mice	Agonist	Antagonists	Therapeutic indication
Locomotion	Unchanged	Unchanged	Dose-dependent		
Anxiety	Increased	Unchanged	Anxiolytic		Agonist for stress/PTSD
Learning/ memory	Unchanged	Increased	Decreased		Agonist for PTSD
Morphine dependence	Decreased	Decreased	Unchanged		
Pain: supraspina			Hyperalgesic	Antinociceptive	Antagonist for
Pain: spinal			Analgesic	Unclear	opiate tolerance
Depression		Decreased		Decreased	Agonist for depression
Feeding			Increased		
Cough			Decreased		Agonist as antitussic

Comparison of the results obtained for the two KO mice strains with those obtained for agonist (mainly OFQ/N) and antagonists (surrogate molecules). The different therapeutic indications stems from the results and are influenced by the author's point of view
PTSD post-traumatic stress disorder

leviate two hallmarks of PTSD. Developing an agonist to a neuropeptide receptor is sometimes viewed as a very difficult task. However, the number of surrogate agonists already reported shows that this is not the case for the NOP receptor. Furthermore, such an agonist may exhibit a positive effect on chronic peripheral pain and cough. An antagonist on the other hand may be of help in managing depressive states, but, moreover, could attenuate opiate tolerance and thus be of help in cancer treatments. Without doubt the OFQ/N system has not yielded all its secrets but it already shows promising insights for drug discovery.

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Orexins and Orexin Receptors: From Molecules to Integrative Physiology

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Abstract Recent studies have implicated the orexin system as a critical regulator of sleep/wake states, feeding behavior, and reward processes. Orexin deficiency results in narcolepsy-cataplexy in humans, dogs, and rodents, suggesting that the orexin system is particularly important for maintenance of wakefulness. Orexin agonists and antagonists are thought to be promising avenues toward the treatment of sleep disorders, eating disorders, and drug addiction. In this chapter, we discuss the current understanding of the physiological roles of orexins in regulation of arousal, sleep/wake states, energy homeostasis, and reward systems.

1 Introduction

Identification of a transmitter that naturally activates an orphan GPCR is crucial for understanding the physiological roles of a particular ligand-receptor system. Identification of ligands has the potential to define unknown functions and therapeutic targets in novel fields. One of the most compelling cases is the orexin system, in which classical and modern molecular pharmacological approaches have crossed paths, producing spectacular results. Starting from the discovery of molecules (orexins and orexin receptors), identification of the distribution of ligands and receptors predicted the role of this neuropeptide system. Investigations of gene-modified animals led to the finding of previously unknown functions of the orexins (sleep/arousal regulation), as well as the understanding of the pathophysiology of narcolepsy-cataplexy in humans. Anatomical, physiological, and pharmacological approaches to investigating the orexin system resulted in the appreciated knowledge of brain functions and regional networks. Referring to the orexin system is indispensable for research in the field of sleep, feeding, and reward control. Moreover, the possibility of clinical treatment for sleep disorders using antagonists or agonists of orexin receptors has been examined.

Since there are many comprehensive reviews on the orexin system (for a recent review, see Sakurai 2007), in this chapter, we will discuss an overview of its physiological significance, in relation to the possibility of clinical utility of orexin antagonists and agonists in the treatment of eating disorders, sleep disorders, and drug addiction.

2
Orexin and Orexin Receptors

2.1
Identification of Orexin (Hypocretin) by Deorphaning of Two GPCRs

Both orexin A and B were identified by the orphan GPCR strategy using stably expressing HFGAN72 *orexin-1 receptor* (*OX_1R*)-transfected HEK293 cells from rat brain extracts by detecting intracellular Ca^{2+} mobilization (Sakurai et al. 1998). Orexins constitute a novel peptide family with no significant homology with any previously described peptides. Orexin A is a 33-amino-acid peptide of 3562 Da. It has an N-terminal pyroglutamyl residue and C-terminal amida-

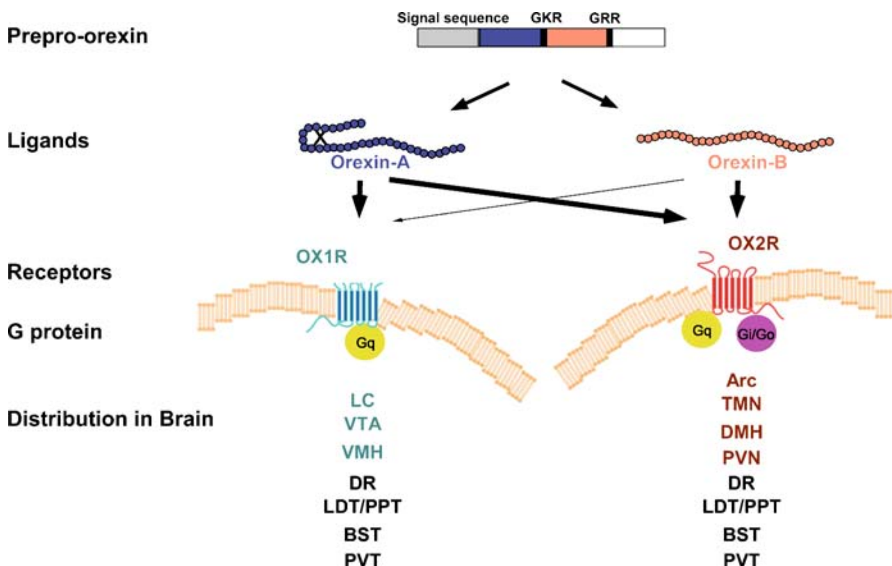

Fig. 1 Overview of orexin system. Orexin A and orexin B are derived from a common precursor peptide, *prepro-orexin*. The actions of orexins are mediated via two G protein-coupled receptors named orexin-1 (OX_1R) and orexin-2 (OX_2R) receptors. OX_1R is selective for orexin A, whereas OX_2R is a nonselective receptor for both orexin A and orexin B. OX_1R is coupled exclusively to the G_q subclass of heterotrimeric G proteins, whereas OX_2R couples to $G_{i/o}$ and/or G_q.

tion (Sakurai et al. 1998). The four Cys residues of orexin A form two sets of intrachain disulfide bonds. The primary structure predicted from the cDNA sequences of the genes is conserved among several mammalian species (human, rat, mouse, cow, sheep, dog, and pig). On the other hand, rat orexin B is a 28-amino-acid, C-terminally amidated linear peptide of 2937 Da, which is 46% (13/28) identical in sequence to orexin A. The C-terminal half of orexin B is very similar to that of orexin A (73%; 11/15), while the N-terminal half is more variable. Mouse orexin B is identical to rat orexin B. Compared to the rodent sequence, human orexin B has two amino acid substitutions within the 28-residue stretch. Other than mammalian species, the structures of fish, xenopus, and chicken orexin A and orexin B have been elucidated, and they have also conserved structures as compared to mammalian sequences. (Alvarez and Sutcliffe 2002; Sakurai 2005; Shibahara et al. 1999).

The *prepro-orexin* cDNA sequences reveal that both orexins are produced from a common precursor polypeptide, prepro-orexin, by usual proteolytic processing (Fig. 1). An mRNA encoding the same precursor peptide was independently isolated by de Lecea et al. as a hypothalamus-specific transcript (de Lecea et al. 1998). De Lecea et al. predicted that this transcript potentially encodes two neuropeptides, named hypocretin-1 and hypocretin-2. The names "hypocretin" and "orexin" are currently used as synonyms in many papers.

2.2 Orexin Receptors

OX_1R was initially identified as an expressed sequence tag (EST) from human brain (US Patent WO96/34877 (Sakurai et al. 1998)). The *Orexin-2 receptor* (OX_2R) was identified by searching an EST database by tBLASTn with the OX_1R sequence as a query (Sakurai et al. 1998). OX_1R has one order of magnitude greater affinity for orexin A than for orexin B. In contrast, orexin A and orexin B bind OX_2R with similar affinity (Sakurai et al. 1998) (Fig. 1). OX_1R is thought to transmit signals through activation of the $G_{q/11}$ class of G proteins, which results in activation of phospholipase C (PLC) with subsequent triggering of the phosphatidylinositol cascade. OX_2R is shown to be coupled to both $G_{q/11}$ and inhibitory G_i proteins (Zhu et al. 2003) (Fig. 1).

2.3 Distribution of Orexin Neurons

Orexin-producing neurons (orexin neurons) are located exclusively in the lateral posterior hypothalamic regions, including the perifornical area (PFA), lateral hypothalamus, and posterior hypothalamus. The lateral hypothalamic area (LHA) has classically been implicated in a wide variety of behavioral and homeostatic regulatory systems, and thus the localization of *orexin*-expressing neurons has generated hypotheses as to their physiological rele-

vance (Bernardis and Bellinger 1996; Elmquist et al. 2005). Specific localization of orexin neurons is regulated by the *orexin* gene promoter (Moriguchi et al. 2002). The human *prepro-orexin* gene fragment, which contains the 3149-bp 5′-flanking region and 122-bp 5′-non-coding region of exon 1, has the ability to express *lacZ* in orexin neurons without ectopic expression in transgenic mice, suggesting that this genomic fragment contains most of the cis-elements necessary for appropriate expression of the gene (Sakurai et al. 1999).

From these *orexin*-expressing regions, these cells widely project to the entire brain, except for the cerebellum (Date et al. 1999; Nambu et al. 1999; Peyron et al. 1998). Dense staining of orexin-immunoreactive nerve endings in the brain was present in the central medial nucleus (CM) and paraventricular nucleus of the thalamus (PVT); arcuate nucleus (Arc), ventomedial nucleus (VMH), dorsomedial nucleus (DMH), and tuberomammillary nucleus (TMN) of the hypothalamus; locus coeruleus (LC); and ventral tegmental area (VTA).

2.4 Distributions of Orexin Receptors

Orexin receptors are expressed in regions which contain dense orexin projections, as described above. OX_1R and OX_2R mRNAs show partially overlapping and partially distinct distribution patterns, suggesting that they play different physiological roles.

OX_1R is expressed in many brain regions, such as the prefrontal and infralimbic cortex (IL), hippocampus (CA2), amygdala and bed nucleus stria terminalis (BST), PVT, anterior hypothalamus, dorsal raphe (DR), VTA, LC, and laterodorsal tegmental nucleus (LDT)/ pedunculopontine nucleus (PPT) (Lu et al. 2002; Marcus et al. 2001). OX_2R is expressed in the amygdala and BST, PVT, DR, VTA, and LDT/PPT (Lu et al. 2002; Marcus et al. 2001). On the other hand, OX_2R is prominent in the Arc, TMN, DMH, paraventricular nucleus (PVN), LHA in the hypothalamus, CA3 in the hippocampus, and medial septal nucleus (Lu et al. 2002; Marcus et al. 2001). These histological findings suggest that orexins and their receptors are involved in feeding, sleep, memory, and reward systems. These regions are major effector sites of orexins, as later described in detail.

3 Neural Circuits of Orexin Neurons

3.1 Neuronal Afferents

Recently, upstream neuronal populations that make innervations to orexin neurons in rodents were revealed in several studies (Sakurai et al. 2005;

Yoshida et al. 2006). These studies showed that orexin neurons are innervated by the lateral parabrachial nucleus (LPB), ventrolateral preoptic nucleus (VLPO), medial and lateral preoptic areas, basal forebrain (BF), posterior/dorsomedial hypothalamus, VTA, and median raphe nuclei (MnR). Many neurons were identified in regions associated with emotion, including the IL, amygdala, shell region of the nucleus accumbens, lateral septum (LS), and BST.

From these regions, neurons send input to orexin neurons and regulate orexin neuronal activity by secretion of particular neuromodulators. Many electrophysiological and histological studies have identified several neurotransmitters and neuromodulators that activate or inhibit orexin neurons (summarized in Table 1).

Hypothalamic regions preferentially innervate orexin neurons in the medial and perifornical parts of the field, but most projections from the brainstem target the lateral part of the field, suggesting functional dichotomy of orexin neurons. Another work also suggested that the lateral part of orexin neurons is strongly linked to preferences for cues associated with drug and food reward (Harris et al. 2005).

Table 1 Factors that influence the activity of orexin neurons

Excitation	***Receptor involved***
Glutamate	AMPA-R, NMDA-R mGluRs
Ghrelin	GHS-R
CCK	CCK-A
Neurotensin	N.D.
Vasopressin	V1a
Oxytocin	OTR
CRH	CRHR1
TRH	TRHR
Ach (muscarinic) (20%)	M3
GLP1	GLP1-R
GALP	N.D.
Dopamine	(D1)
ATP	P2X
Inhibition	***Receptor involved***
Glucose	unknown
GABA	GABA-A, GABA-B
Serotonin	5HT1A
Noradrenaline	$\alpha 2$
Dopamine	D2
Leptin	ObR
Ach (muscarinic) (6%)	N.D.
NPY	Y1 (postsynaptic), Y2/Y5 (presynaptic)
Adenosine	A1

3.2 Local Interneurons

GABAergic input from local interneurons to orexin neurons is also important for organization of neuronal activity, because genetic disruption of this input resulted in marked sleep/wake abnormality (Matsuki and Sakurai 2008, unpublished). Both postsynaptic and presynaptic GABAergic input to orexin neurons strongly inhibits activity of orexin neurons. At the same time, glutamatergic input might be important for regulation of orexin neuronal activity. Overnight fasting promotes the formation of more excitatory synapses and synaptic currents to orexin neurons (Horvath and Gao 2005). Additionally, the fasting-induced increase of miniature excitatory presynaptic currents (mEPSCs) is blocked by leptin signals (Horvath and Gao 2005). Orexin increases local glutamate signaling by facilitation of glutamate release from presynaptic terminals (Li et al. 2002). On the other hand, orexins activate local GABA input to orexin neurons (Matsuki and Sakurai 2008, unpublished). These local GABAergic and glutamatergic interneuron networks might play critical roles in the regulation of orexin neurons.

3.3 Efferents of Orexin Neurons

As discussed earlier, the densest staining of orexin-immunoreactive nerve endings from orexin neurons in the LHA/PFA were found in the PVT, Arc, VMH, VTA, LC, and TMN. There are many reports on the effects of orexins on these neurons (summarized in Table 2). These regions are important for maintenance of arousal, feeding, and reward systems.

4 Roles of Orexins in Regulation of Sleep/Wake States

4.1 Interaction with Sleep and Arousal Centers

The POA, especially the VLPO, appears to play a critical role in initiation of non-rapid eye movement (NREM) sleep and maintenance of both NREM and rapid eye movement (REM) sleep (Sherin et al. 1998). The VLPO sends inhibitory projections to wake-active neurons producing wake-promoting neurotransmitters, including histamine, norepinephrine, 5-HT, and acetylcholine (Lu et al. 2002; Sherin et al. 1998). Neurons in the VLPO fire at a rapid rate during sleep, with attenuation of firing during the waking period. These sleep-promoting neurons in the VLPO mostly contain GABA and/or galanin, and are inhibited by wake-active transmitters, such as noradrenaline and

Table 2 Summary of orexin output effects

Region	Cell type	Effect	Function	Refs.
Prefrontal cortex	Layer V pyramidal neurons (Glu)	Glutamate release	Attention	(Lambe and Aghajanian 2003)
Prefrontal cortex	Layer 6b (Glu)	Activation	Arousal system	(Bayer et al. 2004)
Hippocampus/ Septum (LS)	Acetylcholine GABA Schaffer collateral CA3-CA1	Activation LTP & theta rhythm plasticity	Arousal system REM sleep	(Selbach et al. 2004; Wu et al. 2004, 2002)
Amygdala	Central medial nucleus	Activation	Fear/emotion	(Bisetti et al. 2006)
BST		Activation	Fear/emotion, Autonomic nervous system	(Mullett et al. 2000)
Thalamus	Rhomboid nucleus, centromedial nucleus	Activation	Arousal system	(Bayer et al. 2002; Huang et al. 2006)
Basal forebrain	Acetylcholine	Activation	Arousal system	(Eggermann et al. 2001)
Arc	POMC	Inhibition	Feeding/energy regulation	(Ma et al. 2007)
Arc	NPY	Activation	Feeding/energy regulation	(Li et al. 2002; van den Top et al. 2004)
Arc	GABA	Activation	Feeding/energy regulation	(Burdakov et al. 2003)
VMH	(Glucose-responsive neurons)	Activation	Autonomic responses	(Monda et al. 2005; Muroya et al. 2004)
PVN	CRF	Activation	Stress/ autonomic response	(Kuru et al. 2000; Sakamoto et al. 2004)
TMN	Histamine	Activation (direct and indirect)	NREM sleep, Arousal system	(Bayer et al. 2001; Yamanaka et al. 2002)
LHA	Orexin	Activation (indirect)	Feeding, Arousal, Reward systems	(Li et al. 2002; Li and van den Pol 2006)
LHA	MCH	Activation (direct and indirect)	Feeding/energy regulation	(Li and van den Pol 2006; van den Pol et al. 2004)
LHA	Glu	Activation	Feeding, Arousal, Reward	(Li et al. 2002; van den Pol et al. 1998)
LHA	GABA	Activation	Feeding, Arousal, Reward	(van den Pol et al. 1998; Matsuki 2008, unpublished)

Table 2 (continued)

Region	Cell type	Effect	Function	Refs.
SNr	GABA	Inhibition	Locomotion	(Thorpe and Kotz 2005)
VTA/NAc	Dopamine GABA	Activation	Locomotion, Reward system	(Borgland et al. 2006; Boutrel et al. 2005; Harris et al. 2005; Korotkova et al. 2003; Nakamura et al. 2000; Narita et al. 2006)
LC	Noradrenaline	Activation	Arousal system	(Hagan et al. 1999; Horvath et al. 1999; van den Pol et al. 2002)
DR	Serotonin	Activation	Arousal system	(Brown et al. 2001, 2002)
LDT/PPT	Acetylcholine	Activation (direct and indirect)	Locomotors, REM sleep	(Burlet et al. 2002; Takakusaki et al. 2005; Xi et al. 2001)
Spinal cord	Lamina 1 & X	Activation	Pain and thermal sensation	(van den Pol et al. 2002; Yamamoto et al. 2002)
Spinal cord	Sympathtic preganglionic neurons	Activation	Heart rate, blood pressure	(Antunes et al. 2001; van den Top et al. 2003)

Abbreviations:
BF; basal forebrain, BST; bed nucleus stria terminalis, CM; central medial nucleus, PVT; paraventricular nucleus of the thalamus, Arc; arcuate nucleus, VMH; ventomedial nucleus, DMH; dorsomedial nucleus, LHA; lateral hypothalamic area, PFA; perifornical area, PVN; paraventricular nucleus, TMN; tuberomammillary nucleus of the hypothalamus, LC; locus coeruleus, VTA; vetral tegmental area, NAc; nucleus accumbens, LS; lateral septum, DR; dorsal raphe, LDT; laterodorsal tegmental nucleus, PPT; pedunculopontine nucleus, DRG; dorsal root ganglion, GABA; γ-aminobutyric acid, POMC; proopiomelanocortin, NPY; neuropeptide Y, MCH; melanin concentrating hormone, CRF; corticotropin-releasing factor, Glu; glutamate

acetylcholine (Gallopin et al. 2000). GABAergic neurons in the POA densely innervate orexin neurons (Sakurai et al. 2005; Yoshida et al. 2006). Orexin neurons are strongly inhibited by both a $GABA_A$ receptor agonist, muscimol, and a $GABA_B$ receptor agonist, baclofen (Xie et al. 2006; Yamanaka et al. 2003a). These observations suggest that VLPO neurons send GABAergic inhibitory projections to orexin neurons (Fig. 2). This pathway might be important in turning off orexin neurons during sleep. Also, orexin neu-

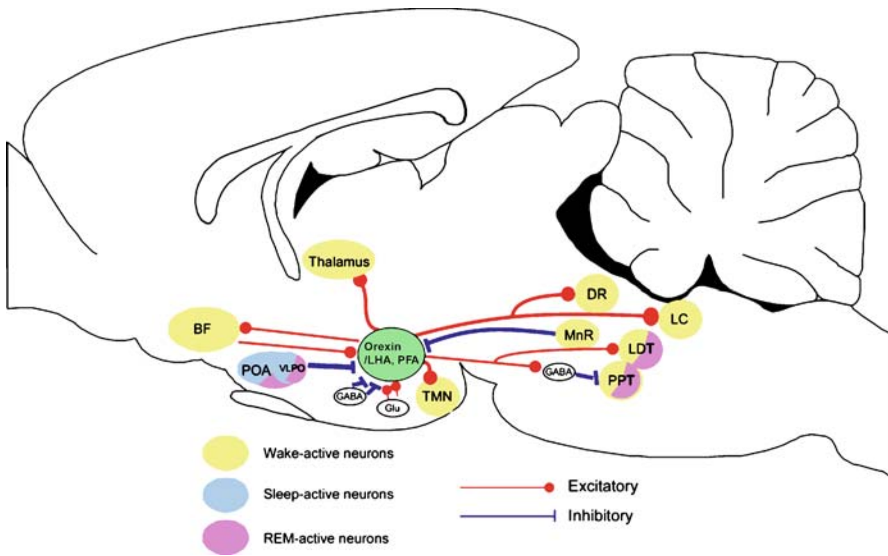

Fig. 2 Input and output of orexin neurons in sleep/wake regulation. Orexin neurons regulate cortical arousal and promote wakefulness through the aminergic nuclei and other arousal-related nuclei. On the other hand, in the sleep center, VLPO-GABAergic neurons innervate orexin neurons. BF-cholinergic neurons also project to orexin neurons.

rons are innervated by BF-cholinergic neurons (Sakurai et al. 2005) (Fig. 2). A cholinergic agonist, carbachol, activates some populations of orexin neurons (Sakurai et al. 2005). This cholinergic input to orexin neurons probably plays a role in stabilization of wakefulness (Sakurai et al. 2005). Furthermore, serotonergic neurons send inhibitory projection to orexin neurons (Muraki et al. 2004; Sakurai et al. 2005). Noradrenergic neurons might also have inhibitory effects on orexin neurons (Yamanaka et al. 2006). These inhibitory feedback mechanisms might also be important for the stability of orexin neuronal activity (Fig. 2).

4.2 Interaction with Waking Centers

How do the orexins regulate sleep/wake states, and why does lack of orexin signaling result in narcolepsy-cataplexy?

The activity of monoaminergic neurons in the hypothalamus and brain stem, including neurons in the TMN, LC, and DR, is known to be synchronized and strongly associated with sleep/wake states. These groups play a significant role in arousal maintenance (Saper et al. 2005). They fire tonically during the awake state, less during NREM sleep, and not at all during REM sleep (Vanni-Mercier et al. 1984). Since orexin neurons innervate and

their receptors are expressed in these regions, it is suggested that orexin neurons might be activated during the wakeful period, and that they exert an excitatory influence on these wake-active neurons to sustain their activity (Fig. 2). Consistent with this hypothesis, orexin neurons discharge during active waking, and virtually cease firing during sleep, including the NREM and REM periods, in vivo (Lee et al. 2005). In vitro, noradrenergic cells of the LC (Hagan et al. 1999), dopaminergic cells of the VTA (Nakamura et al. 2000), serotonergic cells of the DR (Brown et al. 2002; Liu et al. 2002), and histaminergic cells of the TMN (Yamanaka et al. 2002) are all activated by orexins (Fig. 2). Orexins also have a strong direct excitatory effect on cholinergic neurons of the BF (Eggermann et al. 2001), which also play an important role in regulating arousal (Alam et al. 1999). In addition, orexin neurons project to LDT/PPT cholinergic neurons. Injection of orexin A into the LDT of cats results in an increase in waking time and a decrease in REM sleep time (Xi et al. 2001). In addition, orexin A induces long-lasting excitation of cholinergic neurons in the LDT (Takahashi et al. 2002). On the contrary, orexin A indirectly inhibits cholinergic neurons in the PPT via activation of GABAergic local interneurons and GABAergic neurons in the substantia nigra pars reticulata (Takakusaki et al. 2005). These results suggest that hypothalamic orexin neurons affect the activity of LDT/PPT cholinergic neurons directly and/or indirectly to appropriately regulate the ability of these cells to control wakefulness and REM sleep (Fig. 2).

Some reports have shown that the effect of orexins on awake time is largely mediated by activation of the TMN histaminergic system through OX_2R. In rats, intracerebroventricular (icv) injection of orexin during the light period potently increases the awake period, and this effect is markedly attenuated by the H_1 antagonist, pyrilamine (Yamanaka et al. 2002). Furthermore, the pharmacological effect of orexin A on waking time in mice is almost completely absent in histamine H_1-*receptor*-deficient mice (Huang et al. 2001). OX_2R is abundantly expressed in the TMN, while OX_1R is strongly expressed in the LC. Therefore, the TMN-histaminergic pathway seems to be an important effector site of orexin for sleep/wake regulation.

Several findings indicate that signaling through OX_1R is also important for the proper regulation of vigilance states. However, OX_2R knockout mice exhibit characteristics of narcolepsy (Willie et al. 2003), and OX_1R knockout mice do not have any overt behavioral abnormalities and exhibit only very mild fragmentation of the sleep/wake cycle (Willie et al. 2001). Interestingly, the phenotype of OX_2R knockout mice is less severe than that found in *prepro-orexin* knockout mice and double receptor knockout (OX_1R- and OX_2R-null) mice, which appear to have the same phenotype as *prepro-orexin* knockout mice. Importantly, both OX_2R knockout and *prepro-orexin* knockout mice are similarly affected by behaviorally abnormal attacks of NREM sleep ("sleep attacks") and show a similar degree of disrupted wakefulness (Willie et al. 2003). In contrast, OX_2R knockout mice are only mildly affected by cataplexy

and direct transitions to REM sleep from an awake state, whereas *prepro-orexin* knockout mice and OX_1R/OX_2R-double knockout mice are severely affected. These observations suggest that OX_1R also has additional effects on sleep/wake regulation, especially inhibition and gating of REM sleep. These findings suggest that despite the lack of an overt OX_1R phenotype, loss of signaling through both receptor pathways is necessary for emergence of a complete narcoleptic phenotype. It is reasonable to think that the lack of obvious phenotype in OX_1R knockout mice might result from compensatory effects of OX_2R, while lack of OX_2R cannot be compensated by OX_1R.

These observations suggest that the profound dysregulation of wakefulness in the narcolepsy syndrome emerges from loss of signaling through both OX_1R-dependent and OX_2R-dependent pathways.

5 Roles of Orexins in Feeding Behavior

The finding of decreased caloric intake combined with an increased body mass index in narcolepsy patients suggests that they have a feeding abnormality with reduced energy expenditure, or a low metabolic rate (Lammers et al. 1996; Schuld et al. 2000). Consistently, orexin neuron-ablated mice also show hypophagia and late-onset obesity, although the degree of the obese phenotype critically depends on their genetic background (Hara et al. 2001, 2005). The altered energy homeostasis in narcolepsy patients and mouse narcolepsy models suggests a role of orexin in the regulation of energy homeostasis (Honda et al. 1986; Schuld et al. 2000).

5.1 Interaction with Hypothalamic Neurons

The LHA has long been recognized as a "feeding center", because electrical stimulation of this area causes hyperphagia and obesity, whereas lesions yield the opposite results (Anand and Brobeck 1951). Other hypothalamic regions, including the Arc, VMH, DMH, and PVN, are also involved in energy homeostasis (Elmquist et al. 1999). Leptin-responsive or leptin-sensitive neurons exist in the Arc, VMH, DMH, and LHA regions, in which the functional leptin receptor (*ObRb*) and *STAT3* are expressed (Elmquist et al. 2005; Hakansson et al. 1999; Hakansson and Meiste 1998).These regions reciprocally project to LHA neurons.

Intracerebroventricular injection of orexin increases feeding, and *orexin* mRNA is increased upon fasting (Sakurai et al. 1998). POMC neurons and NPY neurons in the Arc have been shown to innervate to orexin neurons (Elias et al. 1999). Injection of agouti related protein (Agrp), which is an endogenous antagonist for MC3/4Rs, resulted in the activation of orexin neu-

rons, but not MCH and NPY neurons (Zheng et al. 2002). These findings suggest that the orexin system is involved in the hypothalamic neuronal network that regulates feeding behavior and energy homeostasis.

5.2 Regulation of Orexin Neurons by Humoral Factors

Orexin neurons respond to humoral and neuroendocrine factors as indicators of energy balance. Changes in extracellular glucose concentration produce electrophysiological changes in orexin neurons (Yamanaka et al. 2003a). Increasing extracellular glucose concentration, as well as leptin, induces marked hyperpolarization and cessation of action potentials in orexin neurons. Conversely, decreasing the glucose concentration induces depolarization and increases the frequency of action potentials in these same neurons (Burdakov et al. 2005; Yamanaka et al. 2003a). Orexin neuron-ablated *ataxin-3* Tg mice cannot respond to fasting by increased locomotor activity and waking time. *Prepro-orexin* mRNA level is also increased in hypoglycemic conditions, suggesting that expression of the gene is also regulated by plasma glucose level (Griffond et al. 1999; Moriguchi et al. 1999). Importantly, this mechanism is sufficiently sensitive to encode variations in glucose levels, reflecting those occurring physiologically between normal meals (Burdakov et al. 2005). A recent study demonstrated that inhibition of orexin neurons by glucose is mediated by tandem-pore K^+ (K_{2P}) channels (Burdakov et al. 2006).

When applied in superfused solution, the orexigenic peptide, ghrelin, activated 60% of dispersed orexin neurons, with depolarization and an increase in action potential frequency (Yamanaka et al. 2003a). These findings are consistent with the idea that orexin neurons act as sensors for the nutritional status of the body (Sakurai et al. 1998). Consistently, *prepro-orexin* expression of normal and *ob/ob* mice is negatively correlated with changes in blood glucose, leptin, and food intake (Yamanaka et al. 2003a).

5.3 Mechanism of Orexin-mediated Feeding

Supporting the physiological relevance of orexin in the control of feeding, icv administration of anti-orexin antibody or an OX_1R-selective antagonist reduced food intake (Haynes et al. 2000; Yamada et al. 2000). *Prepro-orexin* knockout mice and transgenic mice lacking orexin neurons ate less than control wild-type mice (Hara et al. 2001; Willie et al. 2001). Moreover, an OX_1R selective antagonist reduced food intake and ameliorated obesity of leptin-deficient ob/ob mice (Haynes et al. 2002), suggesting that leptin deficiency at least partly activates the orexin pathway to increase food intake. This is consistent with electrophysiological findings showing that activity of orexin neurons is inhibited by leptin. Orexin neurons densely project to the Arc

(Date et al. 1999; Peyron et al. 1998; Yamanaka et al. 2000), and *Fos* expression was induced in NPY neurons of the arcuate nucleus by icv injection of orexin, suggesting that orexin-stimulated feeding may occur at least partly through NPY pathways (Yamanaka et al. 2000). Electrophysiological data showed that orexin directly and indirectly activated NPY neurons (Li and van den Pol 2006; van den Top et al. 2004), but inhibited proopiomelanocortin (POMC) neurons (Ma et al. 2007; Muroya et al. 2004). Furthermore, the orexin A-induced increase in food intake was partly inhibited by prior administration of BIBO3340, an NPY-Y1 receptor antagonist, in a dose-dependent manner (Yamanaka et al. 2000). These experiments suggest that orexin-stimulated food intake is at least partially mediated by activation of NPY neurons.

Recent reports also showed that infusion of orexin A into the shell of the nucleus accumbens (NAc) increased feeding behavior (Thorpe and Kotz 2005). In addition, infusion of the $GABA_A$ receptor agonist, muscimol, into the NAc shell strongly induced food intake, and it simultaneously increased *Fos* expression specifically in orexin neurons (Baldo et al. 2004). These findings indicate that interactions between the orexin and limbic systems have a role in the regulation of feeding.

Orexin-mediated maintenance of consolidated wake states might also be important in supporting feeding behavior, because proper maintenance of arousal during food searching and intake is essential for an animal's survival. For example, when faced with reduced food availability, animals adapt with a longer awake period, which disrupts the normal circadian pattern of activity (Challet et al. 1997; Itoh et al. 1990). Consistently, transgenic mice with ablated orexin neurons fail to respond to fasting with increased wakefulness and activity (Yamanaka et al. 2003a). This suggests that orexin neurons have a critical role in maintenance of arousal during the period in which the energy balance is negative. These mechanisms may modulate the activity of orexin neurons according to energy stores in order to maintain wakefulness.

5.4
Orexin as Effector of Food Entrainable Oscillator (FEO)

The activity of orexin neurons also contributes to the promotion and maintenance of food anticipatory activity (FAA) (Akiyama et al. 2004; Mieda et al. 2004). Daily restricted feeding produces an anticipatory locomotor activity rhythm and entrains the peripheral molecular oscillator, which is independent of the central clock located in the suprachiasmatic nucleus (SCN). Restricted feeding was shown to shift the peak of *Fos* expression of orexin neurons from night to the period of restricted feeding (Akiyama et al. 2004; Mieda et al. 2004). Formation of the FAA is severely impaired in orexin neuron-ablated, *orexin/ataxin-3*, transgenic mice (Akiyama et al. 2004; Mieda et al. 2004). Expression of *mNpas2* mRNA, a transcription factor thought to be involved in regulation of the FEO, as well as *mPer1* and *mBmal1* mRNA, is

reduced in *orexin/ataxin-3* mice. These observations suggest that orexin neurons convey an efferent signal from the putative FEO or oscillators to increase wakefulness and locomotor activity. The DMH was shown to have marked oscillation of *mPer* expression only under restricted feeding (Mieda et al. 2006). Consistently, Gooley et al. also demonstrated that lesions in the DMH in rats blocked food entrainment of wakefulness, locomotor activity, and core body temperature (Gooley et al. 2006). However, Landry et al. reported that complete ablation of the dorsomedial hypothalamic nucleus does not affect food anticipatory activity rhythms in rats (Landry et al. 2006). The nature of the discrepancy between these reports is unclear. However, taken in conjunction with our recent finding that DMH neurons directly project to orexin neurons (Sakurai et al. 2005), these findings indicate a possibility that a link between DMH neurons and orexin neurons might play a key role as a central FEO in the feeding-mediated regulation of circadian behavior.

The circuit for neurons in the hypothalamus and other regions in energy homeostasis is summarized in Fig. 3.

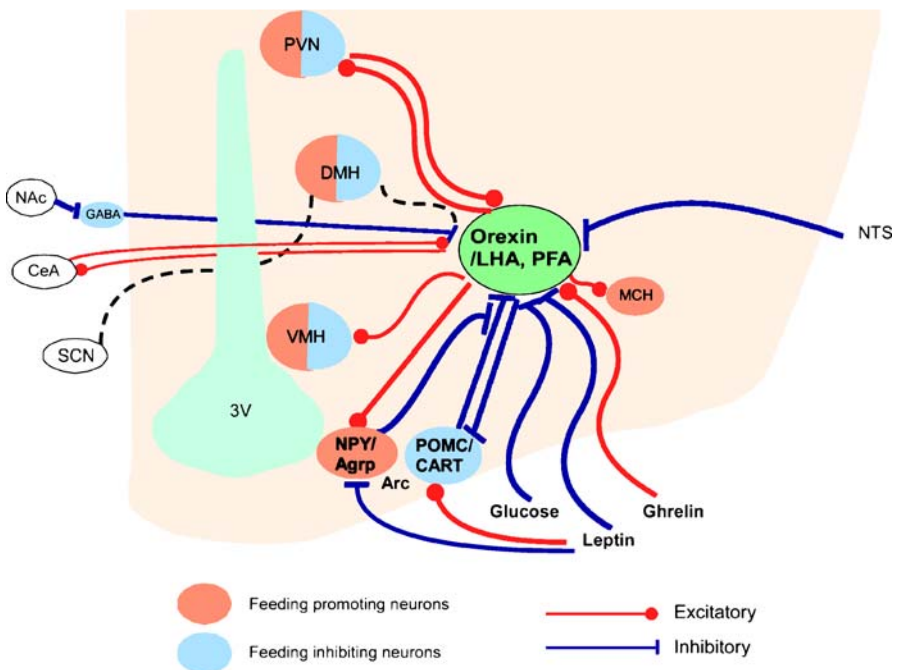

Fig. 3 Input and output of orexin neurons in energy homeostasis. Orexin neurons regulate the hypothalamic nuclei involved in feeding behavior. Peripheral metabolic signals, leptin, ghrelin, and glucose, and circadian rhythms influence orexin neuronal activity to coordinate arousal and energy homeostasis. Input from the limbic system may also influence feeding behavior.

6 Roles of Orexins in Reward Systems

6.1 Input from Reward Systems

Orexin neurons receive projections from the VTA, NAc, and LS, regions involved in reward systems (Yoshida et al. 2006). Dopamine inhibits orexin neurons (Yamanaka et al. 2003b). In the LHA/PFA, dopamine has an inhibitory influence on food intake and the reward pathways (Yang et al. 1997). On the contrary, VTA neurons receive a projection from a part of the LHA/PFA including orexin neurons (Fadel and Deutch 2002; Marcus et al. 2001). These reciprocal interactions might constitute regulatory mechanisms of reward systems (Fig. 4).

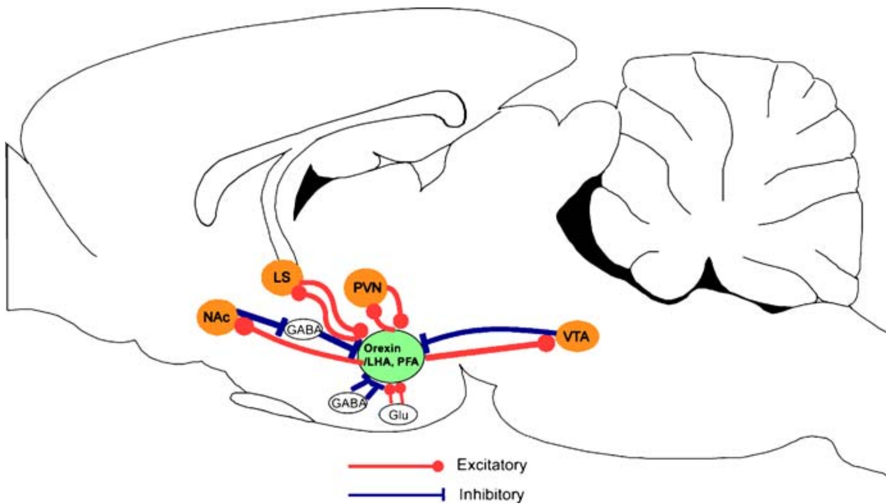

Fig. 4 Input and output of orexin neurons in reward systems. Stimulation of dopaminergic, limbic, and cholinergic centers by orexins can modulate reward systems, motor activity, and emotional arousal.

6.2 Output to Reward Systems

Orexin directly activates VTA dopaminergic neurons (Korotkova et al. 2003; Nakamura et al. 2000), and orexin neurons abundantly innervate the VTA (Fadel and Deutch 2002; Peyron et al. 1998). The dopamine receptor antagonist, haloperidol, blocks hyperlocomotion and stereotypy induced by icv orexin (Nakamura et al. 2000). Intracerebroventricular or local VTA infu-

sion of orexin is shown to reinstate drug-seeking or food-seeking behavior in rodents (Boutrel et al. 2005; Harris et al. 2005). On the other hand, injection of an orexin antagonist into the VTA blocks the development of heroin-conditioned place preferences (Narita et al. 2006). Recent work has also shown that orexin A input to the VTA potentiates *N*-methyl-D-aspartate receptor (NMDAR)-mediated neurotransmission via PLC/PKC-dependent recruitment of NMDARs in VTA dopamine neuron synapses in slice preparations (Borgland et al. 2006). Furthermore, in vivo administration of an OX_1R antagonist blocks locomotor sensitization to cocaine and occludes cocaine-induced potentiation of excitatory currents in VTA dopamine neurons (Borgland et al. 2006). These results suggest a critical role of orexin signaling in the VTA in neural plasticity, and they imply that orexins play a critical role in cocaine-induced psychomotor sensitization and reward-seeking. These findings suggest roles of orexin in the mechanisms of reward systems and drug addiction (Fig. 4).

These mechanisms are similar to CRF-induced sensitization of the activation of dopaminergic neurons by glutamate. Changes in synaptic efficacy, such as those induced by orexin and CRF, are likely to underlie arousal responses to the environment. CRF activates orexin neurons directly (Winsky-Sommerer et al. 2004). Increased activity of orexin neurons could also lead to a state of hyperarousal and excitement propitious to drug craving, or could contribute to the susceptibility to relapse of drug seeking during protracted abstinence (de Lecea et al. 2006) (Fig. 4).

7 Orexins in Emotion, Stress Responses and Autonomic Nervous System

7.1 Input from Mesolimbic System

Input from the limbic system to orexin neurons might be involved in the regulation of feeding and/or motivating behavior, because some of the affective content of the perception of food is thought to be processed in the amygdala and limbic system (Morton et al. 2006). In narcoleptic dogs, cataplexy is triggered by recognition of highly palatable food and excited play, but neither noxious stimuli nor unfamiliar environments (John et al. 2004; Siegel and Boehmer 2006). In rodents, cataplexy is most frequently linked to exploration, burrowing, and investigation of the environment (Chemelli et al. 1999; Hara et al. 2001). In humans, cataplexy is most frequently elicited by laughter, but not sadness or pain (Guilleminault and Gelb 1995; Guilleminault et al. 1998; Siegel and Boehmer 2006). A recent report showed that orexin neurons have maximal activity during exploratory behavior, compared to grooming and eating behavior in unanesthetized and freely moving rats (Mileykovskiy

et al. 2005). They also reported that firing of orexin neurons decreased during food aversion, a state characterized by high levels of attention and motor activity.

On the other hand, the PFA, a region in which orexin neurons exist, has been known as the center for defense responses, or "fight or flight" response (Geerling et al. 2003; Jansen et al. 1995). The defense response is characterized by a coordinated rise in arterial blood pressure, heart rate, and respiratory frequency. Pioneering work showed that electrical stimulation of the posterior hypothalamus in cats elicited behavioral rage, along with specific autonomic responses. These defense responses are induced by activation of the BST and amygdala, or other regions involved in emotional responses. Furthermore, the BST and amygdala nearly project to orexin neurons (Sakurai et al. 2005). These pathways might be important for regulation of the activity of orexin neurons upon emotional stimuli to evoke emotional arousal or fear-related responses. The importance of these inputs is readily apparent in the defense response. In an awake and freely moving condition, *prepro-orexin* knockout mice showed diminished cardiovascular and behavioral responses to emotional stress in the resident-intruder paradigm (Kayaba et al. 2003). These findings suggest that emotional states modulate the activity of orexin neurons (Fig. 5).

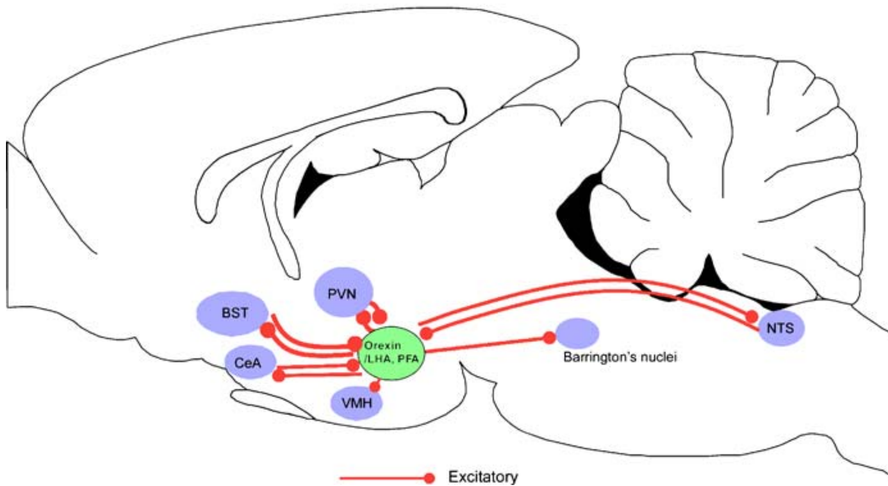

Fig. 5 Input and output of orexin neurons in stress response and autonomic regulation. Orexin neurons regulate the PVN and BST nucleus, which are involved in stress responses and the autonomic nervous system. Orexin A administered into the LH significantly increase cFos-immunoreactivity in the lateral septal area, CeA, shell of NAc, and BST. The CeA, BST, and NAc also strongly project to orexin neurons. Input from the limbic system may be important to regulate the activity of orexin neurons upon emotional stimuli to evoke emotional responses.

7.2
Stress Response

Orexin neurons are activated by cold exposure or immobilized stress (Sakamoto et al. 2004). Orexin also enhances the pituitary release of adrenocorticotropic hormone (ACTH). This response is mediated by CRF and AVP in the PVN (Engelmann et al. 2004). The CRF peptidergic system in the PVN provides an anatomical input to orexin neurons expressing *CRF receptors CRFR1* and *CRFR2* (Winsky-Sommerer et al. 2004). CRF directly activates a population of orexin neurons by acting on CRFR1. The number of orexin neurons expressing *c-Fos* was markedly reduced in *CRFR1* knockout mice in both foot-shock challenge and restraint stress (Winsky-Sommerer et al. 2004). These results suggest that CRFR1 signaling is important for the activation of orexin neurons by physical stress.

Orexin neurons innervate the PVN, in which OX_2R is abundantly expressed (Lu et al. 2002; Marcus et al. 2001). Orexin enhances *c-Fos* expression, *CRF*, and *AVP* mRNA expression in the PVN (Al-Barazanji et al. 2001; Brunton and Russell 2003; Kuru et al. 2000). Orexin A strongly activates *CRF*-expressing neurons in the PVN and the central nucleus of the amygdala (CeA) (Sakamoto et al. 2004). These interactions between the orexin system and CRF neurons might regulate arousal in stressful environments.

7.3
Orexin in Autonomic Nervous System

Intracerebroventricular orexin injection increases blood pressure and heart rate, and these effects are abolished by prior administrations of an α or β blocker (Shirasaka et al. 1999). Moreover, orexin-deficient mice show 10–15 mmHg lower blood pressure than wild-type littermates (Kayaba et al. 2003; Zhang et al. 2006). These results suggest that orexins physiologically stimulate sympathetic outflow. Therefore, *orexin* deficiency might decrease sympathetic tone, which might result in decreased energy expenditure. As suggested from the effects on sympathetic tone, although orexins stimulate feeding behavior, they do not slow the metabolic rate, as might be expected in a system geared for weight gain. Instead, orexins both increase food intake and increase the metabolic rate (Lubkin and Stricker-Krongrad 1998). Because animals must be aware and active when they seek and eat food, this function might be important for feeding behavior. This suggests that the function of orexins might support reward-seeking behavior with an increase in vigilance, awareness, and sympathetic outflow.

Barrington's nucleus in the midbrain also has dense innervations of orexin neurons, and these neurons express OX_2R (Marcus et al. 2001; Nambu et al. 1999; Peyron et al. 1998). These neurons are the center for the autonomic nervous system involved in urinary function (Mitsuyoshi 2005). It remains

unknown how these physiological functions are influenced by the orexin system.

These networks for emotion and autonomic responses are summarized in Fig. 5.

8 Clinical Implications

8.1 Orexin Deficiency and Narcolepsy-Cataplexy

Narcolepsy-cataplexy is a disabling sleep disorder affecting 0.02% of adults worldwide (Dauvilliers et al. 2007). It is characterized by severe, irresistible daytime sleepiness and sudden loss of muscle tone (cataplexy), and can be associated with sleep-onset paralysis and hypnagogic hallucinations, frequent movement and awakening during sleep, and weight gain. Sleep monitoring during night and day shows rapid sleep onset and abnormal, shortened REM sleep latency. The onset of narcolepsy-cataplexy usually occurs during teenage and young adulthood and persists throughout life. Since narcolepsy-cataplexy has no cure, its management relies on symptomatic treatment. This includes psycho-stimulants, including modafinil, methylphenidate, amphetamine, and caffeine for excessive daytime sleepiness and sleep attacks, and tricyclic antidepressants and selective serotonin reuptake inhibitors (SSRI) for cataplexy and other REM-associated symptoms. Hypnotics are also used for disturbed night-time sleep. Pathophysiological studies have shown that the disease is caused by early loss of orexin-producing neurons in the hypothalamus. The cause of neuronal loss could be autoimmune, since most patients have the HLA DQB1*0602 allele, which predisposes to the disorder. Because narcolepsy-cataplexy is a disorder of organization of the sleep/wake cycle, resulting from an absence of orexin, replacement therapy using orexin receptor agonists may provide an effective treatment for narcolepsy.

8.2 Orexin Agonists

Chronic overproduction of orexin peptides from an ectopically expressed transgene prevented the development of narcolepsy syndrome in orexin neuron-ablated *orexin/ataxin-3* mice (Mieda et al. 2004) (schematic model in Fig. 6A). Acute administration of orexin A also maintained wakefulness, suppressed sleep, and inhibited cataplectic attacks in narcoleptic mice (Mieda et al. 2004). However, chronic overexpression of orexin in an unregulated fashion results in fragmentation of non-REM sleep. Therefore, if orexin agon-

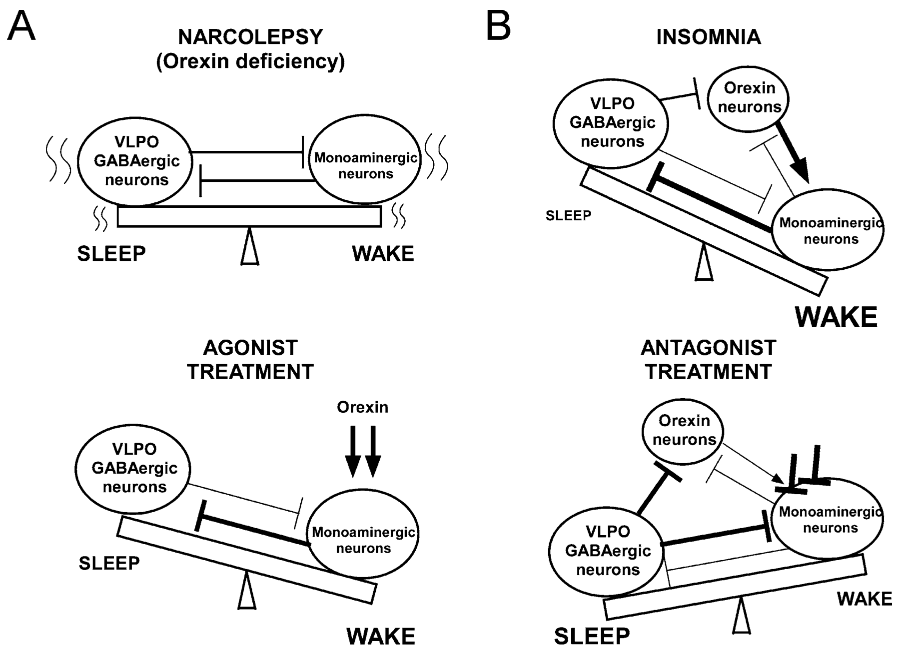

Fig. 6 Mechanism by which orexin system stabilizes sleep and wakefulness. Schematic summary of interactions between orexin neurons and monoaminergic waking center and VLPO sleep center under normal, agonist, and antagonist treatment states. Arrows show excitation and bars show inhibition. **A** Agonist treatment. In narcolepsy, monoaminergic neurons and VLPO neurons set up a mutually inhibitory circuit, which can cause unwanted abrupt transitions between each state. Administration of orexin agonists activates and has an increased excitatory influence on monaminergic cells to maintain their activity. These monoaminergic cells send excitatory projections to the forebrain cortex, and inhibitory projections to the VLPO sleep center. These mechanisms maintain awake states and a balanced sleep/awake state. **B** Antagonist treatment. Orexin neurons send excitatory influences to monaminergic neurons, and monoaminergic neurons send inhibitory feedback projections to orexin neurons. This system might maintain the activity of monoaminergic neurons. Then, administration of an orexin antagonist results in inhibition of monoaminergic neurons. This effect maintains sleep states.

ists were available, a short half-life (<12 hr) might be desirable. Attempts at using orexin-based treatment after peripheral administration have been disappointing, since the peptides do not cross the blood—brain barrier (Mignot and Nishino 2005). Unfortunately, currently there are no reported non-peptide orexin receptor agonists. Orexin-based therapy, such as direct use of orexin receptor agonists (Mieda et al. 2004) and orexin neuron transplantation, is currently under investigation in animal models.

Orexin-immunoreactive fibers innervate the spinal cord, especially dorsal root ganglion (DRG) neurons and lamina I and X surrounding the central canal (van den Pol 1999). OX_1R is localized on C-fibers in the spinal cord

(Hervieu et al. 2001). These data suggest that the spinal orexin system is involved in transmission of nociceptive information. Several studies have shown that an orexin receptor agonist produces an analgesic effect in a rat model (Bingham et al. 2001; Yamamoto et al. 2002). Thus, orexin receptor agonists could possibly also be useful for pain control (Kajiyama et al. 2005; Mobarakeh et al. 2005).

8.3 Orexin Antagonists

There is much evidence suggesting that the orexin system is involved in the regulation of feeding, wakefulness, and reward. Several pharmaceutical companies have shown interest in the potential therapeutic application of non-peptide, low molecular weight orexin receptor antagonists (review; (Bingham et al. 2006). Orexin receptor antagonists might be effective for inducing sleep and treating insomnia patients (schematic model in Fig. 6B). Recently, it was reported that a new dual orexin receptor antagonist (ACT-078573) selectively blocks both OX_1R and OX_2R at nanomolar concentrations (Brisbare-Roch et al. 2007). The drug is orally active and rapidly enters the brain. Although the drug thoroughly blocks orexin signaling and produces sleepiness, it does not appear to produce cataplexy. This drug was effective for promoting sleep when given to rodents and dogs during the active period, but it had no effect when given during the rest period. Accordingly, this drug may be very effective in shift workers or people with jet lag trying to sleep when their biological clock is signaling wakefulness. The company indicated that the compound was suitable for use as a sleep quality improver, and the drug has progressed to phase II clinical trials for insomnia (Actelion Ltd. Press Release 2006).

Another possible use of orexin receptor antagonists includes withdrawal from drug addiction. A selective OX_1R antagonist, SB334867A, significantly suppressed morphine-induced place preference and hyperlocomotion (Harris et al. 2005; Narita et al. 2006), and it blocked the reinstatement of previously extinguished cocaine-seeking behavior and locomotor sensitization to cocaine (Borgland et al. 2006; Boutrel et al. 2005). These studies indicate that the orexin system is directly implicated in reward systems.

Several research groups have demonstrated that an effect of the orexin-induced increase of food consumption is blocked by an OX_1R antagonist (SB-334867) (Bingham et al. 2006). Studies using strains of mice and rats that differ in susceptibility to diet-induced obesity have also demonstrated the anorexic effect of SB334867A. Furthermore, SB334867A blocks 2-DG- and orexin A-induced gastric acid secretion in rats. Although to date there is no report of an OX_1R antagonist in clinical development, OX_1R antagonists may have therapeutic utility in the treatment of obesity.

9 Conclusion

Symptoms of narcolepsy unequivocally show that orexins and orexin receptors play highly important roles in regulating sleep/wake states and the maintenance of arousal by regulating monoaminergic/cholinergic nuclei in the brain. At the same time, this system is also related to the limbic system, reward systems, and hypothalamic mechanisms that regulate energy homeostasis.

The link between the limbic system and orexin neurons might be important for emotional arousal and sympathetic responses during emotional events. On the other hand, the responsiveness of orexin neurons to peripheral metabolic cues, leptin, and glucose suggests that these cells might act as a sensor for the metabolic status of animals. These findings indicate that orexin neurons provide a crucial link between energy balance, emotion, reward systems, and arousal.

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Prolactin-Releasing Peptide

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Abstract Prolactin-releasing peptide (PrRP) was initially isolated from the bovine hypothalamus as an activating component that stimulated arachidonic acid release from cells stably expressing the orphan G protein-coupled receptor hGR3 (Hinuma et al. 1998) [also known as GPR10 (Marchese et al. 1995), or UHR-1 for the rat orthologue (Welch et al. 1995)]. Initially touted as a prolactin-releasing factor (therefore aptly named prolactin-releasing peptide), the perspective on the function of this peptide in the organism has been greatly expanded. Over 120 papers have been published on this subject since its initial discovery in 1998. Herein I review the state of knowledge of the PrRP system, its putative function in the organism, and implications for therapy.

1 Molecular Pharmacology

1.1 PrRP

Using reverse pharmacology Hinuma et al. (1998) reported the identification of two peptides from bovine hypothalamus that specifically activated arachidonic acid release from Chinese hamster ovary (CHO) cells stably expressing GPR10 (Hinuma et al. 1998). It became apparent that both peptides are derived from the same preproprotein, a 20 amino acid isoform as a c-terminal cleavage product of a larger 31 amino acid peptide (named PrRP20 and PrRP31, respectively). The cDNA encodes a protein of 98 amino acids with a prototypical N-terminal sequence signal peptide preceding Ser 23, a proteolytic cleavage motif consisting of basic amino acid repeat at Arg 55, Arg 56, Arg 57, and a conserved domain of Arg 52, Phe 53, and Gly 54, with Gly 54 as the amide donor, reminiscent of peptides with C-terminal amidation at the glycine residue found in the large family of RFamide peptides found most abundantly in insects and invertebrates (Dockray 2004). The peptide is highly conserved in vertebrate animals, and has been identified and characterized from various teleost fish [Tilapia (Seale et al. 2002), carp (Moriyama et al. 2002), salmon (Montefusco-Siegmund et al. 2006), gold fish (Kelly and Peter 2006), and guppy (Amano et al. 2007)], am-

A.

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Human          ----------------------------------------------MKVLRAWLLCLLM 13
Bovine         ----------------------------------------------MKAVGAWLLCLLL 13
Rat            -----------------------------------------------MALKTWLLCLLL 12
Chicken        ----------------------------------------------MKLGATCLLCLLL 13
Tilapia        ---------------------------MLPVRAAD----VRHCVLTSRWLPAALALLLL 28
Salmon         MNNLARGKLSTDPPQQATNVYVPCRPSTMTPETTAACPVMVRECVLGSRWLMAALTILLL 60
Carp           ---------------------------MLPTAITQP---VTKCLIGSRLGTIAFLLLLI 29
Xenopus        ---------------------------MLNYDPWPS----SHQCLSKPKLCTVYILFLL 28
                                                                        :*:

Human          LGLALRGAASRTHR------HSMEIRTPDINPAWYASRGIRPVGRFGRRRATLGDVP--- 64
Bovine         LGLALQGAASRAHQ------HSMEIRTPDINPAWYAGRGIRPVGRFGRRRAAPGDGP--- 64
Rat            LSLVLPGASSRAHQ------HSMETRTPDINPAWYTGRGIRPVGRFGRRRATPRDVT--- 63
Chicken        TCMALP-AAGRLRE------RSMEIRNPDIDPSWYTGRGIRPVGRFGRRRALGESAQ--- 63
Tilapia        LSSSFSRAHSTTVEHDFHIVHNVDNRSPEIDPFWYVGRGVRPIGRFGKRHSSLEALD--S 86
Salmon         LSTTVTCFHSTTVEHNFHIVHNVDNRSPEIDPFWYVGRGVRPIGRFGKRQSGGGGSGGLR 120
Carp           LSATESNAHGTTVEHDLHIVHNVDNRSPEIDPFWYVGRGVRPIGRFGKRQSG-GGLQ--- 85
Xenopus        VSFTIVSAKSRSFN------HQIDNRSPEIDPYWYVGRGVRPIGRFGKRQLKFRKTFQPR 82
                        .   .      :.::  *.*:*:*  **..**:**:****:*:

Human          KPGLRPRLTCFPLEGGAMSS--QDG----------- 87
Bovine         RPGPRRVPACFRLEGGAEPSRALPGRLTAQLVQE-- 98
Rat            GLG---QLSCLPLDGRTKFS--QRG----------- 83
Chicken        PRRAALHPACIPPH--TQPSREQRSA---------- 87
Tilapia        DGMPVVRTLELLLSS-LRNKENLGKVLDGEDA---- 117
Salmon         HPVAMVSTLEILLDI-IRNQENIGKTLSGEDADWLP 155
Carp           ---PVVKSLEILLNT-LRNKESLRSALAQEESDWLP 117
Xenopus        LRFLLEQTLEIMKKHGVLNIENIHSW---------- 108
                         :
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B.

```
Human       tpdinpawyasrgirpvgrf-amide
Bovine      tpdinpawyagrgirpvgrf-amide
Rat         tpdinpawytgrgirpvgrf-amide
Chicken     npdidpswytgrgirpvgrf-amide
Tilapia     speidpfwyvgrgvrpigrf-amide
Carp        speidpfwyvgrgvrpigrf-amide
Salmon      speidpfwyvgrgvrpigrf-amide
Xenopus     speidpywyvgrgvrpigrf-amide
```

C.

```
Human       Srthrhsmeirtpdinpawyasrgirpvgrf-amide
Bovine      Srahqhsmeirtpdinpawyagrgirpvgrf-amide
Rat         Srahqhsmetrtpdinpawytgrgirpvgrf-amide
Chicken     Grlrersmeirnpdidpswytgrgirpvgrf-amide
Tilapia     Dfhivhnvdnrspeidpfwyvgrgvrpigrf-amide
Carp        Dlhivhnvdnrspeidpfwyvgrgvrpigrf-amide
Salmon      Nfhivhnvdnrspeidpfwyvgrgvrpigrf-amide
Xenopus     Srsfnhqidnrspeidpywyvgrgvrpigrf-amide
```

phibians [*Xenopus laevis* (Sakamoto et al. 2006), euryhaline mudskippers (Sakamoto et al. 2005)], chicken (Tachibana et al. 2005), and mammals (cattle, rat, mouse, human) (Fig. 1A). The last 20 amino acids are much better conserved between species compared to the 31 amino acid peptide, with mostly conservative amino acid changes that are clearly phylogenetically separated between lower vertebrates versus the higher vertebrates (Fig. 1B,C).

◀ **Fig. 1** **A** Protein sequence alignment of PrRP preproprotein between various species. Alignment was performed using the alignment tool ClustalW (http://www.ebi.ac.uk/clustalw/#). *Gray box* indicates PrRP20 sequence. Degree of alignment is defined by the software as: "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment, ":" means that conserved substitutions have been observed, according to the conservation of hydrophobic, acidic, basic, or hydroxyl/amine residues, and "." means that semi-conserved substitutions are observed. Peptide alignment of processed peptides of PrRP20 (**B**) and PrRP31 (**C**) are as shown. Well-conserved basic, acidic, hydroxyl residues are designated by light *gray boxes*, and less-conserved residues between species are designated by *darker gray boxes*

Gene structure analysis reveals that the rat PrRP gene spans approximately 2.4 kilobases containing three exons and two introns (Yamada et al. 2001). 5′- and 3′-RACE determined a polyadenylation signal at 103 dp downstream from the stop codon and possibly three transcriptional start sites at 92, 199, and 325 bp upstream to the translational start site. A 1.6 kb element upstream of the +1 start site was sufficient to specifically induce reporter gene expression in neuronal and pituitary cell lines. Putative binding sites such as SP1-1, AP-2, Oct-2A, and three TATA boxes are found within this region, suggesting a bona-fide promoter element.

1.2 PrRP Receptor

The PrRP receptor was originally an orphan G protein-coupled receptor called GPR10 [also known as hGR3 (Hinuma et al. 1998), or UHR-1 for the rat orthologue (Welch et al. 1995)], cloned by low stringency PCR using degenerate primers to the conserved transmembrane domains 2 and 6 of somatostatin receptors (Marchese et al. 1995). GPR10 bore some resemblance to NPY receptors (31% overall and 46% in the transmembrane domains), but it could not be activated with NPY, NPY-related peptides, such as pancreatic polypeptide (PP) or Peptide YY (PYY), or any other peptides known at the time (Marchese et al. 1995). Identification of PrRP as the ligand for GPR10 revealed the receptor to be related instead to NPFF-2, the receptor for the first RFamide peptide NPFF found in mammals. Both PrRP31 and PrRP21 bind with high affinity to CHO cells expressing either hGR3/GPR10 or UHR-1 (2.5×10^{-11} M) that is completely dependent on the C-terminal amidation. Interestingly, hPrRP20 and hPrRP31 have nearly equal affinity for NPFF-2 (Ki = 23 and 19 nM, respectively) as NPFF has for NPFF-2 (Ki = 5.2 nM). While NPFF-2 has promiscuity in its binding with high affinity to other peptides such as PrRP, FMRFamide (Ki = 13 nM), RFRP-1 (Ki = 15 nM) and RFRP-3 (Ki = 55 nM), the PrRP receptor is highly discriminate for binding to PrRP peptides (Ki = 1.0 nM PrRP20, Ki = 0.68 nM PrRP31, Ki > 100 000 NPFF).

As for signal transduction, PrRP in CHO cells stably expressing the PrRP receptor stimulates Ca^{2+} influx and inhibits forskolin-stimulated cyclic AMP

(Hinuma et al. 1998). In GH3 rat pituitary tumor cells, PrRP activates Akt through Gi/Go since it could be blocked by the PI3K inhibitor wortmannin, the *c*-terminal tail of beta-adrenergic receptor kinase I (which blocks beta gamma subunits of G proteins), and pertussis toxin (Hayakawa et al. 2002). PrRP could also stimulate extracellular signal-regulated protein kinase (ERK) and Jun N-terminal kinase (JNK), an effect that is completely abrogated by pertussis toxin, an inhibitor of Gai/o, but not by protein kinase C inhibitor or changes in intra- or extracellular Ca^{2+} concentration, suggesting a mechanism independent of Gaq/PKC (Kimura et al. 2000). The release of prolactin by PrRP is dependent on the differential activation of both ERK and JNK, with both cascades being necessary to activate the prolactin promoter in an Ets transcription factor-dependent mechanism (Kimura et al. 2000). These results suggest that the PrRP receptor activates downstream events via Gαq and Gαi/o in a cell-type specific manner that differentially controls downstream events.

Although PrRP has been isolated and the gene cloned from mammals, fish, and amphibians, the receptor has not been fully cloned from the lower vertebrates, with the exception of partial sequences found in public databases. The PrRP receptor is well conserved among mammals, with protein sequence identity of 90% or better (Fig. 2A). However, the PrRP receptor in chickens is only 54% related to its mammalian counterpart, suggesting phylogenetic divergence between birds and mammals. The sequence of the C-terminal tail is also highly conserved, especially in the last six amino acids, being nearly 100% identical ranging from mouse to human (Fig. 2B). It has been discovered that the PrRP receptor may interact with proteins via this conserved sequence (Lin et al. 2001). The last four amino acids-SVVI are very similar to canonical sequences found in the C-terminus of α-amino-3-hydroxy-5-methylisoxazole-4-propionic Acid (AMPA) glutamatergic receptors (-SVKI). This motif interacts with PSD-95/Discs-large/ZO-1 (PDZ) domains from a host of synaptic proteins that are critical for AMPA receptor clustering and signaling in the microdomains of the postsynaptic membrane (Bredt and Nicoll 2003). The PrRP receptor binds very specifically with GRIP, ABP, and PICK-1 and not PSD-95, a protein that interacts with NMDA receptors. Alteration of a single amino acid residue in three of the four amino acids (– SVXI; X = any amino acid) completely abrogates binding to these proteins (Lin et al. 2001). Clustering of the PrRP receptor by PICK-1 is also mediated via this binding motif. Since the PrRP receptor is co-expressed in the same brain regions as these proteins, this suggests that this interaction may mediate signaling and regulation in macromolecular protein complexes at the postsynaptic membrane. Indeed, there is evidence to suggest that PrRP receptor activation can modulate synaptic transmission through modulation of AMPA receptor signaling (Lin et al. 2002). The exact mechanism by which this regulation occurs is currently unknown.

The human PrRP receptor gene spans approximately 2.0 kb and contains two exons and one intron. Several putative transcriptional start sites are lo-

A.

```
Human           MASSTTRGPRVSDLFSGLPPAVTTPANQSAEASAGNGSVAGADAPAVTPFQSLQLVHQLK 60
Chimpanzee      MASSPTRGPRVSDLFSGLPPAVTTPANQSAEASAGNGSVAGADAPAVTPFQSLQLVHQLK 60
Dog             MASLPTQGPSVPDLFSGLPPAASIPANQSSEASAGNGSAAGAGAQAVTPFQSLQLVHQLK 60
Rat             MTSLPPGTTGDPDLFSGPSPAGSTPANQSAEASESNVSATVPRAAAVTPFQSLQLVHQLK 60
Mouse           MTSLSTETTGDPDLSSGLLPASSTPANQSAEASEGNLSATVPRAAAVTPFQSLQLVHQLK 60
Chicken         MADDKRREMMNSDNLTSQSFLSAIHSNASN------------------LFSGLQFVQSFK 42
                *:.        .*  :.     :  :* *                    *..**:*:.:*

Human           GLIVLLYSVVVVVGLVGNCLLVLVIARVRRLHNVTNFLIGNLALSDVLMCTACVPLTLAY 120
Chimpanzee      GLIVLLYSVVVVVGLAGNCLLVLVIARVRRLHNVTNFLIGNLALSDVLMCTACVPLTLAY 120
Dog             GLIVLLYSVVVVVGLVGNCLLVLVIARVRRLHNVTNFLIGNLALSDVLMCTACVPLTLAY 120
Rat             GLIVMLYSIVVVVGLVGNCLLVLVIARVRRLHNVTNFLIGNLALSDVLMCAACVPLTLAY 120
Mouse           GLIVMLYSIVVVVGLVGNCLLVLVIARVRRLHNVTNFLIGNLALSDVLMCAACVPLTLAY 120
Chicken         PLIIPCYSLVVFVGVIGNYLLIYVICKTKKMHNVTNFLVGNLAFSDMLMCATCVPLTLAY 102
                 **:  **:**.**: **  **: **.:.:::*******:****:**:***::********

Human           AFEPRGWVFGGGLCHLVFFLQPVTVYVSVFTLTTIAVDRYVVLVHPLRRRISLRLSAYAV 180
Chimpanzee      AFEPRGWVFGGGLCHLVFFLQPVTVYVSVFTLTTIAVDRYVVLVHPLRRRISLRLSAYAV 180
Dog             AFEPRGWVFGGGLCHLVFFLQPVTVYVSVFTLTTIAVDRYVVLVHPLRRRISLRLSAYAV 180
Rat             AFEPRGWVFGGGLCHLVFFLQPVTVYVSVFTLTTIAVDRYVVLVHPLRRRISLKLSAYAV 180
Mouse           AFEPRGWVFGGGLCHLVFFLQPVTVYVSVFTLTTIALDRYVVLVHPLRRRISLRLSAYAV 180
Chicken         AFEPRGWVYGRFMCYFVFLMQPVTVFVSVFTLTVIAVDRYYATVYPFRRRLTIPVCAYIL 162
                ********:*  :*::**::*****:*******.**:*** . *:*:***::: :.** :

Human           LAIWALSAVLALPAAVHTYHVELKPHDVRLCEEFWGSQERQRQLYAWGLLLVTYLLPLLV 240
Chimpanzee      LAIWALSAVLALPAAVHTYHVELKPHDVRLCEEFWGSQERQRQLYAWGLLLVTYLLPLLV 240
Dog             LAIWALSAVLALPAAVHTYHVELKPHRVRLCEEFWGSQERQRQLYAWGLLLFTYLLPLLV 240
Rat             LGIWALSAVLALPAAVHTYHVELKPHDVRLCEEFWGSQERQRQIYAWGLLLGTYLLPLLA 240
Mouse           LGIWALSAVLALPAAVHTYHVELKPHDVRLCEEFWGSQERQRQIYAWGLLLGTYLLPLLA 240
Chicken         AAIWLLSCTLAAPALVHTYHAEFPELDFSICEEFWFHMRRDRLAYAYSTLIITYVLPLAV 222
                 .** **..** ** *****.*:    . :*****   .*:*  **:. *: **:*** .

Human           ILLSYVRVSVKLRNRVVPGCVTQSQADWDRARRRRTFCLLVVVVVVFAVCWLPLHVFNLL 300
Chimpanzee      ILLSYVRVSVKLRNRVVPGCVTQSQADWDRARRRRTFCLLVVVVVVFAVCWLPLHVFNLL 300
Dog             ILLSYVRVSVKLRNRVVPGCVTQSQADWDRARRRRTFCLLVVVVVVFAVCWLPLHVFNLL 300
Rat             ILLSYVRVSVKLRNRVVPGSVTQSQADWDRARRRRTFCLLVVVVVVFALCWLPLHIFNLL 300
Mouse           ILLSYVRVSVKLRNRVVPGSVTQSQADWDRARRRRTFCLLVVVVVVFAVCWLPLHIFNLL 300
Chicken         ISLSYLRISVKLKNRVVPGNVTQGQAEWDRARRRKTFRLLVLVVAAFGVCWLPLHIFNMM 282
                * ***:*:****:****** ***.**:*******:** ***:**..*.:******:**::

Human           RDLDPHAIDPYAFGLVQLLCHWLAMSSACYNPFIYAWLHDSFREELRKLLVAWPRKIAPH 360
Chimpanzee      RDLDPHAIDPYAFGLVQLLCHWLAMSSACYNPFIYAWLHDSFREELRKLLVAWPRKIAPH 360
Dog             RDLDPHAIDPYAFGLVQLLCHWLAMSSACYNPFIYAWLHDNFREELRKLLLTWPRKIAPH 360
Rat             RDLDPRAIDPYAFGLVQLLCHWLAMSSACYNPFIYAWLHDSFREELRKMLLSWPRKIVPH 360
Mouse           RDLDPRAIDPYAFGLVQLLCHWLAMSSACYNPFIYAWLHDSFREELRKMLLSWPRKIVPH 360
Chicken         KDIDISLIDKQYFNFIQLLCHWFAMMSACTNAFLYAWLHDSFRGELKKMFAWRKKKIGPA 342
                :*:*   **   *.::******:** *** *.*:******.** **:*::    :** *

Human           GQNMTVSVVI 370
Chimpanzee      GQNMTVSVVI 370
Dog             GQTVTVSVVI 370
Rat             GQNMTVSVVI 370
Mouse           GQNMTVSVVI 370
Chicken         TNCIMASVVL 352
                 : : .***:
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B.

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Human          YAWLHDSFREELRKLLVAWPRKIAPHGQNMTVSVVI.
Chimpanzee     YAWLHDSFREELRKLLVAWPRKIAPHGQNMTVSVVI.
Dog            YAWLHDNFREELRKLLLTWPRKIAPHGQTVTVSVVI.
Rat            YAWLHDSFREELRKMLLSWPRKIVPHGQNMTVSVVI.
Mouse          YAWLHDSFREELRKMLLSWPRKIVPHGQNMTVSVVI.
Chicken        YAWLHDSFRGELKKMFAWRKKKIGPATNCIMANVVL.
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Fig. 2 **A** Protein sequence alignment of PrRP receptor between various species. Alignment was performed using ClustalW and alignment definitions are described in Fig. 1. **B** The c-terminal 36 amino acid residue of the cytoplasmic tail of PrRP receptor is shown as compared between various species. Well-conserved basic, acidic, hydroxyl residues are designated by *light gray* boxes, and less-conserved residues between species are designated by *darker gray* boxes

cated 5′ to the translational start site (Kishimoto et al. 2000; Ozawa et al. 2002) and two potential polyadenylation sites are located 510 and 714 bp downstream from the stop codon (Ozawa et al. 2002). The regulation of the PrRP receptor gene reportedly is located within a 4.0 kilobase pair section upstream. No TATA or CAAT box is found, but a GC box located between −467 and −457 is required for basal expression. Putative transcription factor binding sites for Pit-1, pituitary hemeobox 1 (Ptx1), AP-1, Sp1, and cAMP response element (CRE) are found within this 4.0 kb fragment (Kishimoto et al. 2000; Ozawa et al. 2002), but it is still inconclusive whether this is sufficient for tissue-specific expression, since luciferase reporter assay experiments demonstrate promoter activity in this region in both pituitary (GH3, GH4C1) and non-pituitary cell lines (Kishimoto et al. 2000). There is evidence to suggest that regulation of PrRP receptor expression is mediated through these upstream elements. Treatment of GH4C1 cells with bromocriptine, a dopamine D2 receptor agonist used clinically for the treatment of prolactinoma, inhibits PrRP receptor promoter stimulation (Ozawa et al. 2002). Although the PrRP receptor promoter activation by bromocriptine was dependent on the CRE binding protein (CREB), this was not caused by the direct binding of CREB to the DNA but rather mediated by the binding of an unknown 60-kDa protein to a consensus sequence of 5′-cccacatcat-3′ located at the −663 to −672 region (Ozawa et al. 2002).

2 Anatomical Distribution

2.1 PrRP

In-situ hybridization and immunohistochemical studies indicate that PrRP cell bodies reside within the nucleus tractus solitarius (NTS), the ventrolateral medulla (VLM), the reticular nucleus of the medulla, the ventromedial hypothalamus (VMH), and dorsomedial hypothalamus (DMH) (Chen et al. 1999; Ibata et al. 2000; Iijima et al. 1999; Lee et al. 2000; Maruyama et al. 1999a). PrRP expression in the brainstem is restricted to the catecholaminergic neurons of the NTS and VLM and its fibers reach several brain regions. One major contact is to the paraventricular nucleus, where it synapses with cells containing various neuropeptides, such as CRH (Matsumoto et al. 2000) and oxytocin (Maruyama et al. 1999a,b). Synapses also form on oxytocin/vasopressin neurons of the supraoptic nucleus (Maruyama et al. 1999a) and somatostatin neurons of the periventricular nucleus (Iijima et al. 2001). Other sites of PrRP projections are also found in the dorsomedial hypothalamus, area postrema, pontine parabrachial area, preoptic areas, bed nucleus of the stria terminalis, amygdala, mediodorsal nucleus of the thalamus, sep-

tal nucleus, and regions surrounding the ventricular lining and blood vessels (Ibata et al. 2000; Iijima et al. 1999; Maruyama et al. 1999a). These projections suggest that PrRP is involved in other central functions besides prolactin release.

Strangely enough, no PrRP immunoreactivity is found in the median eminence or in neurosecretory cells of the hypothalamus, classically associated with releasing hormones for anterior pituitary function (Maruyama et al. 1999a; Morales et al. 2000). Although it has been demonstrated that PrRP is able to stimulate release of prolactin from both rat pituitary adenoma cells and pituitary primary cells, how PrRP reaches the pituitary to mediate prolactin release is a mystery. In the human, the role of PrRP in prolactin release is even less clear. Regional distribution of PrRP in the human brain determined by radioimmunoassay finds the highest concentration in the hypothalamus, followed by medulla oblongata, and thalamus, without any detectable peptide in the frontal lobe or temporal lobe. However, the level of immunoreactive PrRP in the human hypothalamus is much lower than the levels of other neuropeptides with prolactin-releasing ability, such as TRH and VIP (Takahashi et al. 2000). PrRP is found in other locations besides the brain. PrRP immunoreactivity is also found in human placenta, human decidua, rat adrenal gland, and in the plasma (Fujiwara et al. 2005; Matsumoto et al. 1999a; Reis et al. 2002; Yasui et al. 2001). PrRP cells are specifically located in the adrenal medulla among tyrosine hydroxylase (TH) and phenylethanolamine *N*-methyltransferase (PNMT) cells (Fujiwara et al. 2005). Taken together these data suggest that PrRP has alternative roles within the central nervous system and in the peripheral tissues.

2.2
PrRP Receptor

Initial northern blot analysis revealed PrRP receptor expression to be highest in the pituitary and detectable in the cerebellum, hypothalamus, pons, hippocampus, and adult mouse suprachiasmatic nucleus (SCh) and E18 fetal rat SCh cells (Welch et al. 1995). By RT-PCR, the PrRP receptor is found in abundance in the rat anterior pituitary and in normal human pituitary and pituitary adenomas (Fujii et al. 1999; Zhang et al. 1999). Immunohistochemistry on human pituitary using PrRP receptor antibodies finds PrRP receptor to be expressed in ACTH cells rather than prolactin secreting cells (Abe et al. 2003). Binding experiments using [125I]-labeled PrRP finds specific binding in the reticular nucleus of the thalamus, a site with the highest mRNA expression in the brain, with light binding in the periventricular nucleus (Roland et al. 1999). With in situ hybridization, the PrRP receptor is distributed in several specific brain regions (Ibata et al. 2000; Roland et al. 1999) (unpublished results, Fig. 3). Detailed analysis using emulsion-dipped slides in comparison to sense probe controls (unpublished data, summa-

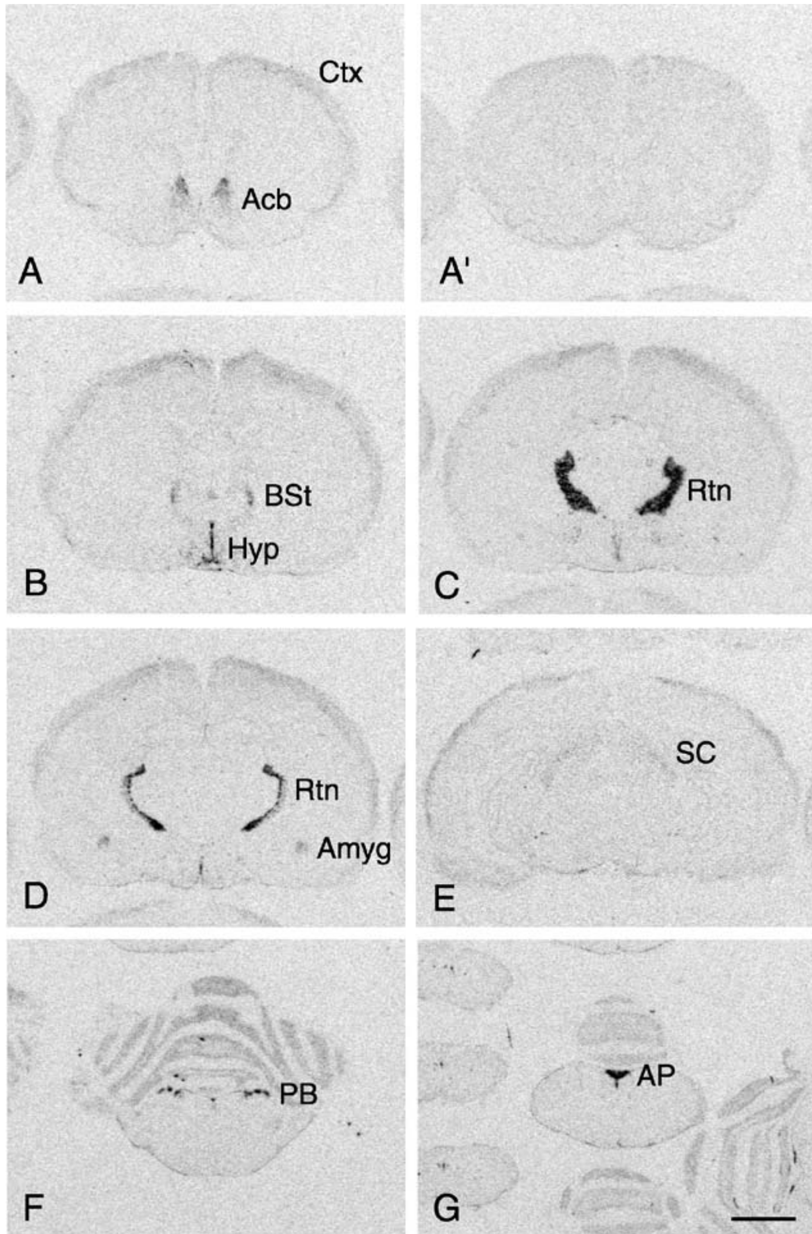

Fig. 3 Film autoradiograms of coronal sections through the adult rat brain. **A–G**: Sections hybridized with antisense [^{35}S]-UHR-1 probes. **A′**: A representative control section showing hybridization with sense [^{35}S]-UHR-1 probe. Acb, nucleus accumbens; Ctx, cortex; BSt, bed nucleus of the stria terminalis; Hyp, hypothalamus; Rtn, reticular thalamic nucleus; Amyg, amygdala; SC, superior colliculus; PB, parabrachial nucleus; AP, area postrema. *Scale bar* = 250 μm

rized in Table 1) reveals specific expression in the cortex, shell of the nucleus accumbens, lateral septal nucleus, bed nucleus of the stria terminalis and central nucleus of the amygdala. In the hypothalamus, the PrRP receptor mRNA is expressed in several preoptic nuclei, suprachiasmatic, supraoptic, ventrolateral hypothalamic, lateral hypothalamic, parvocellular paraventricular, periventricular, dorsomedial, ventromedial, arcuate, ventral premammillary, and ventral tuberomammillary nuclei. The highest expression was found in the reticular thalamic nucleus (RTN), the only thalamic nucleus expressing PrRP receptor mRNA. In the midbrain, very low but specific expression is found in the superior colliculus and the periaqueductal gray. In the brainstem, strong signal occurs on the lateral parabrachial nucleus (PB), moderate signal in the dorsal tegmental nucleus, dorsal raphe, and very low expression in the locus coeruleus. Low expression was also found in the nucleus tractus solitarius and high expression in the area postrema. Extranuclear sites such as ependyma lining of the 3rd, 4th ventricles and central canal is specifically and strongly labeled with PrRP receptor expression (unpublished results). In the peripheral tissues, PrRP receptor is highly expressed in the adrenal medulla in rats (Nieminen et al. 2000; Roland et al. 1999) and cows within the TH positive cells of the adrenal medulla (unpublished data). By RT-PCR, PrRP receptor expression is also found in the stomach and femur (Fujii et al. 1999).

2.3 Comparison Between PrRP and PrRP Receptor Expression

A comparison between PrRP receptor mRNA expression and PrRP expression in the brain is shown in Table 1. Dense PrRP fiber networks are found in some areas of receptor expression, such as the bed nucleus of the stria terminalis, the septal nucleus, the supraoptic nucleus, the paraventricular nucleus, the dorsomedial nucleus (and cell bodies), the amygdala, the nucleus tractus solitarius (and cell bodies), and the ependymal lining of the ventricles. However, there are also great disparities between areas of receptor expression and the distribution of its cognate peptide ligand. No immunoreactive PrRP fibers are detectable in the nucleus accumbens, the cortex, in most of the preoptic regions, in the reticular thalamic nucleus, and in the various hypothalamic nuclei, such as the lateral hypothalamus (LH). No fibers have ever been described to exist in the midbrain, such as the superior colliculus or the periaqueductal gray zones. In the brain stem, no fibers are described to exist in the parabrachial nuclei or the area postrema, or in the cerebellum. PrRP-immunoreactive fibers also exist in regions without receptor expression, such as the areas surrounding the fornix, capillary wall in the septal region, and the mediodorsal thalamic nucleus (Iijima et al. 1999). Receptor/ligand mismatches, although uncommon in classic neurotransmitter synapses such as glycinergic, glutamatergic, and cholinergic synapses, are seen quite commonly in many neuropeptide transmitters (Herkenham

Table 1 Distribution of PrRP receptor mRNA in adult rat brain in comparison to PrRP-ir fibers. Nomenclature according to Paxinos and Watson, 4th edition (1999). PrRP-ir fiber distribution data from Ibata et al. 2000; Iijima et al. 1999; Lee et al. 2000; Maruyama et al. 1999a. "?" = indiscernable from data presented; "*" = cell bodies. The intensity of PrRP receptor mRNA was scored in the following manner: +++++, very-strong expression; ++++, strong expression; +++, moderate expression; ++, low expression; +, very-low expression or small number of high expressing cells; –, no expression

Abbreviations:

A1	A1 noradrenaline cells
A2	A2 noradrenaline cells
ac	anterior commissure
Acbc	nucleus accumbens, core
AcbSh	nucleus accumbens, shell
AH	anterior hypothalamic nucleus
AI	agranular insular
AMPO	anterior medial preoptic nucleus
AP	area postrema
ArcD	arcuate nucleus, dorsal
ArcL	arcuate nucleus, lateral
ArcM	arcuate nucleus, medial
AVPe	anterior ventral periventricular
AVPO	anterior ventral preoptic nucleus
Bar	Barrington's nucleus
BLA	basolateral amygdala
BSTl	bed nucleus of stria terminalis, lateral
BSTld	bed nucleus of stria terminalis, laterodorsal
BSTlj	bed nucleus of stria terminalis, lateral juxtacapsular
BSTlp	bed nucleus of stria terminalis, lateral posterior
BSTlv	bed nucleus of stria terminalis, lateral ventral
BSTma	bed nucleus of stria terminalis, medial anterior
BSTmv	bed nucleus of stria terminalis, medial ventral
BSTv	bed nucleus of stria terminalis, ventral
C1	C1 adrenaline cells
C2	C2 adrenaline cells
cc	corpus callosum
CeC	central amygdaloid nucleus, capsular
CeL	central amygdaloid nucleus, lateral
CeM	central amygdaloid nucleus, medial
Cg	cingulate gyrus
CIC	central nuc. inf. colliculus
CPu	caudate putamen
DCIC	dorsal cortex inf. colliculus
DLO	dorsolateral orbital cortex
DMD	dorsomedial nucleus of hypothalamus
DMDC	dorsomedial nucleus, lateral
DMDV	dorsomedial nucleus, ventral
DpG	deep gray layer, sup. colliculus
DpWh	deep white layer, sup. colliculus
DRC	dorsal raphe, caudal
DTg	dorsal tegmental nucleus
ECIC	external cortex inf. colliculus
Ent	entorhinal cortex
GI	granular insular cortex
HDB	horizontal diagonal band of Broca
IL	infralimbic cortex
InG	intermed. gray layer sup. colliculus
InWh	intermed. White layer sup. colliculus
IRT	intermed. reticular nucleus
LA	lateral anterior hypothalamus
LC	locus coeruleus
LH	lateral hypothalamus nucleus
LO	lateral orbital cortex
LPB	lateral parabrachial nucleus
LPO	lateral preoptic nucleus
LRt	lateral reticular nucleus
LSD	lateral septal nucleus, dorsal
LSI	lateral septal nucleus, intermediate
LSV	lateral septal nucleus, ventral
MCPO	magnocellular preoptic nucleus
ME	median eminence
Me5	mesencephalic 5 nucleus
MeAD	medial amygdaloid nucleus, anterior dorsal
MeAV	medial amygdaloid nucleus, anterior ventral

Table 1 (continued)

MM	medial mammillary bodies	PRL	prelimbic cortex
MnPO	median preoptic nucleus	PT	paratenial thalamic nuc.
MO	medial orbital cortex	RCh	retrochiasmatic area
Mo5	motor nucleus 5	RSA	retrosplenial agranular cortex
MPA	medial preoptic area	Rt	reticular thalamic nuc.
MPB	medial parabrachial nucleus	SCh	suprachiasmatic nucleus
MPO	medial preoptic nucleus	scp	superior cerebellar peduncle
MPOC	medial preoptic nucleus, central	SO	supraoptic nucleus
Op	optic nuc. layer, sup. colliculus	SolC	nucleus solitary tract nuc., commissural
Pa	paraventricular nucleus		
PaAM	Pa, anterior magnocellular	SolL	nucleus solitary tract nuc., lateral
PaAP	Pa, anterior parvicellular	SolM	nucleus solitary tract nuc., medial
PaLM	Pa, lateral magnocellular	SuG	superficial gray, sup. colliculus
PaMP	Pa, medial parvicellular	TC	tuber cinereum area
PaPo	Pa, posterior part	TT	tenia tecta
PaV	Pa, ventral part	VLPO	ventrolateral preoptic nucleus
Pe	periventricular nucleus	VMH	ventromedial hypothalamus
Pir	piriform cortex	VMHDM	VMH, dorsomedial
PMCo	posteromedial cortical amygdaloid nucleus	VMHVL	VMH, ventrolateral
		VO	ventral orbital cortex
PMD	premammillary nucleus, dorsal	VTM	ventral tuberomamillary nuc.
PMV	premammillary nucleus, ventral	ZI	zona incerta
Pr5	principle sensory 5 nucleus		

Region	PrRPR mRNA	PrRP-ir fiber	Region	PrRPR mRNA	PrRP-ir fiber
Neocortex		No	GI	++	
Frontal			IL	++	
Layer II	+		LO	+	
Layer III	+		MO	+	
Parietal			PRL	+	
Layer II	+		Pir	–	
Layer III	+		RSA	–	
Temporal			TT	–	
Layer II	++		VO	+	
Layer III	++		*Telencephalon*		
Occipital			Ventral forebrain		
Layer II	++		ac	–	?
Layer III	++		AcbC	–	?
Other cortical regions		No	AcbSh	+++	?
AI	++		HDB	+	?
cc	–		Septum		Yes
Cg	–		LSD	+	
DLO	+		LSI	+/–	
Ent	–		LSV	+	
MS	–		ME	–	No

Table 1 (continued)

Region	PrRPR mRNA	PrRP-ir fiber	Region	PrRPR mRNA	PrRP-ir fiber
Basal Ganglia		No	MM	–	?
Cpu	+/–		Pa	++	Yes
Bed Nucleus			PaAM	+	Yes
BSTl	+++	Yes	PaLM	+/–	Yes
BSTld	+++	Yes	PaAP	+++	Yes
BSTlj	–	?	PaMP	+	Yes
BSTp	+	?	PaPo	++	Yes
BSTlv	+++	Yes	PaV	+	Yes
BSTma	+	?	Pe	++	Yes
BSTmv	++	Yes	PMD	–	?
BSTv	+++	Yes	PMV	++	?
Amygdala			RCh	+	?
BLA	–	Yes	SCh	+	?
CeL	++	No	SO	+	Yes
CeM	+	No	TC	+	?
CeC	++	No	VMH	+	?
MeAD	+	No	VMHDM	–	?
MeAV	+	No	VMHVL	++	?
PMCo	+	No	VTM	++	?
Diencephalon			Thalamus		
Preoptic			PT	–	Yes
AMPO	+++	Yes	Rt	+++++	No
AVPO	+++	Yes	ZI	–	No
AVPe	++	Yes	*Mesencephalon*		
LPO	++	Yes	Inferior Colliculus		No
MCPO	+	?	CIC	–	
MnPO	++	?	DCIC	–	
MPA	+	?	ECIC	+	
MPO	++++	?	Superior Colliculus		No
MPOC	++++	?	DpG	–	
Pe	++	Yes	DpWh	–	
VLPO	+++	?	InG	+	
Hypothalamus			InWh	–	
AH	+	?	Op	–	
ArcD	++	?	SuG	+/–	
ArcM	+/–	?	*Cerebellum*		No
ArcL	+/–	?	Cortex		
DMD	+++	Yes*	Molecular	–	
DMDV	++	Yes*	Purkinje	+/–	
DMDC	+	Yes*	Granule cell	–	
LA	–	?	*Metencephalon*		
LH	++	?	Bar	–	No
DTg	+	?	Pr5	+	No
DRC	++	?	scp	–	No

Table 1 (continued)

Region	PrRPR mRNA	PrRP-ir fiber	Region	PrRPR mRNA	PrRP-ir fiber
IRT	+/–	No	*Myelencephalon*		
LC	+/–	Yes	A1	–	No
LPB		Yes	A2	+	No
dorsal	++++	?	AP	++++	Yes
external	+	?	C1	–	No
internal	+++	?	C2	+	No
ventral	++++	?	LRt	–	Yes*
Me5	–	No	SolC	++	Yes*
Mo5	+	No	SolL	+	Yes*
MPB	+++	?	SolM	+	Yes*

1987) such as substance P (Duggan et al. 1990; Liu et al. 1994; Nakaya et al. 1994), somatostatin (Leroux et al. 1993), vasointestinal polypeptide (Hof et al. 1991), and the endogenous opioids (Elias et al. 2000). In fact, many peptide systems have significant mismatches between the distribution of the peptides and their respective binding sites (Herkenham 1987; Mantyh et al. 1984). Taking these into consideration, PrRP may reach its target through the following routes: (1) PrRP could be released at distant sites and reach the receptor by diffusion through tissue, as seen for substance P (Duggan et al. 1990), or (2) PrRP is released into the cerebrospinal fluid (CSF) through fibers terminating at ventricular zones (Iijima et al. 1999) and reaches receptor sites through this route of transport. These issues could be addressed by studying the temporal and spatial patterns of PrRP release into the CSF or diffusion through tissue utilizing antibody microprobe technology (Duggan et al. 1990). A remote possibility includes a receptor subtype that is yet to be identified, which has been suggested in one pharmacological study of peripheral tissues (Satoh et al. 2000).

3 Functional Implications

The overall distribution of PrRP receptor mRNA in the brain suggests that PrRP is involved in various brain functions, such as stress modulation, energy hemeostasis and food intake, nociceptive, sexual and reproductive behaviors, sleep and arousal, and cardiovascular regulation. The basis of these functions will be discussed in relation to expression patterns of the PrRP receptor and PrRP in the brain and periphery, what is known about these regions of receptor and peptide expression with respect to the function of the organism,

and the current state of knowledge of the PrRP system with regards to these specific functions.

3.1 Prolactin Release

Functional studies searching for the in vivo relevance of PrRP as a central mediator of prolactin release were initially controversial. Those that showed positive results were relying on intravenous injections of PrRP into rats during certain estrous phases of the female estrous cycle (Matsumoto et al. 1999b; Tokita et al. 1999). However, studies that used intracerebroventricular (ICV) injection of PrRP reported no prolactin release (Jarry et al. 2000; Samson et al. 2003). This discrepancy was recently resolved with the demonstration that PrRP is able to elicit prolactin release when ICV injected during the proestrous phase, an action not evident in females in other phases or in males (Hizume et al. 2000). These results suggest that PrRP functions as a prolactin-releasing factor at specific times in the estrous cycle. How PrRP gets to the anterior pituitary still remains a mystery. There's the possibility that peripheral sources of PrRP, such as those found in the adrenal medulla, pancreas, testis (Nieminen et al. 2000) and placenta (Yasui et al. 2001), may directly impinge upon the anterior pituitary to affect prolactin release. PrRP may influence prolactin secretion indirectly through central sites, such as the paraventricular nucleus and/or other regions. Alternatively, PrRP is an ancient system for prolactin release that has lost this ability in higher order species. In teleost fish, immunoreactive fibers of C-RFa (a mammalian orthologue of PrRP) are found to project to the pituitary and terminate close to prolactin cells in the rostal pars distalis and the somatolactin (SL) cells in the pars intermedia. IP injection of C-RFa into rainbow trout causes release of prolactin and SL without affecting growth hormone release (Moriyama et al. 2002; Seale et al. 2002). This function is recapitulated in the amphibious euryhaline mudskippers, with coexpression of PrRP with cells expressing prolactin (Sakamoto et al. 2005). In humans, however, PrRP receptor expression no longer resides with prolactin-secreting cells, but is found with ACTH-secreting cells (Abe et al. 2003), suggesting a shift in the role of PrRP in humans versus the lower vertebrates.

3.2 Stress and Anxiety Response

PrRP receptor mRNA is found in several nuclei involved in modulating stress responses. These include the lateral septal nucleus, lateral and ventral bed nucleus of the stria terminalis, central nucleus of the amygdala, shell of the nucleus accumbens, parvocellular paraventricular nucleus of the hypothalamus, and nucleus tractus solitarius, in addition to its low expression in the

locus coeruleus (Lin et al. 2002). Various stressors are able to induce neuronal activation (as assessed by c-Fos activation) in all of these regions (Beck and Fibiger 1995; Cullinan et al. 1995), and lesion studies have implicated these regions in the stress response (Feldman et al. 1990; Kopchia et al. 1992). PrRP mRNA expression in the brainstem exist in cell groups that often are activated by stressful stimuli.

In concordance with the view that the PrRP system may regulate the stress response, ICV injection of PrRP causes marked induction of c-Fos expression in the paraventricular nucleus (PVN), some of which are on CRH-positive neurons (Matsumoto et al. 2000). This is correlated with the concomitant release of ACTH and corticosterone into peripheral circulation that is partly dependent on CRH release (Maruyama et al. 2001; Matsumoto et al. 2000; Seal et al. 2002) and not a direct action on the ACTH cells of the rat pituitary (Seal et al. 2002). A dense fibrous network of PrRP is found clustered in the PVN (some of which synapse onto CRH neurons) as well as in the amygdala, bed nucleus of stria terminalis (BST), and septum (Ibata et al. 2000; Iijima et al. 1999; Maruyama et al. 1999a). Some of the fibers also make synaptic connections with oxytocin-containing cells in the paraventricular hypothalamus (Maruyama et al. 1999a). Intracerebroventricular injection of PrRP elicits release of oxytocin (Maruyama et al. 1999b), which likely contributes to PrRP's role in CRH release. By retrograde analysis PrRP neurons innervating the paraventricular hypothalamus originate in the dorsomedial medulla, within the tyrosine hydroxylase containing A1/A2 cell groups, sites with the highest PrRP expression levels. PrRP and norepinephrine from the same cells act synergistically to elicit ACTH release, since both given together at subthreshold doses are able to cooperatively increase ACTH levels (Maruyama et al. 2001). Experimental stress models such as water immersion-stress (Maruyama et al. 2001), conditioned-fear stimuli (Zhu and Onaka, 2003), and footshock stress (Morales and Sawchenko 2003) elicit c-Fos activation in the PrRP neurons in the medulla but this is not the case with hemorrhage or suckling stimuli, suggesting a differential sensitivity of PrRP neurons to acute stressors (Morales and Sawchenko 2003). This is further supported by the finding that running stress, although stimulating c-Fos activation of PrRP neurons in the brainstem, paradoxically inhibits ACTH reversible with neuralizing antibody to PrRP (Ohiwa et al. 2007), an effect opposite to that seen during restraint stress or with ICV injection of low dose PrRP (Ohiwa et al. 2007). The stress modulatory function of PrRP through hormonal intermediaries could also be seen in the periphery, with high levels of PrRP receptor expression in the adrenal medulla among TH positive cells (Roland et al. 1999). More recently, PrRP cell bodies have been found in the adrenal medulla among epinephrine secreting cells, as well as in pheochromocytoma cells (Fujiwara et al. 2005). It's unclear whether PrRP stimulation would elicit adrenaline release under conditions of stressful stimuli. Taken together, these data suggest that PrRP may play an important role in the stress response, both centrally and peripherally.

3.3
Food Intake and Energy Homeostasis

Several hypothalamic and extrahypothalamic sites of receptor expression indicate a role of PrRP in food intake regulation. In the hypothalamus, receptor expression is found in the lateral hypothalamus, ventrolateral hypothalamus (VLH), ventromedial hypothalamus, dorsomedial hypothalamus, and arcuate nucleus (Arc). The VLH and the VMH are classically considered as part of the "dual center" in feeding and satiety in that physical or chemical lesioning of the "feeding center" VLH causes aphagia (absence of eating) (Anand and Brobeck 1951), ablation of the "satiety center" VMH causes hyperphagia (overeating) (Shimizu et al. 1987) and electrical stimulation of VMH inhibits feeding (Ruffin and Nicolaidis 1999). The arcuate nucleus is also implicated in feeding behavior because many of the neuropeptides that regulate feeding, such as neuropeptide Y, alpha-melanocortin-stimulating hormone (a-MSH), agouti-related protein (AGRP), and Cocaine-and-Amphetamine Regulated Transcript (CART) are largely expressed there (Flier and Maratos-Flier 1998). Leptin, a hormone released by adipocytes in the periphery and acts centrally to regulate energy homeostasis, activates brain regions involved in the feeding response, such as the VMH, DMH, PVN, arcuate, and retrochiasmatic area (Elias et al. 2000). All of these sites contain PrRP receptor expression.

In accordance with the role of PrRP in the feeding response, 24-hour fasting reduces the amount of PrRP mRNA expression in the NTS and DMH (Lawrence et al. 2000), two of the major sites of peptide synthesis. Intracerebroventricular injection of PrRP into rats significantly reduces the fasting-induced re-feeding response and nocturnal food intake without effect on water intake (Lawrence et al. 2000). This leads to reduction in body weight gain, due not only to a reduction in food intake but to alteration in energy homeostasis by increasing overall energy expenditure in brown adipose tissue that is centrally mediated (Ellacott et al. 2003). Such negative regulation of food intake has been reproduced in fish (Kelly and Peter 2006), but an opposite effect of increased intake was seen in chickens (Tachibana et al. 2004). Physiologically, PrRP may mediate satiety signaling of both the gut via CCK (Lawrence et al. 2002) and adipose tissue via leptin (Ellacott et al. 2002) since both are able to strongly activate PrRP neurons in the brainstem. In fact, over 90% of PrRP neurons are leptin-receptor positive (Ellacott et al. 2002). Mice with targeted knockout of the PrRP receptor are hyperphagic, obese, with significant increases in body fat, insulin and leptin levels, and reduced glucose tolerance (Gu et al. 2004). Interestingly, the strong satiety signal mediated through CCK is completely abrogated in the knockout mice, suggesting that PrRP is the main central mediator of the gut-released satiety signal (Bechtold and Luckman 2006). Consistent with experimentally generated deletion of the PrRP receptor, the naturally arising obese diabetic

Otsuka Long-Evans Tokushima Fatty (OLETF) strain of rats harbors a single nucleotide change in the initiating codon of PrRP receptor from ATG to ATA, rendering the PrRP receptor nonfunctional (Watanabe et al. 2005). While centrally administered PrRP suppressed food intake in cogenic rats, the OLETF rats have no satiety response to centrally administered PrRP (Watanabe et al. 2005). It is likely that the single point mutation of the PrRP receptor produces the hyperphagic phenotype, leading to obesity, dyslipidemia, and diabetes in the OLETF rats. So far the human equivalent genetic mutation leading to the similar phenotype has not been reported. The data strongly suggests that the PrRP system is a key negative regulator of energy storage in the organism.

3.4 Pain Processing

PrRP receptor mRNA is highly expressed in the lateral and medial parabrachial nucleus, key nuclei involved in the central processing of visceral, nociceptive, and gustatory information (Spector 1995). PB is a major afferent relay center for afferents of nocireceptive neurons from the spinal cord originating in laminae I–II and spinal trigeminal cells as well as of medullary NTS and Area Postrema (AP). Noxious stimuli cause significant c-Fos expression in the Lateral Parabrachial Nucleus (LPB) (Bellavance and Beitz 1996; Bester et al. 1997) and electrophysiological recordings from forebrain sites involved in nociceptive processing receive connections from the LPB (Bernard et al. 1994; Bester et al. 1995; Mao et al. 1993; Menendez et al. 1996). Nociceptive information are mostly relayed through dorsal, external, and internal lateral subnuclei (Cechetto et al. 1985; Slugg and Light 1994), whereas some visceral/gustatory inputs restrict to the ventral lateral, waist, and medial subdivisions with some overlap with nociceptive inputs in the dorsal and external lateral zones (Chamberlin and Saper 1992; Herbert et al. 1990).

The role for PrRP in nociception is just beginning to be explored. Intrathecal and intracerebral injection of PrRP produces significant antinociception in normal rats and an antiallodynic effect in neuropathic rats (Kalliomaki et al. 2004). However, this is exactly opposite in another study where ICV injection of PrRP instead promoted hyperalgesia and reversed morphine-induced antinociception. Consistent with this finding, the pain threshold for receptor knockout mice was significantly higher and the stress-induced analgesia stronger compared to wild-type animals, an effect that was reversed with naloxone treatment (Laurent et al. 2005). In the mutant animals, accentuation of morphine-mediated analgesia and reduction of morphine tolerance was seen (Laurent et al. 2005). These data indicate that PrRP is a negative regulator of the analgesic/opioid system.

3.5 Sexual and Reproductive Functions

The medial preoptic nucleus (MPO), a site with high PrRP receptor mRNA expression, influences the behavioral and neuroendocrine activities related to reproduction and sexual behavior (Kartha and Ramakrishna 1996). This nucleus is sexually dimorphic in both neuropeptide content as well as size. Male animals tend to have MPOs that are twice as large as their female counterpart (Gorski et al. 1980; Swaab and Fliers 1985), which is influenced by testosterone exposure during prenatal development in utero (Hoepfner and Ward 1988). Lesion of this nucleus severely disrupts both the masculine mating and maternal behaviors (Arendash and Gorski 1983; Christensen et al. 1977; Klaric and Hendricks 1986; Robertson et al. 1991), and c-Fos activation of this nucleus occurs in sexually active males and females demonstrating maternal behaviors (Coolen et al. 1998; Fleming et al. 1994; Robertson et al. 1991). Interestingly, another hypothalamic nucleus with some PrRP receptor expression, the ventromedial nucleus of the hypothalamus, controls feminine sexual behavior, such as lordosis reflex (Mathews and Edwards 1977; Matsumoto and Arai 1986). Although no evidence of this is seen in the literature, it will be interesting to determine whether there is sexual dimorphism in the expression of PrRP receptor mRNA in these nuclei.

Besides the importance of MPO in eliciting sexual behaviors, its neurochemical interaction with the pituitary critically determines the production of gametes and sex hormones. MPO is a major site of synthesis of gonadotrophic releasing hormone (GnRH), which when released into the median eminence and into the portal blood system, mediates the release of leutenizing hormone (LHO) or follicular stimulating hormone (FSH) which are important for spermatogenesis, ovum maturation, and sex hormone production (Frohman 1983). Its release, however, depends on a functional connection with the suprachiasmatic nucleus (SCh) which regulates the GnRH neurons in the preoptic area (Gray et al. 1978; Van der Beek et al. 1997; Wiegand et al. 1980).

Two recent studies showed that injection of PrRP could elicit a significant LHO (Hizume et al. 2000; Seal et al. 2000) and FSH (Seal et al. 2000) release, and injection of neutralizing antibody to PrRP attenuates the normal LHO surge seen in the afternoon of proestrus of the estrous cycle (Hizume et al. 2000). These results suggest a tonic activation of PrRP in stimulating LHO release. PrRP's effect on LHO/FSH release is most possibly mediated through the release of GnRH from the MPO (Watanobe 2001). Additional evidence supporting PrRP's role in sexual function comes from studies examining the changes in PrRP expression and action towards prolactin release between male and female rats. These studies showed a sexual dimorphism in PrRP expression in the NTS and VLM, the expression of which is highly regulated by estrogen and progesterone levels (Kataoka et al. 2001; Tokita

et al. 1999). Female rats during the proestrous phase had significantly higher PrRP expression in the NTS and VLM, but not in the DMH, than females in diestrous or normal males (Kataoka et al. 2001). In accordance to these findings, the sensitivity of the anterior pituitary to elicit prolactin secretion was also dependent on the estrous phase and estrogen concentration in the blood (Kawamata et al. 2000; Tokita et al. 1999). No prolactin release to PrRP was evident in ovariectomized females, females in diestrus, proestrus, or males, but only in females in estrous phase or treated with estrogen. The in vivo significance of this sexual dimorphism in PrRP-stimulated prolactin release is unclear at this point.

3.6 Sleep and Arousal

PrRP-receptor expression occurs in several brain regions that are important for the regulation of sleep and control of circadian rhythm. Abundant expression is found in various preoptic nuclei, such as the median preoptic nucleus (MnPO), medial preoptic area (MPA), ventromedial preoptic nucleus (VMPO), lateral preoptic nucleus (LPO), ventrolateral preoptic nucleus (VLPO), and the medial preoptic nucleus (MPO), all of which are heavily interconnected and actively contribute to sleep. More specifically, the VLPO has been promoted as the sleep-promoting center (Gallopin et al. 2000; Sherin et al. 1996). Lesioning of the VLPO leads to long-term insomnia (Lu et al. 2000), possibly because VLPO activity is critical for sleep induction (Alam et al. 1995; Novak et al. 1999; Sherin et al. 1996; Szymusiak et al. 1998). VLPO extends GABAergic and galaninergic projections to several nuclei important for arousal, such as the monoaminergic neurons of the ventral tuberomammillary nucleus (vTMN) (Sherin et al. 1998), dorsal and median raphe nuclei (Trulson and Trulson 1985), and the locus coeruleus (Aston-Jones and Bloom 1981; Aston-Jones et al. 1986), all of which express the PrRP receptor. The vTMN is interesting in that this group of neurons provides all the histaminergic inputs in the brain (Schwartz et al. 1991), and is a major cell group promoting arousal (Lin et al. 1988, 1994). Blocking histamine action either pharmacologically or by lesioning the TMN promotes sleepiness (Monti et al. 1986; Nicholson et al. 1985; Swett and Hobson 1968) and augmenting histamine action either chemically or by stimulating this nucleus induces wakefulness (Kalivas 1982; Monnier et al. 1970; Tasaka et al. 1989). The placement of the PrRP receptor in the various preoptic nuclei, the histaminergic ventral tuberomammillary nucleus, as well as the noradrenergic locus coeruleus and the serotoninergic dorsal raphe, suggests that PrRP must participate in some aspects of arousal and sleep. Additional support is our finding that the PrRP receptor is also expressed in the suprachiasmatic nucleus, which is the circadian pacemaker that synchronizes sleeping and waking, as well as animal behavior and metabolic functions, to the light-dark cycle (Czeisler et al. 1980;

Rusak and Zucker 1979). The SCh is heavily connected to the various preoptic nuclei (Sun et al. 2000; Watts et al. 1987). These interconnections may be important in the circadian control of sleep. The PrRP receptor is also highly expressed in the reticular thalamic nucleus, a predominantly GABAergic nucleus that acts as a gateway for ascending inputs into the cortex and has an important role in the transition into sleep and the control of vigilance states (Steriade et al. 1986).

These data suggest that PrRP should influence sleep-wake transitions and/or attention and arousal. Zhang et al. reported that the chronic ICV injection of PrRP into animals during the dark cycle (10 hour injections with 12 hour behavioral monitoring) resulted in increased REM and NREM sleep, which they attributed to the somnolence property of prolactin acting on brain sites (Zhang et al. 2001). We find however that acute injection of PrRP into animals normally asleep (during the light cycle) potently promotes rapid and prolonged awakening (Lin et al. 2002). Although some of the awakening effect we have attributed to PrRP's ability to suppress the oscillatory activities generated in the reticular thalamic nucleus as measured electrophysiologically on brain slices (Lin et al. 2002) as well as its ability to suppress the spike-wave activity seen in an absence seizures model (Lin et al. 2002), additional sites of action such as those mentioned above would be obvious candidates for this arousal promoting action of PrRP. In-depth studies using intratissue infusions into these various brain regions would help clarify the relative contribution of each to PrRP's effect on sleep regulation.

3.7 Pressor Effects

One study demonstrated that PrRP injection caused a significant rise in blood pressure without altering fluid intake (Samson et al. 2000). Putative sites of action of PrRP to mediate this action are sites of receptor expression in the brainstem. NTS and AP receive visceral and chemical information from cardiovascular sites in the periphery, and PrRP action at these nuclei could modify the ascending and descending efferent connections affecting blood pressure homeostasis (Aicher et al. 1995; Willette et al. 1984). Interestingly, the AP is known to regulate blood pressure homeostasis under conditions of stress (Qian and Koon 1998), a function that may be related to the overall participation of PrRP in stress response (see above). Additional brain sites in the PVN, DMH, BST, Central Amygdala (CEA), and lateral septum also participate in the central control of cardiovascular function. Pressor and depressor centers are known to exist in the extended amygdala (Gelsema et al. 1993; Roder and Ciriello 1993). Electrical stimulation of selective areas in the BST that contains PrRP receptor expression elicits pressor-like effects in the ventrolateral BST and depressor-like effects in the dorsal lateral subdivision (Ciriello and Janssen 1993; Dunn and Williams 1995). Electrical and chemical

stimulation of the CEA causes a significant increase in systemic blood pressure (Baklavadzhyan et al. 2000; Goren et al. 1997). Electrical and chemical stimulation of the hypothalamic nuclei PVN and DMH also elicits increases in blood pressure, possibly through activation of the sympathetic nervous system and closely mimics the pressor changes occurring during stress (Ciriello and Calaresu 1980; Goren et al. 1997; Martin and Haywood 1992). However, stimulation of the lateral septum leads to lowered blood pressure (Calaresu et al. 1976; Gelsema and Calaresu 1987).

Given the multi-nuclear involvement in the central control of blood pressure, studies using intratissue infusions are needed to determine the sites of action of PrRP in this function. One such study was undertaken to clarify regions in the brainstem that are involved in the pressor response (Horiuchi et al. 2002). Microinjection of PrRP in the most caudal VLM (ventrolateral medulla) resulted in a dose-dependent increase in mean arterial pressure, heart rate, and renal sympathetic activity. This was specific to PrRP since injection of TRH, CRH, or angiotensin II are unable to elicit the same response. Surprisingly, injection of PrRP into more rostral locations in the depressor area of caudal VLM and pressor area of the rostral VLM, as well as NTS and AP, had no such pressor responses (Horiuchi et al. 2002). Exactly how PrRP acts to modulate BP in the caudal VLM, an area with minimal receptor expression and not create an effect in an area with high receptor expression such as the AP is a mystery. From human studies, there is now some epidemiological evidence to suggest that PrRP may have a role in blood pressure regulation. Eight polymorphisms of the PrRP receptor gene have been identified, with the most common noncoding (G-62A) and coding (C914T, P305L) polymorphisms typed in 1084 U.K. Caucasians (Bhattacharyya et al. 2003). While there was no association of any of the polymorphisms to body mass index (BMI) score, the P305L variant is significantly associated with lower systolic and diastolic blood pressure compared to the control group without the genetic altercation. In vitro study demonstrated lower calcium signaling in this receptor variant compared to the wild-type receptor, suggesting a potential mechanism for the clinical association (Bhattacharyya et al. 2003). Another recent study has demonstrated a significant association between PrRP receptor polymorphism and the association of physical activity energy expenditure score with blood pressure (Franks et al. 2004).

3.8 Other Actions

PrRP receptor mRNA is also expressed in the periventricular nucleus of the hypothalamus (Pe) and supraoptic nucleus, both of which mediate the release of various hormones. Pe is known to contain somatostatin, and one recent study found immunoreactive fibers making contact with somatostatin-

neurons of the Pe, which also exhibits PrRP receptor expression (Iijima et al. 2001). Injection of PrRP causes a reduction in GH release into the systemic circulation, suggesting a functional correlate to this anatomical prediction (Iijima et al. 2001). However, PrRP receptor expression in Pe only occurs in discrete locations and not throughout the entire rostral-caudal extent of this nucleus. It will be interesting to see the reason for this distinct localization. PrRP infusion into the brain also elicits release of oxytocin and vasopressin (Maruyama et al. 1999b), presumably from magnocellular cells of the supraoptic and paraventricular nucleus since PrRP fibers make contact with those cells. Very few PrRP receptor mRNA-expressing cells are found in the magnocellular division of the paraventricular nucleus or in the supraoptic nucleus. Whether these cells are sufficient to support the release of oxytocin or vasopressin is unclear. Alternatively, PrRP could activate other brain regions to transynaptically stimulate release of these hormones.

PrRP receptor expression is also highly expressed on the ependymal cells of the ventricles and the central canal, as well as the subfornical organ (SFO), another circumventricular organ in addition to its expression in the area postrema. PrRP fibers are known to make contact with the ventricular lining (Iijima et al. 1999), suggesting either PrRP is released to act on receptors present on ependymal cells or into the CSF to act on distant sites. By acting directly on the ependymal cells PrRP may regulate the permeability of substances across the CSF into brain tissue by changing the permeability properties of these cells, or may regulate ion and water homeostasis of the CSF as seen in other systems (Pilgrim 1978; Pollay and Curl 1967; Rosenberg et al. 1986). PrRP could also be released into the CSF or the blood through circumventricular organs (CVO) such as the SFO or the AP, as seen for numerous other peptides (Black 1982; Kozlowski 1986). It would be interesting to see whether PrRP is released into the CSF under certain conditions, such as stress, sleep, or during reproductive behaviors.

4 Conclusion

What is the role of PrRP in the organism? Since the peptide is well conserved among species, from fish and amphibians, to birds (chickens) and mammals, its function is likely primitive but it modulates an important function for the organism. Our understanding of the PrRP system has evolved from its initial characterization as a prolactin-releasing factor to our present view of a peptide with multiple functions within the brain. Considering what is now known about the PrRP system, it likely helps the animal adapt to various environmental (either internal or external) alterations. These may include adjustment in the emotional responses to stressors, promotion of a heightened arousal state, stimulation of the release of hormones necessary for the adapta-

tion to stress or nociception, changes in energy metabolism, either indirectly by modulating hormone release or regulating food intake, and adaptation of the cardiovascular system. The PrRP system itself is also adjusted and regulated by external cues, as well as by the internal state such as the energy, reproductive and hormonal state of the animal.

From a therapeutic standpoint, small molecule antagonists against the PrRP receptor should mimic the phenotype of the gene knockout of the PrRP receptor. Tantalizing therapeutic possibilities include treatment of anorexia or cachectic states of cancer patients, and at the same time, it may improve antinociception and reduce tolerance of opioid medications in these same patients. Receptor agonists, on the other hand, could have a role in improving arousal states for sleep disorders and the treatment of absence seizures. With the further development of other model systems such as PrRP knockout or conditional knockouts, many of the putative functions could be more accurately delineated and our knowledge refined. There is still much to learn about PrRP and its definitive roles in the CNS. Since much of the groundwork has been established future research on the PrRP system promises to be rewarding.

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Structure and Function of Ghrelin

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Abstract The endogenous ligand for growth-hormone secretagogue receptor (GHS-R) was purified from the stomach and we named it "ghrelin", after a word root ("ghre") in Proto-Indo-European languages meaning "grow", since ghrelin has potent growth hormone (GH) releasing activity. In addition, ghrelin stimulates appetite by acting on the hypothalamic arcuate nucleus, a region known to control food intake. Ghrelin is orexigenic; it is secreted from the stomach and circulates in the blood stream under fasting conditions, indicating that it transmits a hunger signal from the periphery to the central nervous system. Taking into account all these activities, ghrelin plays important roles for maintaining growth hormone release and energy homeostasis in vertebrates. The diverse functions of ghrelin raise the possibility of its clinical application for GH deficiency, eating disorder, gastrointestinal disease, cardiovascular disease, osteoporosis and aging, etc.

1 Introduction

In recent years, searches for novel ligands using orphan GPCR-expressing cells have resulted in the discovery of several novel bioactive peptides, such as nociceptin/orphanin FQ (Reinscheid et al. 1995), orexin/hypocretin (Sakurai et al. 1998), prolactin-releasing peptide (Hinuma et al. 1998), apelin (Tatemoto et al. 1998), metastin (Ohtaki et al. 2001), neuropeptide B (Fujii et al. 2002; Tanaka et al. 2003), and neuropeptide W (Shimomura et al. 2002; Tanaka et al. 2003). For the orphan-receptor strategy used to identify the endogenous ligands, we first established a cell line that stably expresses an orphan GPCR. Then, a peptide extract is applied to the cell line and a second messenger response is measured. If a target orphan GPCR is functionally expressed on the cell surface and the extract contains the endogenous ligand that can activate the receptor, the second messenger response, as usually monitored by the levels of cAMP or intracellular Ca^{2+} concentration, will increase or decrease. Through monitoring of this assay system, the endogenous ligand can be purified through several chromatographic steps. In this way, orphan receptors represent important tools for the discovery of novel bioactive molecules and for drug development (Civelli et al. 2001).

Among the numerous orphan GPCR receptors, GHS-R (growth hormone secretagogue receptor) attracted the attention of many academic and industrial scientists, since its endogenous ligand could potentially be used directly for treatment of GH deficiency. Many groups tried unsuccessfully to isolate the endogenous GHS-R ligand from extracts of brain, pituitary, or hypothalamus, the known sites of GHS-R expression. Unexpectedly, we succeeded in the purification and identification of the endogenous ligand for the GHS-R from the stomach, and named it "ghrelin" (Kojima et al. 1999). Ghrelin is a growth-hormone-releasing and appetite-stimulating peptide.

In this review, we review the structure, distribution, and physiological functions of ghrelin.

2
Discovery and Structure Determination of Ghrelin

A cultured cell line expressing the GHS-R was established and used to identify tissue extracts that could stimulate the GHS-R, as monitored by increases in intracellular Ca^{2+} levels. After screening several tissues, very strong activity was unexpectedly found in stomach extracts (Kojima et al. 1999). The peptide that stimulated GHS-R was purified from the rat stomach through four steps of chromatography: gel-filtration, two ion-exchange HPLC steps, and a final reverse-phase HPLC (RP-HPLC) procedure. The second ion-exchange HPLC yielded two active peaks (P-I and P-II), from which ghrelin and des-Gln14-ghrelin were purified, respectively (Hosoda et al. 2000b). The active peaks were finally purified by RP-HPLC. The name ghrelin is based on "ghre", a word root in Proto-Indo-European languages for "grow", in reference to its ability to stimulate GH release. Ghrelin is a 28-amino-acid peptide, in which the serine 3 (Ser3) is *n*-octanoylated and this modification is essential for ghrelin's activity (Fig. 1). Ghrelin is the first known and only case of a peptide hormone modified by a fatty acid.

In rat stomach, a second type of ghrelin peptide has been purified and identified as des-Gln14-ghrelin (Hosoda et al. 2000b). Except for the deletion of Gln14, des-Gln14-ghrelin is identical to ghrelin, even retaining the *n*-octanoic acid modification. Des-Gln14-ghrelin has the same potency of activities as that of ghrelin. The deletion of Gln14 in des-Gln14-ghrelin arises due to the usage of a CAG codon to encode Gln, which results in its recognition as a splicing signal. Thus, two types of active ghrelin peptide are produced in rat stomach, ghrelin and des-Gln14-ghrelin. However, des-Gln14-ghrelin is only present in low amounts in the stomach, indicating that ghrelin is the major active form.

In mammals, ghrelin homologues have been identified in human, rhesus monkey (Angeloni et al. 2004), rat, mouse, mongolian gerbil (GenBank Accession number: AF442491), cow (GenBank Accession number: AB035702), pig (GenBank Accession number: AB035703), sheep (GenBank Accession num-

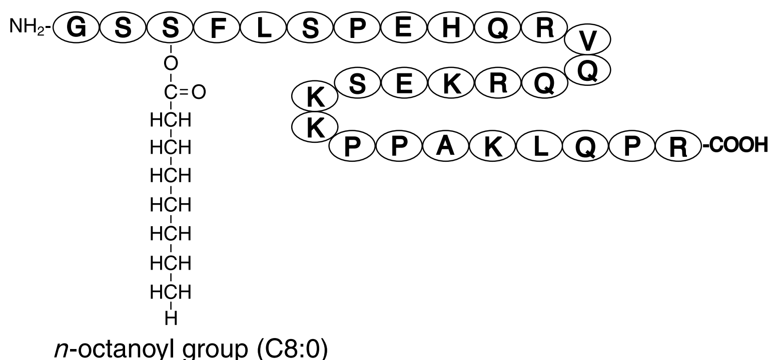

Fig. 1 Structures of human ghrelins. Human ghrelin is a 28-amino-acid peptide, in which Ser3 is modified by a fatty acid, primarily *n*-octanoic acid. This modification is essential for ghrelin's activity

ber: AB060699), dog (Tomasetto et al. 2001), and cat (Ida et al. 2007), etc. The amino-acid sequences of mammalian ghrelins are well conserved; in particular, the ten amino acids are with respect to their N-termini identical (Fig. 2). This structural conservation and the universal requirement for acyl-modification of the third residue indicate that this N-terminal region is of central importance to the activity of the peptide.

Ghrelin has also been identified and the structures determined in birds, fishes, amphibians and reptiles (Kojima and Kangawa 2005) (Fig. 2). All vertebrate ghrelins are mainly produced in stomach, or stomach-like organs, and modified by medium-chain fatty acid. The fatty acids used for acyl-modification are *n*-octanoic, *n*-decanoic acid or other minor medium-chain fatty acids. The characteristic features of non-mammalian ghrelins are their multiple forms in tissues: ghrelins could be classified by the type of acyl-modification and amino acid length.

3
Des-Acyl Ghrelin

A non-acylated form of ghrelin, des-acyl ghrelin, also exists at significant levels in both stomach and blood (Hosoda et al. 2000a). In blood, des-acyl ghrelin circulates in amounts far greater than acylated ghrelin. Des-acyl ghrelin does not replace radiolabelled ghrelin at the binding sites of acylated ghrelin in hypothalamus and pituitary and shows no GH-releasing and other endocrine activities in rats. Moreover, des-acyl ghrelin does not possess endocrine activities in humans.

One question is whether there is a specific receptor for des-acyl ghrelin. Baldanzi and coworkers have suggested the existence of another ghrelin re-

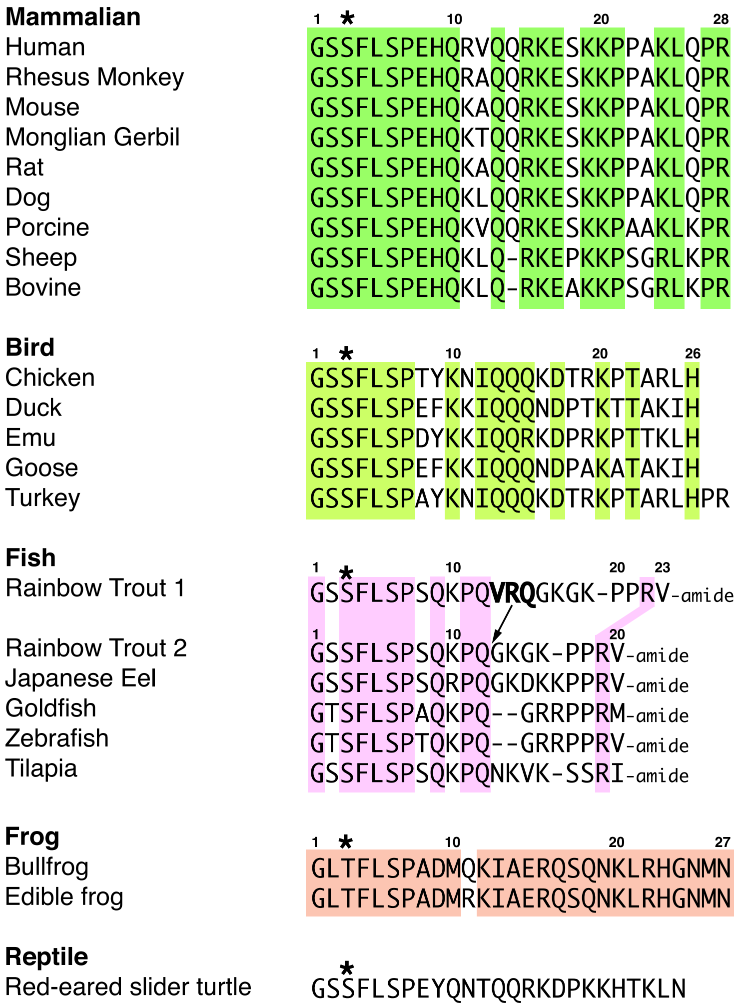

Fig. 2 Sequence comparison of vertebrate ghrelins. Identical amino acids in each species of mammal, bird, fish and frog are *colored*. The *asterisks* indicate acyl-modified third amino acids. N-terminal cores with acyl-modification sites are well conserved among all vertebrate ghrelins. A unique third residue (Thr3) in two frog ghrelins differs from the Ser3 in ghrelins of other species. Because serine and threonine both possess hydroxyl groups on their side chains, they can both be modified by fatty acids. Indeed, the frog ghrelins are modified by either *n*-octanoic or *n*-decanoic acids. Fish ghrelins were found to contain an amide structure at their COOH-terminal ends, though these amide structures are not necessary for activity

ceptor in the cardiovascular system (Baldanzi et al. 2002). They showed that ghrelin and des-acyl ghrelin both recognize common high-affinity binding sites on H9c2 cardiomyocytes, which do not express the ghrelin receptor,

GHS-R. However, BLAST searches of the human genome using ghrelin receptor (GHS-R) cDNA as a search sequence have not revealed any ghrelin receptor homologues. Further study is required to determine whether desacyl ghrelin is biologically active and binds to an as-yet-unidentified receptor.

4 Ghrelin Gene and the Structure of the Ghrelin Precursor

The human ghrelin gene is localized on the chromosome 3p25–26. The human ghrelin receptor gene has also been identified on chromosome 3, at position q26–27 (Smith et al. 1997).

The human ghrelin gene, like the mouse gene, comprises five exons (Kanamoto et al. 2004; Tanaka et al. 2001). The short first exon contains only 20 bp, which encode part of the 5′-untranslated region. There are two different transcriptional initiation sites in the ghrelin gene; one occurs at –80 and the other at –555 relative to the ATG initiation codon, resulting in two distinct mRNA transcripts (transcript-A and transcript-B). Transcript-A is the main form of gastric ghrelin mRNA.

The 28 amino acids of the functional ghrelin peptide are encoded in exons 2 and 3. In the rat and mouse ghrelin genes, the codon for Gln14 (CAG) is used as an alternative splicing signal to generate two different ghrelin mRNAs (Hosoda et al. 2000b). One mRNA encodes the ghrelin precursor, and another encodes a des-Gln14-ghrelin precursor. Des-Gln14-ghrelin is identical to ghrelin, except for the deletion of Gln14.

The amino-acid sequences of mammalian ghrelin precursors are well conserved. In these precursors, the 28-amino-acid active ghrelin sequence immediately follows the signal peptide. The cleavage site for the signal peptide is the same in all mammalian ghrelins. Although propeptides are usually processed at dibasic amino acid sites by prohormone convertases, the C-terminus of the ghrelin peptide sequence is processed at an uncommon Pro-Arg recognition site. The Pro-Arg sequence is also used for the C-terminal processing of atrial natriuretic peptide (ANP) (Seidah and Chretien, 1999; Steiner, 1998). Zhu et al. reported that the protease that acts at the Pro-Arg site is prohormone convertase 1/3 (PC1/3) (Zhu et al. 2006).

5 Enzyme for Acyl-Modification of Ghrelin

An enzyme that catalyzes the acyl-modification of ghrelin has not yet been identified. The universal incorporation of *n*-octanoic acid in mammals, fish, birds, and amphibians suggests that this putative enzyme is rather specific in its choice of medium-chain fatty acid substrates.

Ingestion of either medium-chain fatty acids (MCFAs) or medium-chain triacylglycerols (MCTs) specifically increases production of acyl-modified ghrelin without changing the total (acyl- and des-acyl-) ghrelin level (Nishi et al. 2005). When mice ingested either MCFAs or MCTs, the acyl group attached to nascent ghrelin molecules corresponded to that of the ingested MCFAs or MCTs. Moreover, *n*-heptanoyl (C7:0) ghrelin, an unnatural form of ghrelin, was produced in the stomach of mice following ingestion of *n*-heptanoic acid or glyceryl triheptanoate. These findings indicate that ingested fatty acids are directly utilized for acyl-modification of ghrelin.

A number of acyltransferases have previously been identified in mammals; the only reported enzymes that use MCFAs as substrates are carnitine octanoyltransferases, which function in the β-oxidation of fatty acids (Ramsay and Naismith 2003). Members of the serine acyltransferase family that transfer acyl groups to serine residues of target molecules have been identified, including two serine palmitoyltransferases functioning in the biosynthesis of sphingolipids in mammals (Hanada 2003) and a plant Ser *O*-acetyltransferase gene family in *Arabidopsisthaliana* (Howarth et al. 2003). The putative ghrelin Ser *O*-acyltransferase may have structural homology with these acyltransferases. Further investigations characterizing the putative ghrelin Ser *O*-acyltransferase are required to elucidate the mechanism of the unique acyl modification seen in ghrelin.

6
Ghrelin Receptor Family

The ghrelin receptor, or GHS-R, is a typical G protein-coupled receptor with seven transmembrane domains (7-TM). Two distinct ghrelin receptor cDNAs have been isolated: GHS-R type 1a mRNA and type 1b mRNA (Howard et al. 1996). The first, GHS-R Type 1a, encodes a 7-TM GPCR with binding and functional properties consistent with its role as ghrelin's receptor. This Type 1a receptor has features characteristic of a typical GPCR, including conserved cysteine residues in the first two extracellular loops, several potential sites for post-translational modifications (*N*-linked glycosylation and phosphorylation), and an aromatic triplet sequence (E/DRY) located immediately after TM-3 in the second intracellular loop. Another GHS-R cDNA, type 1b, is produced by an alternative splicing mechanism. The GHS-R gene consists of two exons; the first exon encodes TM-1 to 5, and the second exon encodes TM-6 to 7. Type 1b is derived from only the first exon and encodes only five of the seven predicted TM domains. The type 1b receptor is thus a C-terminal truncated form of the type 1a receptor and is pharmacologically inactive.

The ghrelin receptor (GHS-R) has several homologues, whose endogenous ligands are gastrointestinal peptides or neuropeptides. This receptor superfamily contains receptors for ghrelin, motilin (Feighner et al. 1999),

neuromedin U (Howard et al. 2000; Kojima et al. 2000) and neurotensin (Vincent et al. 1999). All of these peptides are found in gastrointestinal organs and regulate gastrointestinal movement and other functions. This family also contains an orphan receptor, GPR39, whose endogenous ligand was identified as obestatin, a ghrelin precursor-derived peptide (Zhang et al. 2005). However, negative results have been reported against obestatin as the ligand for GPR39.

The ghrelin receptor is most homologous to the motilin receptor; the human forms share 52% identical amino acids (Inui 2001; Smith et al. 2001). Moreover, their ligands, ghrelin and motilin peptides, have similar amino-acid sequences. Preliminary studies have shown that motilin can stimulate the ghrelin receptor, albeit at a low level. In contrast, ghrelin does not activate the motilin receptor (Dass et al. 2003).

The ghrelin receptor is well conserved across all vertebrate species examined, including a number of mammals, chicken, and pufferfish (Fugu) (Palyha et al. 2000; Smith et al. 2001). This strict conservation suggests that ghrelin and its receptor serve important physiological functions.

One case of familial short stature associated with a missense mutation in the ghrelin receptor has been reported (Pantel et al. 2006). This mutation changed a single amino acid, resulting in a charge change at a highly conserved extracellular position. This mutated ghrelin receptor shows severely impaired ghrelin binding.

7
Ghrelin and Motilin

The ghrelin receptor is most homologous to the motilin receptor (Feighner et al. 1999; Inui 2001). Accordingly, the amino-acid sequence of ghrelin has homology with that of motilin, another gastric peptide with gastric contractile activity (Asakawa et al. 2001). Alignment of the 28 amino-acid peptide ghrelin and the 19-amino-acid motilin reveal that they share eight identical amino acids. In fact, after our discovery of ghrelin, Tomasetto and coworkers reported the identification of a gastric peptide, the motilin-related peptide (MTLRP) (Tomasetto et al. 2000). They had tried to isolate new protein clones whose expression was restricted to the gastric epithelium using differential screening. The amino-acid sequence of MTLRP turned out to be identical to that of ghrelin[1-18]; however, the putative processing site of MTLRP, Lys–Lys, is not used in ghrelin in gastric cells. Moreover, the sequence data alone could not reveal any potential acyl-modifications (Del Rincon et al. 2001; Folwaczny et al. 2001).

Interestingly, the region of homology between ghrelin and motilin lies not near the N-terminus, where ghrelin's acyl-modification occurs, but in their respective central regions. Ghrelin and motilin play similar roles in the stom-

ach. Both peptides stimulate gastric-acid secretion and gastric movement (Masuda et al. 2000). Thus, ghrelin and motilin are structurally and functionally considered to compose a peptide superfamily, and may have evolved from a common ancestral peptide.

8 Distribution of Ghrelin

8.1 Plasma Ghrelin

Two major forms of ghrelin are found in plasma: *n*-octanoyl-modified and des-acyl ghrelin (Hosoda et al. 2000a). The normal ghrelin concentration of plasma samples in humans is 10–20 fmol/ml for *n*-octanoyl ghrelin and 100–150 fmol/ml for total ghrelin, including both acyl-modified and des-acyl ghrelins. Plasma ghrelin concentration is increased in fasting conditions and reduced after habitual feeding, suggesting that ghrelin may be an initiation signal for food intake or ghrelin secretion is controlled by some nutritional factors in blood (Cummings et al. 2001; Tschop et al. 2001a).

It is not clear what factors are involved in the regulation of ghrelin secretion. Blood glucose level may be critical: oral or intravenous administration of glucose decreases plasma ghrelin concentration (McCowen et al. 2002; Shiiya et al. 2002). Since gastric distention by water intake does not change ghrelin concentration, mechanical distention of the stomach alone clearly does not induce ghrelin release. Plasma ghrelin concentration is sensitive, however, to the makeup of a meal; it is decreased by a high lipid meal and increased by a low protein one.

Plasma ghrelin concentration is low in obese people and high in lean people, indicating that plasma ghrelin concentration is in inverse proportion to BMI (Hanada 2003; Hansen et al. 2002; Shiiya et al. 2002; Tschop et al. 2001b). Related to this fact, the plasma ghrelin level is highly increased in anorexia nervosa patients and returns to normal levels upon weight gain and recovery from the disease (Ariyasu et al. 2001; Cuntz et al. 2002; Otto et al. 2001). Ghrelin concentration is also increased in cachexia due to cancer, heart failure, and chronic fasting disease, etc.

8.2 Gastric and Intestinal Ghrelin

In all vertebrate species, ghrelin is mainly produced in the stomach. In the stomach, ghrelin-containing cells are more abundant in the fundus than in the pylorus (Date et al. 2000a; Yabuki et al. 2004) (Fig. 3A). In situ hybridization and immunohistochemical analyses indicate that ghrelin-containing cells

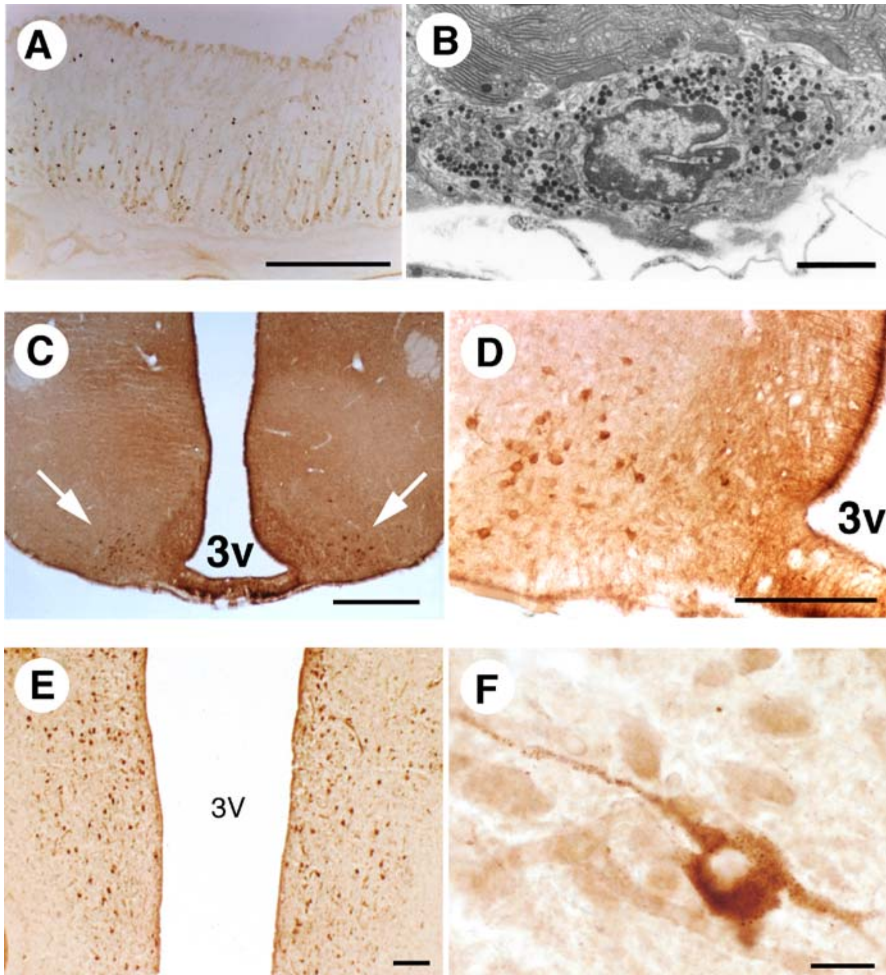

Fig. 3 Ghrelin cells in the stomach and hypothalamus. **A** Ghrelin-immunoreactive cells in the stomach are found from the neck to the base of the oxyntic gland. *Scale bar*: 400 μm. This distribution pattern is typical for gastric endocrine cells. **B** Ghrelin-producing cell has many round, compact, electron-dense granules in its cytoplasm. *Scale bar*: 500 nm. **C** *Arrows* indicate ghrelin neurons in the hypothalamic arcuate nucleus. *Scale bar*: 500 μm. **D** High magnification of (**C**). *Scale bar*: 200 μm. **E** Localization of ghrelin-immunoreactive neurons in the porcine hypothalamic paraventricular nucleus. *Scale bar*: 200 μm. **F** A ghrelin-producing neuron in the paraventricular nucleus. *Scale bar*: 20 μm

are a distinct endocrine cell type found in the mucosal layer of the stomach (Date et al. 2000a; Rindi et al. 2002).

Four types of endocrine cells have been identified in the oxyntic mucosa: ECL, D, enterochromaffin (EC), and X/A-like cells and they show the following relative abundances. The rat oxyntic gland contains approximately

60–70% ECL cells, 20% X/A-like cells, 2–5% D cells, and 0–2% EC cells; in humans, the corresponding percentages are 30%, 20%, 22%, and 7%. The major products in the granules have been identified as histamine and uroguanylin in ECL cells, somatostatin in D cells, and serotonin in EC cells. However, the granule contents of X/A-like cells were unknown until the discovery of ghrelin. The X/A-like cells contain round, compact, electron-dense granules that are filled with ghrelin (Date et al. 2000a; Dornonville de la Cour et al. 2001) (Fig. 3B). These X/A-like cells account for about 20% of the endocrine cell population in adult oxyntic glands. However, the number of X/A-like cells in the fetal stomach is very low and increases after birth (Hayashida et al. 2002). As a result, the ghrelin concentration of fetal stomach is also very low and gradually increases after birth until five weeks of age.

The gastric X/A-like cells can be stained by an antibody that is specific to the N-terminal, acyl-modified portion of ghrelin, indicating that ghrelin in the secretory granules of X/A-like cells has already been acyl-modified. Two major forms of ghrelin are found in the stomach as in plasma: *n*-octanoyl-modified and des-acyl ghrelin.

Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum, and colon (Date et al. 2000a; Hosoda et al. 2000a; Sakata et al. 2002). In the intestine, ghrelin concentration gradually decreases from the duodenum to the colon. As in the stomach, the main molecular forms of intestinal ghrelin are *n*-octanoyl ghrelin and des-acyl ghrelin.

8.3 Pancreatic Ghrelin

The pancreas is a ghrelin-producing organ. Analyses combining HPLC and ghrelin-RIA revealed that ghrelin and des-acyl ghrelin both exist in the rat pancreas (Date et al. 2002b). However, the cell type that produces ghrelin in the pancreatic islets remains controversial, whether it be the α cells, β cells, the newly identified islet epsilon (ε) cells, or a unique novel islet cell type (Prado et al. 2004).

The homeodomain protein Nkx2.2 is essential for the differentiation of islet β cells and α cells, and lack of Nkx2.2 in mice results in replacement of pancreatic endocrine cells by cells that produce ghrelin (Prado et al. 2004). Normal murine pancreas also contains a small number of the newly identified islet cell type, ε cells.

The pancreatic ghrelin profile changes dramatically during fetal development (Chanoine and Wong 2004); pancreatic ghrelin-expressing cells are numerous from midgestation to the early postnatal period, comprising 10% of all endocrine cells, and decrease in number after birth. Ghrelin mRNA expression and total ghrelin concentration are markedly elevated in the fetal pancreas, 6–7 times greater than in the fetal stomach. Thus, the onset of islet ghrelin expression precedes that of gastric ghrelin. Pancreatic ghrelin

expression is highest in the prenatal and neonatal periods. In contrast, gastric ghrelin levels are low during the prenatal period and increase after birth (Hayashida et al. 2002). Moreover, pancreatic ghrelin levels are not affected by fasting.

8.4 Pituitary Ghrelin

GH-releasing somatotrophs in the pituitary gland are the target cells of ghrelin. In an in vivo assay, ghrelin stimulated primary pituitary cells and increased their intracellular Ca^{2+} concentration, indicating that the ghrelin receptor, GHS-R, is expressed in pituitary cells (Bennett et al. 1997; Guan et al. 1997; McKee et al. 1997a). Also, ghrelin has been found in the pituitary gland itself (Korbonits et al. 2001a; Korbonits et al. 2001b), where it may influence the release of GH in an autocrine or paracrine manner. Pituitary tumors, such as adenomas, corticotroph tumors, and gonadotroph tumors contain ghrelin peptides.

8.5 Ghrelin in the Brain

Since the ghrelin receptor, GHS-R, is mainly expressed in the hypothalamus and pituitary, its endogenous ligand is thought to exist mainly in the hypothalamic regions. This is supported by the finding that another growth-hormone-releasing peptide, GHRH (growth-hormone-releasing hormone) is produced in the hypothalamus and is secreted into the hypophyseal portal system to stimulate GH release from the pituitary somatotrophs. However, the ghrelin content of the brain was found to be very low (Hosoda et al. 2000a; Kojima et al. 1999). Ghrelin has been found in the hypothalamic arcuate nucleus, an important region for controlling appetite (Fig. 3C,D). In addition, it has been reported that ghrelin is found in previously uncharacterized hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei (Cowley et al. 2003; Sato et al. 2005) (Fig. 3E,F). Two major ghrelin peptides are identified in the rat hypothalamus: *n*-octanoyl-modified and des-acyl ghrelins (Sato et al. 2005). Thus, in a manner similar to ghrelin in the stomach, the two major forms of ghrelin are also found in the hypothalamus.

9 Physiological Functions of Ghrelin

Ghrelin exerts two main physiological functions: growth hormone releasing activity from the pituitary and increase of food intake by stimulation of

the hypothalamic appetite regulatory region. Ghrelin also shows many other physiological functions.

9.1
Growth Hormone Releasing Activity of Ghrelin

Ghrelin has been shown to induce GH release not only in rats and humans (Kojima et al. 1999), but also in non-mammalian vertebrates, including chicken (Kaiya et al. 2002), fish (Kaiya et al. 2003a,b), and frog (Kaiya et al. 2001). Ghrelin stimulates growth-hormone release both in vitro and in vivo in a dose-dependent manner (Fig. 4). Figure 4A shows the increase of GH

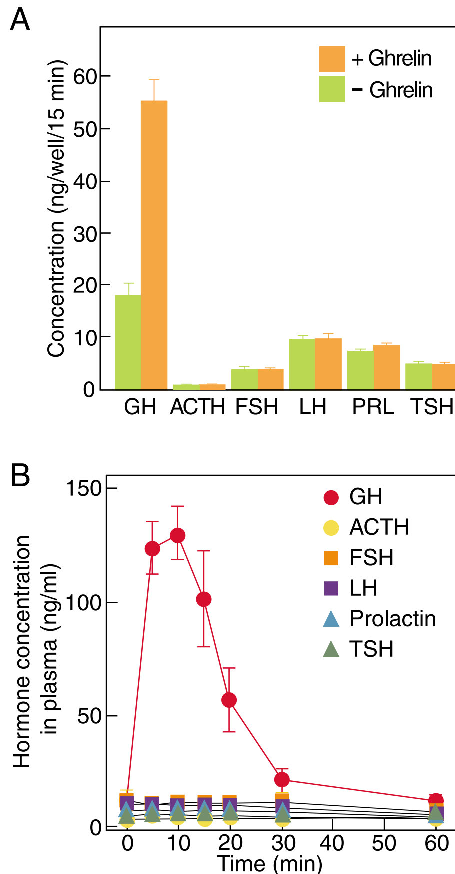

Fig. 4 Effects of ghrelin on pituitary hormone secretion in vitro and in vivo. **A** Effects of a high dose (10^{-6} M) of ghrelin on hormone secretion from rat primary pituitary cells in vitro. ACTH, adrenocorticotropin; FSH, follicle-stimulating hormone; LH, lutenizing hormone; PRL, prolactin; and TSH, thyroid-stimulating hormone. **B** Time courses of plasma hormone concentrations after IV injection of ghrelin into anesthetized male rats in vivo

concentration that was secreted from primary pituitary cultured cells into medium after ghrelin addition (Kojima et al. 1999). Moreover, intravenous injection of ghrelin induces potent GH release in many species. Thus, ghrelin is a potent GH-releasing peptide.

A single intracerebroventricular administration of ghrelin also increased rat plasma GH concentration in a dose-dependent manner, with a minimum dose of only 10 pmol (Date et al. 2000b). Thus, ICV injection appears to be a more potent route of delivery than IV administration.

Co-administration of ghrelin and GHRH had a synergistic effect on GH secretion; that is, co-administration results in more GH release than does either GHRH or ghrelin alone (Hataya et al. 2001). This finding implies that GHRH is necessary for GH release to be maximally effective.

9.2 Appetite Stimulating Activity of Ghrelin

Recent identification of appetite-regulating humoral factors reveal regulatory mechanisms not only in the central nervous system, but also mediated by factors secreted from peripheral tissues (Coll et al. 2007; Stanley et al. 2005). Leptin, produced in adipose tissues, is an appetite-suppressing factor that transmits satiety signals to the brain, while ghrelin, produced in the stomach, is an appetite-stimulating factor that transmits hunger signals to the brain. Ghrelin, thus, is functionally a natural antagonist to leptin.

Ghrelin is produced primarily in gastrointestinal organs in response to hunger and starvation, and circulates in the blood, serving as a peripheral signal telling the central nervous system to stimulate feeding. When ghrelin is injected into the cerebral ventricles of rats, their food intake is potently stimulated (Nakazato et al. 2001; Shintani et al. 2001; Tschop et al. 2000; Wren et al. 2000) (Fig. 5A). Furthermore, chronic ICV injection of ghrelin increases cumulative food intake and decreased energy expenditure, resulting in body weight gain (Fig. 5B). Ghrelin-treated mice also increase their fat mass, both absolutely and as a percentage of total body weight. Not only ICV injection, but also IV and subcutaneous injection of ghrelin have been shown to increase food intake. IV injection of ghrelin (5.0 pmol/kg/min) into human volunteers increased food intake by an average of 28% in every individual (Wren et al. 2001).

The hypothalamic arcuate nucleus is the main site of ghrelin's activity in the central nervous system. The arcuate nucleus is also a target of leptin, an appetite-suppressing hormone produced in adipose tissues, and NPY and AgRP, which are both appetite-stimulating peptides (Morton and Schwartz, 2001). NPY and AgRP are produced in the same population of neurons in the arcuate nucleus, and their appetite-stimulating effects are inhibited directly by leptin. At least part of the orexigenic effect of ghrelin is mediated by upregulating the genes encoding these potent appetite stimulants (Fig. 6).

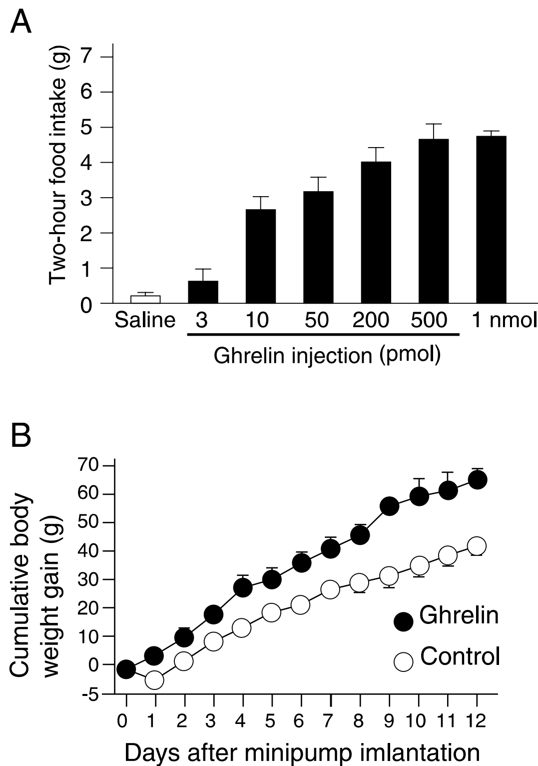

Fig. 5 Stimulation of feeding by ICV administration of ghrelin. **A** Two-hour food intake of free-feeding rats injected with various doses of ghrelin. Control rats were given 0.9% saline. **B** Effect of chronic ghrelin ICV administration on rats. Cumulative body weight gain during an ICV infusion of 250 pmol/day for 12 days

ICV injection of ghrelin induces c-Fos expression in NPY-expressing neurons and increases the amount of NPY mRNA in the arcuate nucleus (Kamegai et al. 2001; Nakazato et al. 2001; Shintani et al. 2001). Moreover, ICV ghrelin injection increases the AgRP mRNA level in the hypothalamus. The appetite-stimulating effects of ghrelin are blocked by an antagonist of NPY receptor 1. ICV injections of an AgRP inhibitor, anti-NPY IgG, and anti-AgRP IgG inhibits the appetite-stimulating effects of ghrelin. Intravenous injection of ghrelin also stimulates NPY/AgRP neurons in the hypothalamus. Immunohistochemical analysis indicated that ghrelin neuron fibers directly contact NPY/AgRP neurons (Cowley et al. 2003). These results indicate that ghrelin exerts its feeding activity by stimulating NPY/AgRP neurons in the hypothalamus to promote the production and secretion of NPY and AgRP peptides. Studies with knockout mice of NPY, AgRP or both confirms these results. Although deletion of either NPY or AgRP caused a modest or no effect on the

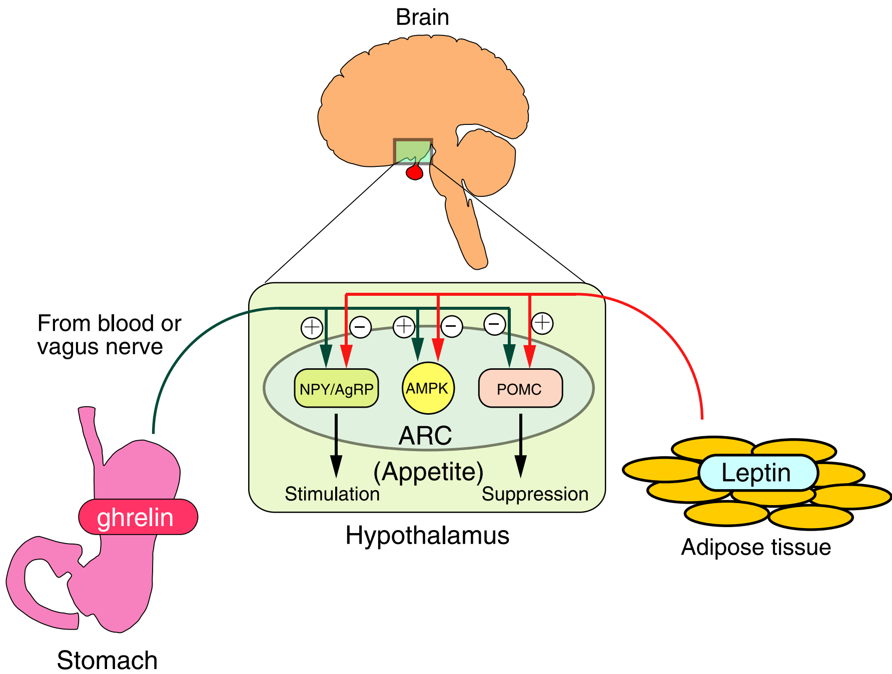

Fig. 6 Hypothalamic appetite regulation by ghrelin and leptin. The arcuate nucleus (ARC) of the hypothalamus is the main target of ghrelin and leptin. Ghrelin is a peripheral orexigenic signal secreted from the stomach, whereas leptin is a peripheral anorexigenic signal secreted from adipose tissue. The effects of ghrelin are opposite to those of leptin. In the ARC, ghrelin stimulates NPY/AgRP neurons and suppresses POMC neurons. On the other hand, leptin suppresses NPY/AgRP neurons and stimulates POMC neurons. Moreover, ghrelin increases AMPK activity in the hypothalamus, whereas leptin decreases AMPK activity

orexigenic action of ghrelin, the double knockout mice lacked the action of ghrelin completely (Chen et al. 2004).

Recently, AMP-activated protein kinase (AMPK) has been shown to be involved in hypothalamic appetite regulation (Minokoshi et al. 2004). Injection of 5-amino-4-imidazole carboxamide riboside, an activator of AMPK, significantly increases food intake. Administration of ghrelin in vivo increases AMPK activity in the hypothalamus (Andersson et al. 2004). By contrast, injection of leptin decreases hypothalamic AMPK activity.

9.3
Pathway of the Ghrelin Signal; from Peripheral Tissues to the Central Nervous System

Peripherally injected ghrelin stimulates hypothalamic neurons and stimulates food intake. In general, peptides injected peripherally do not pass the blood–

brain barrier. Indeed, the rate at which peripheral ghrelin passes the barrier has shown to be very low. Thus, peripheral ghrelin must activate the appropriate hypothalamic regions via an indirect pathway.

The detection of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion suggests that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve (Date et al. 2002a; Zhang et al. 2004). Moreover, the observation that ICV administration of ghrelin induces c-Fos in the dorsomotor nucleus of the vagus and stimulates gastric-acid secretion indicates that ghrelin activates the vagus system (Date et al. 2001).

In contrast, vagotomy inhibits the ability of ghrelin to stimulate food intake and GH release (Date et al. 2002a). A similar effect was also observed when capsaicin, a specific afferent neurotoxin, was applied to vagus nerve terminals to induce sensory denervation. However, the basal level of ghrelin concentration is not affected and a decrease of ghrelin levels is not observed after vagotomy. On the other hand, fasting-induced elevation of plasma ghrelin is completely abolished by subdiaphragmatic vagotomy or atropine treatment (Williams et al. 2003).

Moreover, peripheral ghrelin signaling, which travels to the nucleus tractus solitarius (NTS) via the vagus nerve, increases noradrenaline (NA) in the arcuate nucleus of the hypothalamus (Date et al. 2006). Bilateral midbrain transections rostral to the NTS, or toxin-induced loss of neurons in the hindbrain that express dopamine β-hydroxylase (an NA synthetic enzyme), abolished ghrelin-induced feeding. Thus, the noradrenergic system is necessary in the central control of feeding behavior by peripherally administered ghrelin. These results indicate that the response of ghrelin to fasting is transmitted through vagal afferent transmission.

9.4 Ghrelin and Eating Disorders

Anorexia nervosa (AN) is a syndrome often seen in young women characterized by a combination of weight loss, amenorrhea, and behavioral changes. Some of these changes are reversible with weight gain. Plasma ghrelin levels in AN patients are high and return to control levels after weight gain by renutrition (Ariyasu et al. 2001; Cuntz et al. 2002; Otto et al. 2001). AN patients often show markedly elevated GH levels, which may be due to high circulating levels of ghrelin. Moreover, high ghrelin increases ACTH, prolactin, and cortisol levels in humans (Takaya et al. 2000), which may explain the amenorrhea and behavioral changes observed in AN patients.

High plasma ghrelin concentration is observed in Prader–Willi syndrome (PWS) (Cummings et al. 2002a), cachexia with cancer or chronic diseases. PWS is a complex genetic disorder characterized by mild mental retardation, hyperphagia, short stature, muscular hypotonia, and distinctive behavioral features. Excessive appetite in PWS causes progressive severe obesity. The

PWS genotype is characterized by a loss of one or more paternal genes in region q11–13 on chromosome 15 (Nicholls and Knepper 2001). It has been suggested that this genetic alteration leads to dysfunction of several hypothalamic areas, including appetite regulatory regions.

To treat severe obesity, gastric bypass operations are often performed (Fobi 2004). Recent research has revealed that ghrelin may contribute to the body-weight reduction that occurs following gastric bypass. Total ghrelin secretion was found to be reduced by up to 77% compared to normal-weight control groups and by up to 72% compared to matched obese groups (Cummings et al. 2002b). Furthermore, the normal meal-related fluctuations and diurnal rhythm of ghrelin level were absent in these patients. Thus, the mean plasma ghrelin concentration decreased significantly after gastric bypass surgery, which may have been responsible for their lack of hyperphagia and contributed to their weight loss.

9.5 Cardiovascular Function of Ghrelin

Evidence for a cardiovascular function of ghrelin has been found: expression of mRNA encoding both ghrelin and its receptor has been observed in the heart and aortas (Gnanapavan et al. 2002; Nagaya et al. 2001a). Moreover, an intravenous bolus of human ghrelin decreased mean arterial pressure without changing the heart rate (Nagaya et al. 2001a). Ghrelin increased the cardiac index and stroke volume indices. Rats with chronic heart failure (CHF) that were treated with ghrelin showed higher cardiac output, stroke volume, and LV dP/dt[max] when compared to afflicted, but placebo-treated controls (Nagaya and Kangawa 2003). Furthermore, ghrelin increased the diastolic thickness of the non-infarcted posterior wall, inhibited LV enlargement, and increased LV fractional shortening in these CHF rats (Nagaya et al. 2001b). Ghrelin, thus, improves LV dysfunction and attenuates the development of LV remodelling and cardiac cachexia.

The decrease in mean arterial pressure induced by ghrelin seems not to occur through its direct action on the circulatory system, but through its action on the nucleus of the solitary tract (Lin et al. 2004; Matsumura et al. 2002). Microinjection of ghrelin into this nucleus significantly decreased the mean arterial pressure and heart rate. This injection also suppressed sympathetic activity. It has been reported that ghrelin inhibits apoptosis of primary adult and H9c2 cardiomyocytes and endothelial cells in vitro.

9.6 Gastrointestinal Function of Ghrelin

Intravenous administration of ghrelin dose-dependently increases gastric-acid secretion and stimulates gastric motility (Masuda et al. 2000). The max-

imum response to ghrelin in terms of gastric-acid secretion is almost as high as that elicited by subcutaneous treatment with histamine (3 mg/kg). These responses to ghrelin were abolished by pretreatment with either atropine or bilateral cervical vagotomy, but not by a histamine H_2-receptor antagonist. ICV administration of ghrelin also increases gastric-acid secretion in a dose-dependent manner (Date et al. 2001).

ICV administration of ghrelin was shown to induce c-Fos expression in the nucleus of the solitary tract and the dorsomotor nucleus of the vagus nerve (Date et al. 2001), indicating that ghrelin's ability to stimulate gastric-acid secretion is mediated through activation of the vagus nerve.

9.7 Ghrelin and Pancreatic Function

The role of ghrelin in insulin secretion is likewise under debate. Ghrelin has been shown to inhibit insulin secretion in some experiments and stimulate insulin release in others (Adeghate and Ponery 2002; Broglio et al. 2001; Date et al. 2002b; Lee et al. 2002). These discrepancies may be due to species differences and/or experimental design. Plasma ghrelin and insulin levels are affected by blood glucose level; high glucose suppresses ghrelin secretion and stimulates insulin secretion. Thus, the glucose level in experiments may be important. Date and colleagues reported that ghrelin stimulates insulin release in the presence of high levels of glucose (8.3 mM) that could release insulin from cultured islet cells (Date et al. 2002b). In contrast, ghrelin had no effect on insulin release in the context of a basal level of glucose (2.8 mM). In contrast, Dezaki and colleagues reported that ghrelin inhibits insulin secretion, while administration of ghrelin-receptor antagonists or anti-ghrelin antibodies increases insulin secretion that was induced by glucose injection (Dezaki et al. 2006). Moreover, they reported that an increase of glucose-induced insulin secretion was observed in ghrelin-null mice. Ghrelin knockout mice showed no change in density, size, insulin level and insulin mRNA of pancreatic island.

9.8 Ghrelin and the Process of Learning and Memory

Ghrelin may be involved in the process of learning and memory. Diano et al. reported that circulating ghrelin entered the hippocampus and bound to the hippocampal neurons to promote synapse formation of the dendritic spines and generate long-term potentiation (Diano et al. 2006). This synapse formation may be paralleled by enhanced spatial learning and memory after ghrelin injection. In contrast, ghrelin knockout mice had a decreased number of dendritic spine synapses in the hippocampal CA1 region and were impaired in behavioral memory in the novel object recognition test. Moreover, the de-

crease in synapse formation and impairment of memory test were promptly recovered by ghrelin administration. Further studies are needed to confirm that ghrelin directly acts on the hippocampal cells to enhance learning and memory processes.

10 Obestatin, a Ghrelin Precursor-Derived Peptide?

In November 2005 Zhang and colleagues from Stanford University reported a novel peptide hormone called "obestatin" from the Latin "obedere", meaning to devour, and "statin", meaning suppression, because it suppressed food intake (Zhang et al. 2005). An interesting fact is that obestatin is processed from the ghrelin precursor; this means that the two peptide hormones with opposing action on food intake, orexigenic ghrelin and anorectic obestatin, are derived from the same hormone precursor. They proposed that no obvious phenotypes in ghrelin knockout mice were due to the lack of both ghrelin and obestatin. Moreover, obestatin is the endogenous ligand for GPR39, an orphan GPCR that shows amino-acid sequence homology to ghrelin, motilin, neurotensin and neuromedin U receptors (McKee et al. 1997b).

However, several reports that followed raised objections to obestatin in its action and the matched receptor (Chartrel et al. 2007; Gourcerol et al. 2007; Holst et al. 2007; Lauwers et al. 2006; Nogueiras et al. 2007; Seoane et al. 2006).

The amino acid sequences of mammalian obestatins are well conserved. However, in non-mammalian species the obestatin parts are not conserved, while the ghrelin parts are well conserved. Moreover, the original paper on obestatin reported that the C-terminal amide structure is essential for obestatin to bind and activate GPR39, however, the precursor parts that seem to contain non-mammalian obestatins lack the Gly residue for the amide formation. Thus, non-mammalian obestatins, if they were contained in the stomach, are not of C-terminal amide structure. Furthermore, the general processing sites for the prohormone convertases, such as Arg-Arg or Lys-Arg, were not found in the non-mammalian obestatin parts. In addition, if both ghrelin and obestatin are processed from the same ghrelin precursor protein, the amount and secretion of both ghrelin and obestatin should be of a similar level and manner. However, the plasma content of ghrelin is higher than that of obestatin and after fasting plasma obestatin concentration did not change while ghrelin concentration was increased. Thus, it is likely that obestatin is not produced by a proper processing of the ghrelin precursor, but by a non-specific protease digestion. Future studies are needed to elucidate the role of obestatin.

11 Epilogue

Seven years have past since the discovery of ghrelin and since that time intensive research has been carried out on ghrelin. However, there remain many interesting questions regarding ghrelin-related biology. These include the identification of the pathways regulating ghrelin's production and release from the stomach, the enzyme that catalyzes its acyl-modification, as well as the continuing search for its physiological actions. Further research will answer these questions and elucidate the biochemical and physiological characteristics of this unique hormone.

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GPR54 and Kisspeptins

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Abstract The G-protein coupled receptor GPR54 has an essential role in the initiation and maintenance of mammalian fertility. Humans and mice with mutations in GPR54 have hypogonadotropic hypogonadism characterized by absence of sexual maturation and low levels of gonadotropic hormones (LH and FSH). The ligand for GPR54 is encoded by the *KISS1* gene, which produces a 54-amino-acid peptide (metastin or kisspeptin-54) that can be cleaved into shorter peptides (kisspeptins 14, 13 and 10) with similar potencies. Kisspeptin administration stimulates gonadotropin release in several species by inducing GnRH secretion from hypothalamic GnRH neurons expressing GPR54. Kisspeptins are produced by neurons located in the AVPV and ARC regions of the hypothalamus. Expression of *Kiss1* in these neurons is differentially regulated by sex steroids providing a mechanism by which testosterone or estrogen can regulate GnRH release. The AVPV region is sexually dimorphic with highest expression of kisspeptin in females. Positive feedback by estrogen on expression of *Kiss1* in the AVPV region may be responsible for the pre-ovulatory LH surge during the estrus cycle. Central administration of kisspeptin to immature female rats can induce precocious activation of the gonadotropic axis, causing advanced vaginal opening, elevated uterus weight, increased serum levels of LH and estrogen and induce ovulation. Kisspeptins/GPR54 have also been implicated in regulating the estrus cycle of seasonal breeders and in the control of lactational amenorrhea. Expression of *Gpr54* and *Kiss1* have also been reported in several peripheral tissues including the pituitary, ovary, testes and the placenta raising the possibility that these genes may have additional functions in these tissues. Regulation of kisspeptin expression by peripheral factors such as leptin may be involved in coordinating metabolic status with the reproductive axis.

1 Introduction

Mammalian reproductive function is regulated by hormonal messengers and feedback loops within the hypothalamic-pituitary-gonadal axis (Fig. 1A). At puberty, neurons in the medial preoptic area of the hypothalamus initiate the pulsatile secretion of gonadotropin releasing hormone (GnRH) into the portal blood system for delivery to the anterior pituitary. Within the pituitary, GnRH stimulates gonadotropic cells to release the gonadotropic hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH). The gonadotropins act on the gonads to stimulate synthesis of the sex steroids (testosterone and estrogen) which are required for spermato-

genesis and oogenesis. Hormonal feedback loops exist between the gonads, hypothalamus and the pituitary to regulate gonadotropin production (Fig. 1A).

Defining the physiological processes that initiate the pulsatile secretion of GnRH at puberty have proved elusive. The GnRH neurosecretory system is functional in neonatal primates with pulsatile gonadotropin secretions during the first months of life followed by suppression of hormone secretion until puberty. In primates, pre-pubescent GnRH secretion is suppressed by γ-aminobutyric acid (GABA) and removal of this suppression coincides with puberty. Loss of this suppression allows the reproductive axes of several species to respond to excitatory amino-acids, such as glutamate (Plant et al. 1989; Urbanski and Ojeda 1987). Conversely, antagonists of the glutaminergic NMDA receptor delay the onset of puberty in rats (Lopez et al. 1990; Urbanski and Ojeda 1990). New key molecules have now been identified

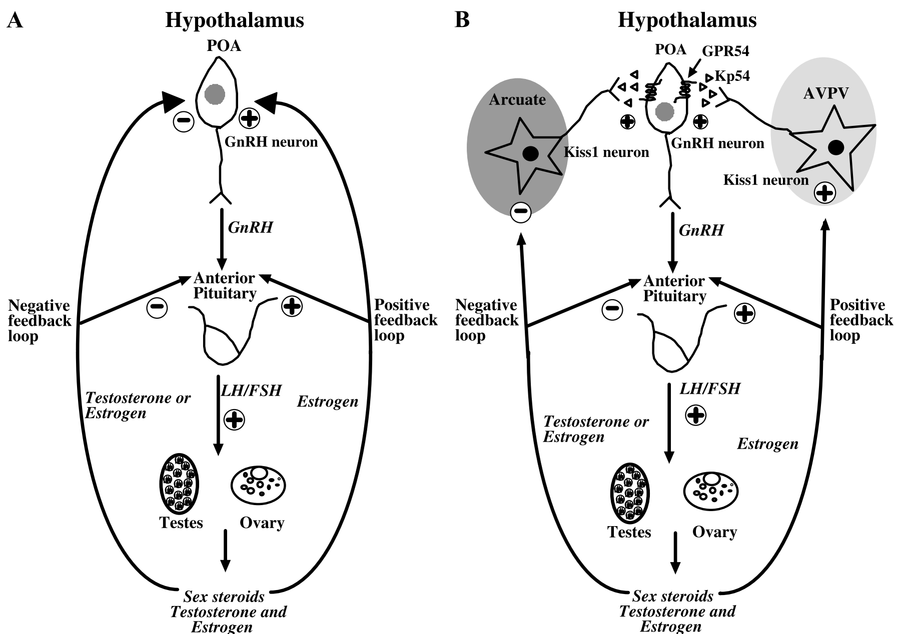

Fig. 1 Hormonal feedback loops in the Hypothalamic-Pituitary-Gonadal axis (**A**) and relationship to kisspeptin producing neurons (**B**). Kisspeptins act as a key mediator of the sex steroid feedback loops that regulate GnRH release from the hypothalamus. Kisspeptins act directly on GnRH neurons via the GPR54 receptor to stimulate GnRH release. Down regulation of kisspeptins in neurons of the arcuate nucleus provide the negative feedback action of sex steroids on GnRH release. Up regulation of kisspeptins by estrogen in neurons of the AVPV region in females stimulates GnRH release to provide the LH surge required for ovulation. GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone, POA, preoptic area; AVPV, anteroventral periventricular nucleus

that are essential for awakening GnRH release at puberty. These molecules are the G-protein coupled receptor, GPR54, and peptide ligands for this receptor encoded by the *Kiss1* gene.

2
The GPR54 Receptor

GPR54 (also called *AXOR12* and *hOT7T175*) was originally amplified from a rat brain cDNA library using degenerate primers to conserved sequences within the third and seventh transmembrane domains of the G-protein coupled receptor family (Lee et al. 1999). *GPR54* consists of five exons and encodes a 398 (395 in mice and 396 in rats) amino-acid protein with slight homology (around 45% amino-acid identity in the transmembrane regions) to galanin receptors but with no functional interaction with galanin (Ohtaki et al. 2001). Chinese hamster ovary (CHO) cells transfected with a GPR54 expression plasmid produced a 75 kDa protein, which is larger than predicted suggesting possible post-translational modification by glycosylation at three potential sites in the extracellular amino-terminus or by carboxy-terminal palmitoylation (Lee et al. 1999; Muir et al. 2001).

GPR54 is most highly expressed in the human pituitary, pancreas and placenta with lower expression in peripheral blood leukocytes, smooth muscle of some blood vessels, testes, spleen, thymus, adrenal glands and lymph nodes (Funes et al. 2003; Mead et al. 2007; Muir et al. 2001; Ohtaki et al. 2001). In the adult brain, expression is found in the superior frontal gyrus, putamen, caudate nucleus, cingulate gyrus, nucleus accumbens, hippocampus, medulla pons and amygdala as well as the hypothalamus (Kotani et al. 2001; Lee et al. 1999; Muir et al. 2001).

3
Kiss1

A physiological ligand for the GPR54 receptor was identified by several groups in 2001 (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001) and is encoded by the *KISS1* gene, which produces a 145 amino-acid protein that is proteolytic cleaved to generate the biologically active 54 amino-acid amidated protein, Kisspeptin 54 (Kp54) also known as metastin (Fig. 2). The carboxy-terminal region of Kp54 is responsible for receptor binding and this region is the most conserved between species (Fig. 3). Carboxy-terminal peptides of 14, 13 or 10 amino-acids (Kp14, Kp13 and Kp10) show similar activities in vitro to Kp54 (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). Binding of kisspeptin to GPR54 stimulates the G-protein Gq to activate phospholipase C and increases intracellular IP_3 and Ca^{2+} and activate the ERK and

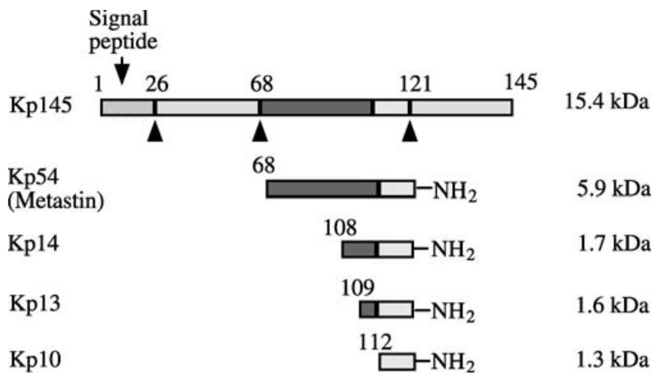

Fig. 2 Processing of KISS1 to produce kisspeptins. The primary protein product of the *Kiss1* gene is cleaved (*arrowheads*) to produce smaller amidated peptides (kisspeptins, Kp) capable of binding to GPR54. All biologically active peptides contain a common carboxy terminal decapeptide sequence (*stippled*)

Human	D	L	P	N	Y	N	W	N	S	F	G	L	R	F-NH_2
Chimp	D	L	P	N	Y	N	W	N	S	F	G	L	R	F-NH_2
Mouse	D	L	***S***	***T***	Y	N	W	N	S	F	G	L	R	***Y***-NH_2
Rat	D	***M***	***S***	***A***	Y	N	W	N	S	F	G	L	R	***Y***-NH_2
Sheep	D	***V***	***S***	***A***	Y	N	W	N	S	F	G	L	R	***Y***-NH_2

Fig. 3 Species comparison of kisspeptin sequences. Amino-acids are indicated by the single letter code and divergence from the human sequence indicated in ***bold italic letters***

p38 MAP kinase pathways. Carboxy-terminal amidation is required for all the peptides to stimulate intracellular Ca^{2+} increase (Muir et al. 2001; Ohtaki et al. 2001).

Kiss1 is highly expressed by syncytiotrophoblast cells in the placenta (Horikoshi et al. 2003). Consequently, plasma kisspeptin levels are raised in patients with molar pregnancies which contain trophoblastic tissue (gestational trophoblastic neoplasia) (Dhillo et al. 2006). Low levels of Kisspeptin-54 are present in the plasma of males and females but this rises dramatically during pregnancy the physiological significance of which is not known (Horikoshi et al. 2003). Lower levels of *Kiss1* are expressed in the testes, liver, pancreas and small intestine (Ohtaki et al. 2001).

4 Role in Metastasis

Kiss1 was originally identified as a differentially expressed cDNA that suppressed the metastatic spread of the human melanoma cell line C8161 after transfection of human chromosome 6 (Lee et al. 1996). As the human *Kiss1* gene is located on chromosome 1, this suggests that a positive regulator of *Kiss1* is located on chromosome 6. Transfection of a chromosome 6 deletion into C8161 cells indicated that this regulator maps between 6q16.3-q23 (Miele et al. 2000). The Sp1-coactivator protein DRIP-130 is a candidate for this regulator as it maps within this region and can increase *Kiss1* transcription in cancer cell lines when co-expressed with the transcription factor Sp1 (Mitchell et al. 2007). Subsequently *Kiss1* has been shown to suppress metastasis of additional human cancer cell lines in xenograft animal models including breast (Lee and Welch 1997) and ovarian carcinomas (Jiang et al. 2005). Reduced expression of *Kiss1* also correlates with increased metastatic potential and poorer prognosis of several human cancers including melanomas (Shirasaki et al. 2001), bladder carcinomas (Sanchez-Carbayo et al. 2003), hepatocarcinomas (Ikeguchi et al. 2003), gastric cancers (Dhar et al. 2004), pancreatic cancer (Masui et al. 2004), oesophageal squamous cell carcinomas (Ikeguchi et al. 2004), malignant pheochromocytomas (Ohta et al. 2005), and breast cancers (Stark et al. 2005). Conversely, some thyroid cancers that do not show loss of *Kiss1* expression are associated with increased expression of the GPR54 receptor (Ringel et al. 2002). Thus, changes in expression of either *Kiss1* or *GPR54* may influence the severity and progression of some types of cancer.

The mechanism by which kisspeptins suppress metastasis is not clear but may not require a change in cell proliferation. The effect of kisspeptins on cell proliferation is unresolved, with some groups reporting no effect (Bilban et al. 2004; Lee et al. 1996) while others report inhibition of cell proliferation (Kotani et al. 2001; Stafford et al. 2002). Kisspeptins also inhibit the migration in culture of cell lines expressing GPR54 such as transfected CHO cells (Ohtaki et al. 2001; Stafford et al. 2002) or primary human trophoblast cells (Bilban et al. 2004). Migratory inhibition may be caused by increased formation of focal adhesion points (Ohtaki et al. 2001) or reduced secretion of proteases such as MMP-2 (Bilban et al. 2004). The inhibitory effect of kisspeptin on migration of the human ovarian cancer cell line SKOV3 can be overcome by phorbol ester stimulation of protein kinase Cα (Jiang et al. 2005) suggesting that continuous GPR54 signalling could reduce cell migration by inhibiting PKCα activity. Kisspeptins also inhibit the intracellular signalling cascade of the pro-metastatic G-protein coupled receptor CXCR4 (Navenot et al. 2005). Metastasis suppression requires kisspeptin secretion as melanoma lines expressing non-secreted forms of the protein are not inhibited (Nash et al. 2007) suggesting an autocrine or paracrine mechanism of action.

5 Role in Reproduction

5.1 GPR54 Mutations in Hypogonadotropic Hypogonadism

5.1.1 Humans

The crucial role that GPR54 plays in human fertility was originally identified by two independent research groups that found loss of function mutations in patients with idiopathic hypogonadotropic hypogonadism (IHH) (de Roux et al. 2003; Seminara et al. 2003). IHH is a clinical condition characterized by absence of pubertal sexual development and low sex steroid and gonadotropin levels in the blood. Some individuals with IHH also have an inability to smell (anosmia, Kallmann syndrome) due to a failure of olfactory bulb neuron development and migration (including GnRH neurons) but individuals with GPR54 mutations are all normosmic. Mutations in several genes can cause hypogonadotropic hypogonadism including the GnRH receptor, FGFR1, DAX-1 and KAL1 although not all cases of IHH have had a specific genetic defect ascribed to them (Iovane et al. 2004).

Most *GPR54* mutations have been identified in individuals from consanguineous marriages between first cousins. De Roux and colleagues studied a family of eight children of whom five were affected by IHH (de Roux et al.

Table 1 GPR54 mutations associated with hypogonadotropic hypogonadism

Mutation	Location	Amount of normal cell signalling	Refs.
Δ155 (Intron 4–142Ex5)	3rd Extracellular Loop termination	Not Described	(de Roux et al. 2003)
L146S (443T > C)	2nd Internal Loop	35%	(Seminara et al. 2003)
R331X (991C > T)	Proximal part COOH terminus	33% 60%	
X339R (1195T > A)	COOH terminal extension		
C223R (667T > C)	5th TMH	20%	(Semple et al. 2005)
R297L (891G > T)	3rd External Loop	85%	
1001–1002insC	Proximal part COOH terminus	Not Described	(Lanfranco et al. 2005)
L102P	1st Extracellular Loop	10%	(de Roux et al. 2003) (Tenenbaum-Rakover et al. 2007)

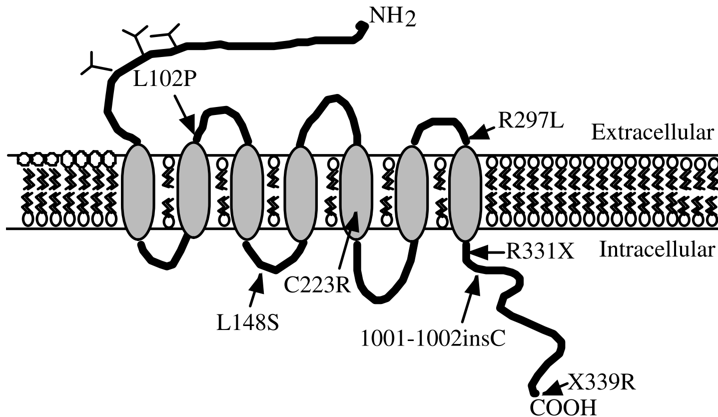

Fig. 4 Location of GPR54 mutations. GPR54 is a G-protein coupled receptor with seven transmembrane domains

2003). A genome-wide linkage scan localized the mutation to a small region of chromosome 19 and candidate genes in this region were sequenced for mutations. A 155 bp deletion was found starting in intron 4 and extending 142 bases into exon 5 of the *GPR54* gene that would produce a truncated protein incapable of G-protein coupling. A missense mutation (leucine to proline at position 102) was also identified in a sporadic case of IHH. Seminara and colleagues used a similar approach to identify GPR54 mutations in a family from Saudi Arabia (Seminara et al. 2003) and found a nonsense mutation leading to a premature stop codon and a nonstop mutation (X399R) that would extend the length of the GPR54 protein. Subsequently, additional mutations in *GPR54* have been identified (Table 1). These mutations are found in different locations of the GPR54 protein and might reduce signalling by different mechanisms (Fig. 4). Mutations within extracellular loops of GPR54 (L102P and R297L) might alter interaction with kisspeptins, while those at the C-terminus (and truncations) would remove interaction with the Gq protein. Indeed, most GPR54 mutations have been expressed in heterologous cell systems and show a reduction in cell signalling compared to wild-type (Table 1).

5.1.2 Mice

The role that GPR54 plays in regulating the fertility of other mammals has been confirmed by the independent generation of three different transgenic mouse lines with disruptions of the *Gpr54* gene (Funes et al. 2003; Kauffman et al. 2007b; Lapatto et al. 2007; Messager et al. 2005; Seminara et al. 2003). In the mice generated by Colledge and colleagues, a segment spanning intron 1 and parts of exon1 and exon 2 of the *Gpr54* coding sequence have been removed and replaced with an IRES-LacZ sequence that allows

the expression pattern of the *Gpr54* gene to be visualized by detection of β-galactosidase activity. Mutant animals of both sexes fail to undergo pubertal sexual development and are sterile. Mutant males have small testes, severe disruption of spermatogenesis and fail to develop secondary sex glands such as the seminiferous vesicles and preputial glands. Mutant females have no estrus cycle, thread-like uteri and ovaries with no mature antral follicle formation. The mammary glands do not show the normal pubertal development of a branched epithelial duct system. Both sexes have low sex steroids and gonadotropic hormones but retain functional pituitary responses to GnRH. Importantly, the mutant mice have normal hypothalamic GnRH content and correct localization of GnRH neurons suggesting a primary defect in GnRH release rather than a developmental defect in GnRH migration.

Mice with a disrupted *Kiss1* gene have also been generated and these show similar reproductive defects to the *Gpr54* mutants (d'Anglemont de Tassigny et al. 2007; Lapatto et al. 2007). *Kiss1* mutant mice have abnormal pubertal maturation of the reproductive system, hypogonadotropic hypogonadism and low sex steroid levels but retain the ability to secrete gonadotropic hormones after kisspeptin injection. It has been reported that the *Kiss1* mutant mice generated by Lapatto and colleagues ($Kiss1^{tm1MGH}$) have a more variable phenotype than *Gpr54* mutant mice (Lapatto et al. 2007). Around half of the $Kiss1^{tm1MGH}$ mice show vaginal opening and ovary weights similar to wild-type. In contrast, the *Kiss1* mutant mice generated by d'Anglemont de Tassigny and colleagues ($Kiss1^{tm1PTL}$) do not show vaginal opening even up to 6 months of age. The reason for this difference is unknown but may reflect the slightly different genetic background of the two lines with the $Kiss1^{tm1MGH}$ mice on a 129S1/SvIMJ background and the $Kiss1^{tm1PTL}$ mice on a 129S6/SvEv background. These mutant mice show that GPR54 and kisspeptins are both essential for the activation of the hypothalamic-pituitary-gonadal axis at puberty.

5.2 Hypothalamic Expression Pattern

The expression pattern of *Gpr54* and *Kiss1* in the hypothalamus is consistent with the function of these genes in the central control of reproduction. The hypothalamus is located just above the brain stem and regulates many important physiological processes including body temperature, hunger, thirst, circadian cycles and reproduction. The hypothalamus is arranged either side of the third ventricle and divided into three functionally distinct zones (periventricular, medial, and lateral). The periventricular and medial zones contain most of the hypothalamic neuronal cell bodies with the lateral zone containing fewer neurons. *Kiss1* and *Gpr54* are expressed in discrete neuronal populations (nuclei) in the hypothalamus (Fig. 5). *Kiss1* expression in rodents has been mapped by in situ

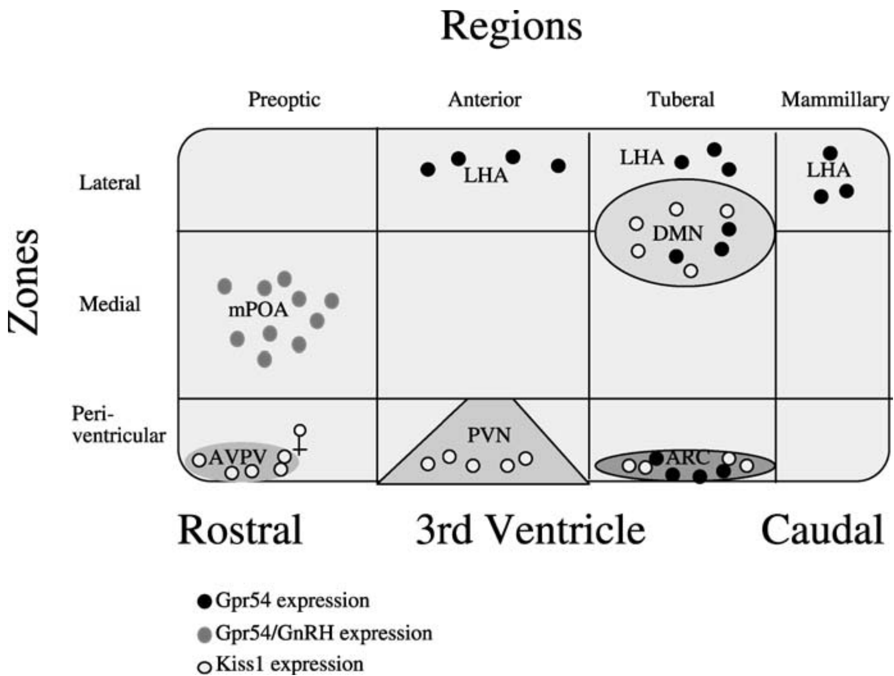

Fig. 5 Consensus expression of *Gpr54* and *Kiss1* in cell bodies in the rodent hypothalamus. Schematic representation of the morphological organization of the hypothalamus. Expression pattern based on in situ hybridization data and KISS1 protein immunohistochemistry. The AVPV region is sexually dimorphic with higher *Kiss1* expression in the female. LHA, lateral hypothalamic area; mPOA, medial preoptic area; DMN, dorsomedial nucleus; AVPV, anteroventral periventricular nucleus; PVN, periventricular nucleus; ARC, arcuate nucleus

hybridization to the arcuate nucleus (ARC), anteroventral periventricular nucleus (AVPV) and the periventricular nucleus (PVN) (Gottsch et al. 2004; Irwig et al. 2004). Immunohistochemical detection of KISS1 protein has not always given concordant results with the in situ expression data possibly due to species differences and antibody specificity. Several anti-KISS1-antibodies have been used by different groups including a commercially available one raised against the human kisspeptin-10 sequence [YNWNSFGLPF-NH_2 (Brailoiu et al. 2005; Pompolo et al. 2006)], a rabbit polyclonal against mouse kisspeptin-10 [YNWNSFGLRY-NH_2 (Franceschini et al. 2006; Clarkson and Herbison 2006)] and a mouse monoclonal against a slightly longer rat kisspeptin sequence [EKDMSAYNWNSFGLRY-NH_2 (Kinoshita et al. 2005)]. The consensus is that kisspeptin immunoreactive cell bodies are found in the ARC, AVPV and PVN of most species (Brailoiu et al. 2005; Clarkson and Herbison 2006; Franceschini et al. 2006; Kinoshita et al. 2005; Pompolo et al. 2006). Kisspeptin-10 immunoreactive cell bodies have

also been found in the dorsomedial hypothalamus of the rat (Brailoiu et al. 2005), mouse (Clarkson and Herbison 2006) and sheep (Franceschini et al. 2006) using two different antibodies but expression in this region has not been found by in situ hybridization. Kisspeptin immunoreactive fibres have been shown to innervate the preoptic area (POA) in sheep (Franceschini et al. 2006; Pompolo et al. 2006) and make intimate connection with GnRH neurons in rodents (Kinoshita et al. 2005; Clarkson and Herbison 2006). Transgenic mice with a *LacZ* targeted *Kiss1* locus have allowed us to visualize *Kiss1* expression by β-galactosidase staining (d'Anglemont de Tassigny et al. 2007). These mice have β-galactosidase expression in the expected regions of the ARC, AVPV and PVN but also show staining in the medial mamillary nucleus which has not previously been reported to express *Kiss1* (d'Anglemont de Tassigny et al. 2007; X. d'AdeT, unpublished).

Gpr54 was first shown to be expressed in the hypothalamus by qRT-PCR at levels approximately 500x less than the housekeeping gene *Gapdh* (Muir et al. 2001). The cellular distribution of *Gpr54* expression in the hypothalamus of rodents as defined by in situ hybridization is localized to the diagonal band of Broca, the medial septum, preoptic areas and the anterior and lateral hypothalamus (Han et al. 2005; Irwig et al. 2004). Of particular significance is the finding that the majority of GnRH neurons in mammals (up to 90%) also express *Gpr54* (Han et al. 2005; Irwig et al. 2004; Messager et al. 2005). Gpr54 expression has also been found in GnRH neurons prior to birth with expression at the 18th day of gestation in rat fetuses (Quaynor et al. 2007). Gpr54 expression by GnRH neurons extends to the cichlid fish suggesting a conservation of function beyond mammals and into other vertebrates (Parhar et al. 2004).

5.3
Stimulation of Gonadotropin Secretion

The function of kisspeptins in vivo has been studied by injection of these peptides into several animal species. These studies have shown that kisspeptins play a crucial role in stimulating gonadotropin release in rats (Castellano et al. 2006b; Irwig et al. 2004; Matsui et al. 2004; Navarro et al. 2004a, 2005a,b; Thompson et al. 2004), mice (Gottsch et al. 2004; Messager et al. 2005), sheep (Messager et al. 2005), primates (Plant et al. 2006; Shahab et al. 2005) and humans (Dhillo et al. 2005) after systemic (intravenous, intraperitoneal or subcutaneous) or intracerebroventricular (ICV) delivery. Kisspeptins are extremely potent agonists of gonadotropin secretion with as little as 1 fmol producing significant release of LH after ICV injection in mice (Gottsch et al. 2004). This is considerably more effective than other substances that stimulate GnRH release (eg., glutamate) and the kisspeptin responses are particularly long lasting often maintaining LH release over several hours. Kisspeptins activate GnRH neurons indicated by an increase in *c-fos* immunoreactivity (Irwig et al. 2004; Matsui et al. 2004) probably by a direct action as GnRH neu-

rons express the GPR54 receptor (Han et al. 2005; Irwig et al. 2004; Messager et al. 2005). Significantly, responses to kisspeptins are absent in *Gpr54* mutant mice showing that GPR54 directly mediates GnRH secretion (Messager et al. 2005). In addition, we have directly shown GnRH secretion in response to Kisspeptin injection in sheep (Messager et al. 2005). Thus, the principle function of kisspeptins in vivo are to act via the GPR54 receptor to stimulate GnRH release and activate the pituitary gonadal axis.

Kisspeptin stimulation of the GPR54 receptor in GnRH neurons may be subject to negative feedback modulation by GnRH. GPR54 and the GnRH-receptor have been shown to form a close association by bioluminescence resonance energy transfer in HEK-293 cells (Quaynor et al. 2007). Moreover, kisspeptin-10 mediated enhancement of GnRH release from the GnRH neuronal cell line GT1-7 can be inhibited by GnRH itself. This suggests that the long recognized ability of GnRH to suppress its own release may be mediated by the GnRH-receptor altering kisspeptin/GPR54 signalling.

As well as these acute effects of kisspeptins on gonadotropin release, the effects of continuous delivery have also been studied. The first chronic delivery study was performed using castrated juvenile male rhesus monkeys intravenously infused with 100 μg/h human Kisspeptin-10 for 4 days (Seminara et al. 2006). Under these conditions, LH secretion was initially stimulated over a 3h period followed by suppression. The monkeys retained LH responses to NMDA and GnRH during the infusion period demonstrating the functional integrity of GnRH neurons and pituitary gonadotrophs. Thus, continuous kisspeptin infusion desensitizes the GPR54 receptor rather than other components of the hypothalamic-pituitary axis in juvenile monkeys. These studies have been extended using non-castrated adult male monkeys (Ramaswamy et al. 2007). A similar increase in LH release immediately after Kisspeptin-10 infusion was found, followed by a decrease at longer time points. In contrast to the juvenile monkeys, the LH responses to NMDA and GnRH injection were reduced, suggesting that in adult monkeys continuous kisspeptin administration desensitizes not only GPR54 but also the GnRH receptor in the pituitary. In rats, chronic subcutaneous administration of Kisspeptin-54 initially increased LH and testosterone after 1 day but this effect was lost by day 2 (Thompson et al. 2006). Longer-term administration of Kisspeptin-54 for 13 days decreased testicular weight and led to degeneration of seminiferous tubules (Thompson et al. 2006).

An early report also claimed that intravenous injection of Kisspeptin-10 increased plasma oxytocin levels in female rats (Kotani et al. 2001). The significance of this observation is not clear and no subsequent reports have confirmed this data. Oxytocin is synthesized by magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus and transported axonally to the posterior pituitary. Oxytocin has a principle role in parturition, lactation and maternal behavior none of which have been yet determined in *Kiss1* mutant mice because of their infertility.

5.4 Activation at Puberty

The absence of puberty in humans and mice with mutations in GPR54 shows that this protein is required for pubertal development but does not prove that GPR54 is a key regulator of this event. Several lines of evidence however, indicate that GPR54 and kisspeptins are more than just downstream mediators of puberty but rather that they are key molecules involved in switching on the HPG axis. *Kiss1* and *Gpr54* expression increase coincidentally with puberty in several species. In the rat, *Kiss1* and *Gpr54* both increased in the hypothalamus as a whole at puberty (Navarro et al. 2004a). More detailed, in situ hybridization studies showed that this increase in *Kiss1* was confined to the AVPV region with little change in the ARC of male mice (Han et al. 2005). Consistent with this, the number of kisspeptin immunoreactive neurons increased in the AVPV/PVN of the mouse during puberty but little change was found in the ARC (Clarkson and Herbison 2006). In primates, *KISS1* and *GPR54* mRNA levels increased in the hypothalamus during the transition from juvenile to mid-puberty stages in intact females (Shahab et al. 2005). In castrated males, *KISS1* expression was 3-fold greater in the pubertal group than in the juvenile animals while *GPR54* expression did not change. Thus, an increase in *Kiss1* expression around the time of puberty is a consistent observation in many species. Consonant with this pubertal increase in *Kiss1* expression, exogenous injection of kisspeptins can advance pubertal development. Chronic administration of Kisspeptin-10 to immature female rats advanced vaginal opening (a sign of puberty) by 5 days (Navarro et al. 2004b) and repetitive injection of Kisspeptin-10 in juvenile primates induced a precocious chain of GnRH pulses similar to those occurring at puberty (Plant et al. 2006).

As well as the expression changes that occur to *Kiss1* and *Gpr54*, it is likely that other functional and anatomical changes are required for proper activation of GnRH release at puberty. For example, the sensitivity of GnRH neurons to kisspeptins alters during pubertal development. Kisspeptins caused a rapid depolarization of over 90% of GnRH neurons in adult mice but only around 30% of neurons in juvenile animals, even though the expression of *Gpr54* was very similar between the age groups (Han et al. 2005). This difference in the sensitivity of GnRH neurons was confirmed in vivo using low doses of kisspeptin which elicited LH release in adults but not juvenile mice after ICV delivery (Han et al. 2005). Similar changes in sensitivity have been found in male rats where ICV injection of low doses of Kisspeptin-10 (1 or 10 pmol) produced better LH secretion in 40-day-old animals than juvenile 15-day-old animals (Castellano et al. 2006b). This developmental change in sensitivity of GnRH neurons may also explain why the immortalized GnRH cell line, GT1-7 does not respond to kisspeptins even though it expresses *Gpr54* (Nazian 2006). It is possible that the GT1-7 line

represents an immature GnRH cell type which may be useful in studying the mechanism by which GnRH cells become responsive to kisspeptins. It should also be remembered however, that there are also changes that occur to the neuronal circuitry at puberty that may be required for kisspeptin signalling. For example, connections between kisspeptin immunoreactive fibres and GnRH neurons increase across post-natal development in male and female mice (Clarkson and Herbison 2006).

5.5 Kiss1 Regulation by Sex Steroids

It is well established that GnRH and gonadotropin secretion are regulated by sex steroids but the mechanism by which this is achieved remains largely unknown. Direct action of estrogen on GnRH neurons is unlikely as they do not express the estrogen receptor alpha (ERα). It now seems likely that *Kiss1* expressing neurons integrate the feedback signals from gonadal steroids to GnRH neurons. *Kiss1* neurons make direct contact with GnRH neurons (Kinoshita et al. 2005; Clarkson and Herbison 2006) and *Kiss1* expression is regulated by sex steroids in a manner consistent with feedback control (Fig. 1B). Gonadectomy of male and female rats increases hypothalamic *Kiss1* expression as measured by RT-PCR, while steroid replacement abolishes this increase (Navarro et al. 2004a). This sex steroid regulation extends to higher primates as testosterone treatment of castrated adult male rhesus monkeys reduces *Kiss1* expression in the mediobasal hypothalamus (Shibata et al. 2007) and ovariectomy of young cynomolgus monkeys increases expression of *Kiss1* in the infundibular nucleus which can be prevented by estrogen replacement (Rometo et al. 2007). In post-menopausal women of average age 72 years with negligible estrogen production, *Kiss1* expression was significantly higher than in pre-menopausal women of average age 32 years (Rometo et al. 2007). This increase in kisspeptin expression may account for the elevation in gonadotropin secretion that occurs after menopause.

Subsequent studies by in situ hybridization and immunohistochemistry have provided more details about the effects of sex steroids on *Kiss1* expression in specific hypothalamic nuclei. In mice, *Kiss1* expression is decreased in the AVPV and PVN and increased in the ARC after gonadectomy (Smith et al. 2005a,b). These changes are eliminated by testosterone (in males) or estrogen (in females) replacement. In sheep, ovariectomy increases expression of *Kiss1* mRNA mainly in the ARC and to a lesser extent the POA (Smith et al. 2007) but immunoreactive kisspeptin only significantly increases in the ARC (Pompolo et al. 2006). *Kiss1* expression in the ARC was returned to the level found in intact sheep by estrogen treatment but progesterone also partially returned the level to normal (Smith et al. 2007). Whether progesterone has an effect on *Kiss1* expression in rodents has not yet been determined.

The effects of gonadal steroids on the expression of *Kiss1* in the hypothalamus would be expected to be mediated by steroid hormone receptors. Indeed, in male mice, around 65% of *Kiss1* neurons in the ARC also express the androgen receptor (AR) and around 90% express the estrogen receptor alpha (ERα) (Smith et al. 2005b). Co-expression data in the AVPV and PVN regions were not given for male mice. In female mice, the majority of *Kiss1* neurons in the ARC, AVPV, PVN express ERα and between 25–40% express ERβ (Smith et al. 2005a). In rats, around 60–70% of *Kiss1* neurons in the ARC and the AVPV express ERα with less (10–20%) expressing ERβ (Smith et al. 2007). In sheep, around 90% of *Kiss1* neurons in the ARC express ERα and 50% in the POA (Franceschini et al. 2006). 86% of *Kiss1* neurons in the sheep ARC also express the progesterone receptor (Smith et al. 2007). Whether *Kiss1* neurons express the progesterone receptor in rodents had not been reported. *Kiss1* neurons in the ARC nucleus of the ewe also express the neuropeptides dynorphin A and neurokinin B (Goodman et al. 2007). Dynorphin A is a 17 amino-acid opioid-like peptide and neurokinin B is a 10 amino-acid peptide of the tachykinin family. Both peptides have been implicated in regulating GnRH release so their co-expression in *Kiss1* neurons may have an important role in modulating the kisspeptin control of GnRH release.

The role of each type of steroid receptor in mediating sex steroid regulation of *Kiss1* expression was assessed by analysis of transgenic mice with mutations in these receptors. Male mice with mutations in either the ERα or the AR still show testosterone-mediated changes in *Kiss1* expression in the ARC, suggesting that this regulation is mediated by both receptor types (Smith et al. 2005b). This observation is also supported by the fact that the effects of testosterone on *Kiss1* expression in castrated mice are completely mimicked by estrogen treatment but only partially mimicked by dihydrotestosterone which cannot be aromatized to estrogen (Smith et al. 2005b). Thus, testosterone modulates *Kiss1* expression in the ARC through the AR and also the ERα after aromatization to estrogen. Female mice with a defective ERα no longer show regulation of *Kiss1* in the ARC and the AVPV, while ERβ mutant mice continue to show estrogen regulation (Smith et al. 2005a). Thus, in female mice ERα but not ERβ, has a crucial role in mediating the estrogen regulation of *Kiss1* expression in the hypothalamus.

The way in which *Kiss1* transcription is regulated is starting to be unravelled. The transcription factors AP-2α and Sp1 act synergistically to positively regulated *Kiss1* expression in breast cancer cell lines (Mitchell et al. 2006). The activity of Sp1 also requires expression of the co-activator protein DRIP-130 (Mitchell et al. 2007). The minimal promoter region that confers estrogen responsiveness on human *Kiss1* expression has been mapped (Li et al. 2007). While the highest induction of promoter activity to estrogen was found for a 1000 bp fragment immediately upstream of the transcription start site, a significant response to estrogen was also obtained with the most proximal

190 bp sequence. This 190 bp region does not actually contain consensus estrogen response elements for direct ERα binding but has four Sp1 binding sites. Sp1 and ERα form a complex to mediate the estrogen-induced activation of the *Kiss1* promoter. All four Sp1 binding sites contribute to the basal promoter activity while the two Sp1 binding sites closest to the transcriptional start site function together to allow estrogen stimulation (Li et al. 2007).

5.6 Kiss1 Expression During the Estrus Cycle

As expected from a gene regulated by sex steroids, *Kiss1* expression varies during the estrus cycle of several species and also shows fluctuations with the breeding season. Given the potency with which kisspeptins stimulate GnRH secretion, it is generally thought that these changes in expression are driving these reproductive cycles rather than simply following them. Significantly, *Kiss1* expression in the AVPV and the ARC regions of the rat hypothalamus show opposite changes during the estrus cycle, probably reflecting the different contributions of these regions in the control of GnRH release during the cycle. *Kiss1* expression is at its peak in the AVPV region during the evening of proestrus (the stage leading up to ovulation) in rats while expression in the ARC is declining at this point (Smith et al. 2006b). *Kiss1* neurons are specifically activated in the AVPV region at proestrus as indicated by an induction in *c-fos* expression (Smith et al. 2006b). This increase in *Kiss1* expression in the AVPV region coincides with the pre-ovulatory LH surge and is also increased during a steroid-induced LH surge in ovariectomized rats (Smith et al. 2006b). Inhibition of kisspeptin action in the POA by local injection of monoclonal antibody abolished the pro-estrous LH surge and inhibited estrous cyclicity in rats (Adachi et al. 2007; Kinoshita et al. 2005).

Consistent with the AVPV nucleus mainly operating in female rodents to induce the LH surge, *Kiss1* expression in this region is sexually dimorphic with female rats having more *Kiss1* neurons than male rats (Kauffman et al. 2007a). This difference is established perinatally by an androgen-mediated reduction in *Kiss1* expression since neonatally androgenized females show male patterns of *Kiss1* expression in the AVPV region at adulthood. The AVPV region of the rodent has been recognized as sexually dimorphic for several years with differences in the number of dopaminergic neurons (Simerly 1998) but the *Kiss1* neurons in this region are distinct from these dopaminergic neurons (Kauffman et al. 2007a). The androgenization process that establishes these sexual dimorphisms requires GPR54, since *Gpr54* knock-out male mice have dopaminergic and *Kiss1* neurons in the AVPV region similar in number to those found in females (Kauffman et al. 2007b). *Gpr54* mutant mice also lack an olfactory-mediated preference for female mice. Thus, GPR54/kisspeptin signalling probably acts during perinatal development to regulate the GnRH-

mediated androgen secretion necessary for the proper development of several sexually dimorphic traits.

Similar changes in *Kiss1* expression occur during the estrus cycle of the sheep but in this species the region of the hypothalamus that controls the pre-ovulatory LH surge is the ARC nucleus. Accordingly, *Kiss1* expression increases in the caudal region of the ARC nucleus in ewes to be highest at the late-follicular stage just before ovulation (Estrada et al. 2006).

5.7 Seasonal Breeding

Kisspeptins probably also regulate the seasonal breeding cycle of species that show circannual changes in reproductive capacity. Many species show seasonal breeding patterns to ensure that offspring are born at a time when environmental conditions are optimal for survival. For example, sheep usually breed in the autumn to give birth in the spring. During the non-breeding season, GnRH secretion is reduced and the estrous cycle shut down. An examination of *Kiss1* expression in the ARC nucleus of ovariectomized ewes by in situ hybridization has found that expression is highest during the breeding season and decreases around 50% in the non-breeding season (Smith et al. 2007). Importantly, because these changes occurred in ovariectomized sheep, this change in *Kiss1* expression is steroid-independent providing further support that kisspeptins drive these changes in reproductive function rather than follow them. Interestingly, it is the number of *Kiss1* positive cells that changes rather than the expression level per cell suggesting that around 50% of the *Kiss1* cells in the ARC do not change expression with breeding season. Why these remaining cells are not capable of driving GnRH release is not known but perhaps they do not make appropriate connections to GnRH neurons.

Siberian hamsters also show seasonal breeding and have been used to examine the effects of photoperiod on reproduction. These hamsters breed during long-day periods and become anestrus during short-days. Kisspeptin immunoreactive neurons were significantly reduced in the AVPV of non-breeding short-day hamsters compared to those kept under long days (Greives et al. 2007). In contrast, kisspeptin immunoreactive neurons dramatically increased in the ARC of short-day animals. These changes were not found in a polymorphic hamster line that does not show seasonal breeding. All hamsters responded to kisspeptin injection irrespective of reproductive state suggesting that the photoperiodic regulation of GnRH release is regulated by local kisspeptin production in the hypothalamus. The regulatory mechanisms that control these seasonal changes in *Kiss1* expression have not been identified. Some changes in *Kiss1* expression may reflect seasonal fluctuations in sex steroid levels but it is likely that other molecules that respond to photoperiodism, such as melatonin, may also play a role.

5.8 Lactation

Suppression of ovulation by lactational suckling is found in most mammalian species due to inhibition of GnRH/LH secretion although the precise mechanism is unknown (McNeilly 2001). Kisspeptins have now been implicated in lactational amenorrhea. Roa et al. (2006) reported a differential sensitivity to low doses of Kisspeptin-10 with lactating rats requiring higher doses of kisspeptins to secrete LH than rats in diestrus (Roa et al. 2006). This observation has been extended by Yamada et al. (2007) who studied the expression level of *Kiss1* in micro-dissected regions of the hypothalamus during lactation by qRT-PCR and immunohistochemistry. A significant reduction in *Kiss1* mRNA and protein expression was found in the ARC-ME region in lactating rats that was independent of sex steroids (Yamada et al. 2007). *Kiss1* expression in the AVPV was very low in both lactating and non-lactating rats with no significant difference between the groups. ICV injection of Kisspeptin-54 stimulated LH secretion indicating that the signalling pathways downstream of kisspeptins are functional in lactating rats. These data are consistent with modulation of *Kiss1* expression in the ARC controlling the suppression of ovulation during lactational suckling.

6 Kisspeptin Action at Non-Hypothalamic Sites

6.1 Pituitary

The role that kisspeptin/Gpr54 signalling plays in regulating hormonal release from the pituitary is equivocal. The pituitary has one of the highest levels of *GPR54* expression of all human tissues (Kotani et al. 2001; Muir et al. 2001) although the specific cell types expressing GPR54 have not been determined. Whether kisspeptins can act on the pituitary to stimulate hormone release is contentious. Navarro and colleagues have reported that mouse Kisspeptin-10 can stimulate LH secretion from adult male rat pituitary explants in a dose-dependent manner although this effect was 4-fold less potent than with GnRH (Navarro et al. 2005b). Similar effects have been found using dispersed pituitary cells from peripubertal male and female rats (Gutierrez-Pascual et al. 2007). In these studies, Kisspeptin-10 stimulated a rise in cytoplasmic Ca^{2+} levels in 63% of gonadotrophs. Weak Kisspeptin-10 stimulation of LH secretion has also been found in dispersed pituitary cells from pig and cows (Suzuki et al. 2007). In contrast, other studies have failed to find any effect of kisspeptins on the pituitary. Anterior pituitary fragments from adult male rats did not respond to Kisspeptin-10 but released LH and FSH

after stimulation with GnRH (Thompson et al. 2004). In another study, primary cultures of female rat anterior pituitary cells did not respond to human Kisspeptin-54 but released both FSH and LH when given GnRH with no potentiation of secretion using both peptides together (Matsui et al. 2004). A comparison of these studies is difficult because of differences in the experimental design but one variable is the time point at which the media was sampled for LH measurement. In the experiments of Navarro et al. (2005), the media was collected after 1 h and 3 h, while Thomson et al. (2004) collected media after 4 h. At the longer time points, the amount of LH in the media of the vehicle control group had increased which may have masked small kisspeptin effects. Alternatively, difficulties in measuring quite modest LH secretion may account for the differences.

Kisspeptin-10 has also been reported to stimulate modest growth hormone (GH) release from pituitary somatotrophs (Gutierrez-Pascual et al. 2007). In these studies, Kisspeptin-10 stimulated GH secretion from dispersed pituitary cells from peripubertal male and female rats but these responses were 20-fold less potent compared to that with the normal agonist GHRH. Kisspeptin-10 also stimulated a rise in cytoplasmic Ca^+ levels in 60% of somatotrophs in the culture. The mechanism of this effect or its physiological significance is not clear. Transgenic mice with null mutations in the GPR54 receptor or the *Kiss1* gene do not have a major growth defect. In these culture experiments, GH secretion is being monitored in a heterogenous population of dispersed pituitary cells. It will be important to determine whether kisspeptins have a direct action on somatotrophs or whether the response is indirect and mediated by kisspeptin action on other cell types in the culture.

6.2 Metabolism

Kisspeptins may also act to integrate the peripheral signals of metabolic status to the central nervous system control of reproductive function. It has been noted that the age of menarche directly correlates with attainment of a minimum body fat composition. It has been suggested that the hormone leptin, a 16-kDa protein produced by adipose tissue, acts as a facilitator of puberty. In rodents, leptin can influence the onset of puberty although its role in primates is not clear. Leptin administration reduces the age at which puberty occurs in rats and mice (Ahima et al. 1997; Carro et al. 1997; Chehab et al. 1997) and female mice with mutations in the leptin gene (ob/ob) are sterile (Chehab et al. 1996). Administration of leptin to ob/ob mice can restore fertility (Mounzih et al. 1997). Similarly, mice with mutations in the leptin receptor (db/db) are sterile and fail to release GnRH (Coleman 1978; Johnson and Sidman 1979).

It has recently been shown that experimentally manipulated changes in the body energy status of rats can alter kisspeptin signalling in the hypothala-

mus. Fasting of prepubertal rats of either sex for 72 h, which is associated with a reduction in peripheral gonadotropin levels, causes a decrease in *Kiss1* and an increase in *Gpr54* in the whole hypothalamus as measured by RT-PCR (Castellano et al. 2005). Fasted animals still responded to central injection of Kisspeptin-10 by release of LH and these responses were larger than in non-fasted animals, perhaps due to the increase in GPR54. In a 30% food restriction model, which prevents puberty (measured by vaginal opening) in peripubertal rats, central injection of Kisspeptin-10 induced puberty in 60% of the animals (Castellano et al. 2005). Interestingly, leptin deficient ob/ob mice have decreased expression of *Kiss1* in the ARC which was increased by intraperitoneal injections of leptin over a period of four days (Smith et al. 2006a). Similar data have been found by Luque et al. (2007) where *Kiss1* and *Gpr54* mRNA levels increased in the whole hypothalamus in ob/ob mice when responses to reduced food intake were taken into account (Luque et al. 2007). These data may represent a direct action of leptin on *Kiss1* neurons as around 40% of *Kiss1* neurons expressed the leptin receptor (Smith et al. 2006a). Neuropeptide Y (NPY) null mice have reduced *Kiss1* expression in the hypothalamus which can be increased by NPY administration (Luque et al. 2007). It will be informative to relate these changes in *Kiss1* expression in the whole hypothalamus to specific changes in *Kiss1* in the AVPV and ARC regions. Nevertheless, these experiments demonstrate an interaction between energy status and the hypothalamic *Kiss1* system.

Kisspeptins may be involved in other metabolic disorders that affect reproductive function. Castellano and colleagues (2006) have studied the role of *Kiss1* in a rat model of type I diabetes that mimics the hypogonadotropic hypogonadism often found in uncontrolled diabetes (Castellano et al. 2006c). In the rat model, the alkylating reagent streptozotocin (STZ) is given to animals which selectively destroys the insulin producing β cells of the pancreas. STZ diabetic rats show a reduction in body weight, very low leptin levels, and defects in gonadotropin release and fertility (Steger et al. 1989) but retain GnRH production in the hypothalamus (Steger et al. 1989) and pituitary gonadotroph responses suggesting a defect upstream of GnRH secretion (Dong et al. 1991). STZ diabetic male rats have reduced *Kiss1* transcripts in the hypothalamus that increase after continuous intracerebral delivery of leptin but not insulin. These diabetic rats retain LH and testosterone secretory responses to ICV injection of Kisspeptin-10. Thus, the hypogonadotropic hypogonadism in this diabetic rat model may be caused by a leptin-related reduction in *Kiss1* expression reducing stimulation of the HPG axis (Castellano et al. 2006c).

Kisspeptins may also act at sites outside the neuroendocrine system. *Gpr54* expression has been detected by RT-PCR in cell lines of both pancreatic α and β-cell origin, while *Kiss1* expression was confined to the latter (Hauge-Evans et al. 2006). In the same study, kisspeptin and GPR54 were localized by immunohistochemistry to α and β cells of mouse pancreatic islets although

the specificity of the antibodies used should be considered. It was also shown that exogenous kisspeptin can enhance glucose-induced insulin secretion, but not glucagon secretion, from purified human or mouse islets while kisspeptin alone had no effect. Co-expression of kisspeptins and GPR54 in islets would allow paracrine or autocrine interactions but how these affect islet responses to glucose metabolism in vivo remains to be determined.

6.3 Ovary/Testes

Gpr54 and *Kiss1* are both expressed in the gonads but it is not known if this has a physiological role in spermatogenesis or ovary function. The cell types that express *Gpr54* in the testes have not been identified. Preliminary examination of heterozygous mice for β-galactosidase expression from the targeted *Gpr54* locus suggests that expression is confined to within seminiferous tubules but we have also detected *Gpr54* expression by RT-PCR in the Leydig cell line, MA-10 (unpublished). A direct action of kisspeptins on the testes is suggested in some studies by the inconsistent relationship between LH and testosterone release after systemic kisspeptin delivery. For example, Thompson et al. (2006) found that subcutaneous delivery of 50 nmol of Kisspeptin-14 in rats produced a very small increase in LH but gave a significant increase in total testosterone levels (Thompson et al. 2006). Similarly, continuous intravenous administration of Kisspeptin-10 in adult male rhesus monkeys gave more testosterone release when normalized to LH responses at higher kisspeptin doses (Ramaswamy et al. 2007). Thus, kisspeptins may enhance the effects of LH on stimulating testosterone production from the testes.

Kiss1 and *Gpr54* mRNAs are expressed in the rat ovary, and *Kiss1* shows a variation in expression during the estrus cycle with a 5-fold increase at proestrus (Castellano et al. 2006a). This increase in *Kiss1* expression correlated with the preovulatory LH surge as blockade of the LH surge prevented this increase which could be restored by injection of LH. Kisspeptin immunoreactivity was detected in the thecal layers of the growing follicles and in the corpora lutea, particularly the areas derived from the invading thecal cells. Thus, it is possible that *Kiss1* expression in the ovary might act as a local regulator of ovulation but is not essential for this as *Gpr54* null mice and a female patient homozygous for a GPR54 mutation (L148S) retain the ability to ovulate (Seminara et al. 2003; Pallais et al. 2006).

6.4 Placenta

Kiss1 is highly expressed by the human placenta and the level of kisspeptins increases at the 8th week of pregnancy from around 1 fmol/ml to more than 2000 fmol/ml for the remainder of the pregnancy (Horikoshi et al. 2003).

The significance of this dramatic rise is not clear, but this level of circulating kisspeptins would be expected to cause agonist suppression of GnRH release from the hypothalamus and shut down of the HPG axis as shown in rats (Thompson et al. 2006) and rhesus monkeys (Seminara et al. 2006). In the human placenta, kisspeptins are expressed only by syncytiotrophoblast cells (Bilban et al. 2004; Dhar et al. 2004; Horikoshi et al. 2003), which represent the cellular interface between the placenta and the maternal blood. In contrast, GPR54 expression is more extensive, being found in syncytiotrophoblast, villous and extravillous cytotrophoblast cells (Bilban et al. 2004). Expression of GPR54 by the highly invasive extravillous cytotrophoblast cells and the anti-metastatic activity of kisspeptins has led to the suggestion that these proteins may control placental invasion but the birth of *Gpr54* knock out mice and patients with homozygous mutations in GPR54 indicates that placentation can take place in the absence of fetal GPR54. Moreover, a female patient with a homozygous mutation in GPR54 (L148S) is reported to have conceived after hormonal treatment and given birth to a healthy child indicating that placentation can also take place in the absence of a functional maternal GPR54 (Pallais et al. 2006). What has not yet been established however, is the effect of loss of GPR54 from both the fetal and maternal placental compartments.

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Neuropeptide S: Anatomy, Pharmacology, Genetics and Physiological Functions

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Abstract Neuropeptide S (NPS) is one of the most recent examples of a neurotransmitter identified by the orphan receptor strategy. Impressive progress has been made in the short time since its identification to determine physiological functions modulated by NPS. The anatomical distribution of NPS and its receptor, NPSR, suggests possible functions in the regulation of vigilance states and modulation of emotional behaviors. Early studies provided evidence that NPS induces behavioral arousal and promotes wakefulness by suppressing all stages of sleep. NPS was also found to produce anxiolytic-like effects in behavioral paradigms that measure fear or responses to novelty. Recent studies have demonstrated that NPS can modulate energy and endocrine homeostasis. Differential regulation of NPS and NPSR transcripts was observed after caffeine or nicotine treatment, indicating complex interactions with adenosine and cholinergic systems. NPS has been found co-localized with other excitatory transmitters such as glutamate, acetylcholine, or corticotropine-releasing factor. Activation of NPSR triggers mobilization of intracellular Ca^{2+} and stimulation of cAMP synthesis, therefore increasing cellular excitability. A functional polymorphism in NPSR has been identified that produces a gain-of-function phenotype by increasing agonist potency up to tenfold. Finally, a gender-specific association of this NPSR polymorphism with panic disorder was found in male patients, indicating that the NPS system might be involved in modulating anxiety responses in humans. Further studies about interactions of the NPS system with other transmitter systems might help to discover additional functions of NPS and define its role within complex neural networks.

Keywords Anxiety · Arousal · Asthma · Feeding · Panic disorder · Sleep · Stress

Abbreviations

CRF	Corticotropin-releasing factor
GPCR	G protein-coupled receptor
HPA axis	Hypothalamus–pituitary–adrenal axis
NPS	Neuropeptide S
NPSR	neuropeptide S receptor
SNP	Single-nucleotide polymorphism

1 Identification and Structure of NPS

Using the orphan receptor strategy, Neuropeptide S (NPS) was identified as the endogenous ligand of the orphan G protein-coupled receptor GPR154

(Sato et al. 2002). NPS is a peptide of 20 amino acids, encoded by a relatively short precursor protein (only 89 amino acids in human). Comparing the primary structures of NPS from various species, it became evident that serine (single amino acid code "S") is always found as the amino terminal residue in all species (Fig. 1) and therefore this structural feature was used to name the peptide accordingly. The NPS precursor protein displays the typical structural characteristics of other neuropeptide precursors with a hydrophobic signal peptide at the amino terminus of the protein and a pair of basic amino acids immediately preceding the immature peptide sequence (Xu et al. 2004; Reinscheid and Xu 2005; Reinscheid 2007). The primary structure of NPS itself shows no homology to any other known neuropeptide. DNA sequences

Sequence	Species	Group
SFRNGVGTGMKKTSFQRAKS	Human	
SFRNGVGTGMKKTSFRRAKS	Chimpanzee	*Primates*
SFRNGVGTGMKKTSFRRAKS	Orang-Utan	
SFRNGVGTGMKKTSFQRAKS	African Elephant	
SFRNGVGTGMKKTSFRRAKS	Cow	
SFRNGVGTGMKKTSFQRAKS	Horse	
SFRNGVGTGMKKTSFRRAKP	Pig	
SFRNGVGTGMKKTSFRRAKS	Dog	*Other*
SFRNGVGTGMRNTSFQRAKS	Dolphin	*Mammals*
SFRNGVGTGMKKTSFRRAKL	Rabbit	
SFRNGVGTGMKKTSFRRAKP	Pika	
SFRNGVGQGIKKTSFRRAKS	Platypus	
SFRNGVGTGLKKTPFPRAKS	Bat	
SFRNGVGTGMKKTSFPRAKP	Guinea Pig	
SFRNGVGSGAKKTSFRRAKQ	Mouse	*Rodents*
SFRNGVGSGVKKTSFRRAKQ	Rat	
SFRNGVGTGMKKTSFRRAKR	Kangaroo Rat	
SFRNGVGTGIKKTSFRRAKS	Wallaby	*Marsupials*
SFRNGVGSGMKKTSFRRAKS	Opossum	
SFRNGVGSGIKKTSFRRAKS	Chicken	*Birds*
SFRNGVGAGIKKTSFRRAKP	Zebra Finch	
SFRNGVGSGMKKTSFRRARL	Green Anole	*Reptiles*
SFRNGVGSGIKKNSFRRAKL	Xenopus	*Amphibians*

Fig. 1 Alignment of NPS peptide sequences deduced from cDNA and genomic DNA sources of representative tetrapods (Reinscheid 2007). The seven amino terminal residues of NPS are perfectly conserved across all species. Amino acid residues different from the human NPS sequence are *shaded*

encoding putative NPS precursor proteins are highly conserved among vertebrates, but are absent from fish or invertebrate genomes (Reinscheid 2007). This indicates that the NPS gene is a relatively recent neuropeptide gene in vertebrate evolution and it is thus far the only example of a neuropeptide that specifically occurs in tetrapods. This peculiar evolutionary distribution suggests that NPS might serve specialized physiological functions in tetrapod vertebrates. Fish might either lack these functions or use alternative transmitter systems. The NPS receptor (NPSR) is a typical G protein-coupled receptor with moderate homology to other peptide receptors. The two most closely related sequences are the V1a and V2 vasopressin receptors, albeit with only 21–23% amino acid identity. NPSR was first identified as the orphan receptor GPR154 and is also known as vasopressin receptor-related receptor 1 (VRR1) (Gupte et al. 2004) or G protein-coupled receptor for asthma susceptibility (GPRA) (Laitinen et al. 2004).

2 Anatomy and Neurochemistry of the NPS System

Distribution of NPS and NPSR gene expression was mapped in detail by in-situ hybridization (Xu et al. 2004, 2007). In the rat brain, expression of NPS precursor is remarkably restricted to only three brainstem structures. A few scattered NPS-expressing cells are found in amygdala and hypothalamus. Among the brainstem structures, a prominent cluster of NPS-expressing cells was found in close proximity to the noradrenergic locus coeruleus (LC). This group of cells in the LC area is also distinct from the neighboring Barrington's nucleus that expresses corticotropin-releasing factor (CRF) as a marker. The NPS-expressing neurons therefore define a previously uncharacterized nucleus in the pericoerulear region. NPS precursor is also expressed in the lateral parabrachial nucleus and the sensory principle 5 nucleus (Pr5) of the rat brainstem.

Analysis of neurochemical markers revealed that NPS appears to be co-localized with other excitatory transmitters (Xu et al. 2007). In the LC area, the majority of NPS-expressing neurons co-express vesicular glutamate transporter mRNA and are thus glutamatergic neurons. In addition, co-expression of choline acetyltransferase was detected in a few cells in this structure. In the lateral parabrachial nucleus, most NPS-producing cells co-express CRF while all NPS-synthesizing neurons in Pr5 are glutamatergic neurons. NPS was never co-localized with markers for GABAergic, noradrenergic, or dopaminergic neurons. NPS precursor mRNA expression appears to be very restricted, with probably less than 200 cells in the rat brain synthesizing NPS.

The restricted distribution pattern of NPS precursor is in contrast to a much broader presence of NPS receptor mRNA throughout the brain. Highest expression of NPSR transcripts is found in the cortex, olfactory nuclei,

thalamus, hypothalamus, amygdala, and parahippocampal formation, such as the subiculum. Only low levels of NPSR expression are detected in the brainstem and no NPSR transcripts were found in NPS precursor-expressing cells (Xu et al. 2007). The pattern of NPSR expression in the central nervous system suggests possible functions in emotional and sensory processing, arousal, stress, energy homeostasis, endocrine regulation, or learning and memory. NPS and NPSR transcripts were also found in peripheral tissues, including thyroid, salivary, and mammary glands, which might indicate additional endocrine functions (Xu et al. 2004), but so far no detailed analysis of these transcripts in peripheral tissues has been reported.

3 Pharmacology and Genetics of NPSR

Human and mouse NPS receptors were studied in heterologous expression systems, showing that NPS induces mobilization of Ca^{2+} and stimulates synthesis of cAMP at EC_{50} values of 4–10 nM. These results imply that NPSR couples to both G_q and G_s proteins and might increase cellular excitability (Xu et al. 2004; Reinscheid et al. 2005). NPSR displays high-affinity saturable and displaceable binding in the subnanomolar range using a radioiodinated NPS analog. High affinity binding and receptor activation in the low nanomolar range are typical hallmarks for neuropeptides and their receptors. Structure–activity relationship studies of NPS have revealed the importance of amino terminal residues for receptor activation, while carboxy-terminal deletions of the last eight amino acids only gradually affect agonist activity (Reinscheid et al. 2005). Alanine- and D-amino acid scanning analogs of NPS demonstrated that residues 2, 3, 4, 6, and 7 contribute critically to NPS agonist activity (Roth et al. 2006; Bernier et al. 2006). These results nicely complement the phylogenetic data because the first seven amino acids of NPS are perfectly conserved across all species analyzed so far (Fig. 1; Reinscheid 2007).

Multiple single-nucleotide polymorphisms (SNPs) and several splice variants have been identified in the human NPSR gene that is located on chromosome 7p14-15. Genetic linkage studies suggested that some SNPs were associated with an increased risk of developing asthma or other allergic diseases characterized by high serum IgE levels (Laitinen et al. 2004). The association study did not provide physiological evidence for any of the NPSR variants. Therefore, we analyzed pharmacological features of two NPSR variants: (i) a SNP that encodes an amino acid change ($Asn^{107}Ile$) in the first extracellular loop of the receptor protein (SNP591694 A>T; ref SNP ID: rs324981), and (ii) a C-terminal splice variant of the receptor that was reportedly overexpressed in human asthmatic airway tissue (Laitinen et al. 2004). We found that the $Asn^{107}Ile$ polymorphism results in a gain-of-function characterized by a five- to tenfold increase in agonist potency at NPSR Ile^{107} compared to

NPSR Asn^{107}. The C-terminal splice variant of NPSR, however, did not appear to cause measurable differences in the pharmacological profile of the receptor (Reinscheid et al. 2005; Bernier et al. 2006).

The pharmacological data demonstrate that the coding polymorphism at Asn^{107}Ile in the NPSR gene causes significantly altered pharmacology of the encoded receptor and might therefore suggest phenotypical changes that could be associated with inheritable disorders. Genetic linkage of the NPSR gene locus with asthma and atopy has been replicated so far in several independent cohorts (Kormann et al. 2005; Melen et al. 2005; Feng et al. 2006; Malerba et al. 2007), although two other studies of asthma patients failed to confirm linkage on chromosome 7p (Immervoll et al. 2001; Shin et al. 2004). In addition, two studies in Northern European individuals found no association of the NPSR risk haplotypes with atopic dermatitis, a skin disorder that is characterized by high serum IgE levels (Söderhäll et al. 2005; Veal et al. 2005). A potential role of NPSR in the pathophysiology of asthma was further questioned by a study using NPSR knockout mice that found no evidence for NPSR function in normal airway physiology or asthma pathophysiology in mice (Allen et al. 2006). In the original paper, Laitinen et al. (2004) reported upregulation of NPSR mRNA in bronchial tissue of human asthma patients and in a mouse model of airway inflammation. However, recent studies could not replicate the presence of NPSR mRNA in either normal or inflamed mouse airway tissue (Allen et al. 2006). The same study also found no evidence for NPSR mRNA expression in human airway tissue. Our own unpublished data confirm the findings of Allen et al. At present, the functional role of NPSR in asthma is therefore unclear. It remains to be seen whether the NPSR chromosomal region contains other thus-far unrecognized genes or regulatory elements that could be involved in asthma or other allergic diseases. Indeed, the original report identified an alternative transcript of unknown function encoded by the complementary DNA strand (Laitinen et al. 2004). Since functional evidence for an involvement of NPSR in airway function has not been found, alternative genes or genetic mechanisms need to be considered to account for the genetic linkage data, indicating that this chromosomal region might confer asthma susceptibility.

Based on the spectrum of behavioral effects produced by NPS (see below), we investigated possible associations of the functional Asn^{107}Ile polymorphism in NPSR with disorders affecting arousal, attention, or anxiety. Analysis of DNA samples from patients diagnosed with schizophrenia, attention-deficit/hyperactivity disorder, or panic disorder revealed a gender-specific association of NPSR genotypes with panic disorder in male patients (Okamura et al. 2007). Homozygous NPSR Asn^{107} carriers were found to be significantly under-represented among male panic disorder patients, indicating that this allele might have protective effects. Female panic disorder patients, as well as the schizophrenia cohort, showed a distribution of NPSR alleles that were indistinguishable from healthy controls. No preferred transmission of specific

NPSR alleles was detected in families with children diagnosed with attention-deficit/hyperactivity disorder. Interestingly, the chromosomal region 7p14-15 had been linked to panic disorder before in two independent studies using genome-wide linkage analysis (Knowles et al. 1998; Crowe et al. 2001). Together with the earlier findings these data suggest that NPSR might therefore be a candidate gene for panic disorder susceptibility. Replication of these findings in independent cohorts will, of course, be necessary to confirm this hypothesis.

4 Modulation of Arousal and Wakefulness by NPS

The regional distribution of NPS and NPSR expression in the brain suggested that activation of the NPS system might influence behavioral arousal and possibly modulate sleep–wakefulness cycles. Studies in our laboratory demonstrated that central administration of NPS produces arousal independent of novelty (Xu et al. 2004). Mice injected with low doses of NPS display

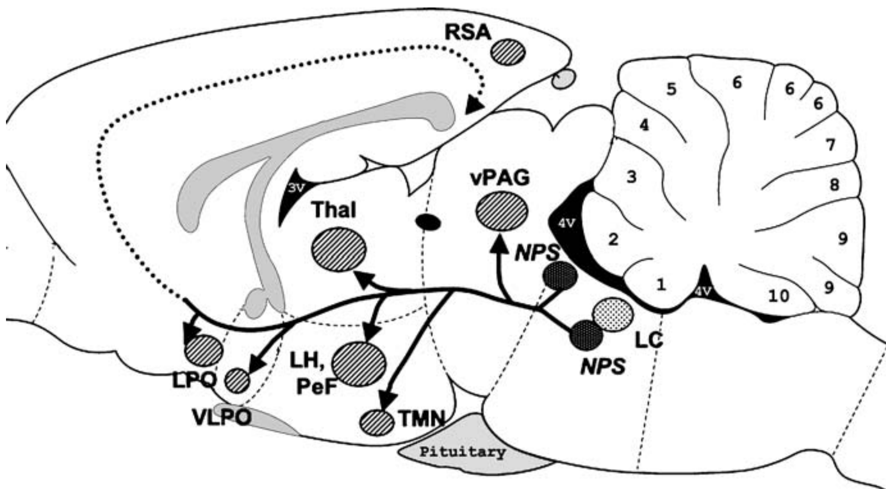

Fig. 2 Schematic drawing of a sagittal section of the rat brain showing possible connections of NPS synthesizing nuclei (*NPS*, *dark shading*) in the brainstem with arousal centers in the brain that express NPS receptor mRNA (*diagonal shading*). The noradrenergic locus coeruleus (*LC*) is depicted for orientation. The *dotted line* represents hypothetical projections of NPS producing neurons to cortical structures, such as the retrosplenial agranular cortex (*RSA*). Cerebral ventricles are shown in *black* and major fiber tracts are *shaded in grey*. Other abbreviations: *LH* lateral hypothalamic area, *LPO* lateral preoptic area, *PeF* perifornical nucleus, *Thal* thalamus, *TMN* tuberomammillary nucleus, *VLPO* ventrolateral preoptic nucleus, *vPAG* ventral part of periaqueductal gray, *3V* third ventricle, *4V* fourth ventricle. The drawing was adapted according to the rat brain atlas of Paxinos and Watson (1997)

increased horizontal and vertical activities for up to 60 min after central administration of 1 nmol NPS. Electroencephalographic (EEG) recordings in rats revealed that NPS produces cortical activation, indicative of enhanced wakefulness. NPS was found to suppress all stages of sleep for up to 1 h when EEG was recorded during the normal time of inactivity in rats (Xu et al. 2004). The NPS-induced state of forced wakefulness was followed later by a rebound in slow wave sleep and REM sleep. These observations suggest an important role of NPS in the modulation of sleep–wakefulness.

A possible anatomical substrate for NPS-induced arousal might be the thalamic midline nuclei that express high levels of NPSR mRNA and have a well-established role as integrators of arousal between the brainstem reticular formation and the cortex (Jones 2003; van der Werf et al. 2002). NPS receptors are ideally located in these thalamic relay nuclei to modulate arousal signals, but are also found in a number of additional brain centers that have been associated with arousal and sleep–wakefulness regulation (Fig. 2). Electrophysiological recordings will be necessary to further examine the functional role of NPSR in these thalamic structures and their contribution to behavioral arousal.

5 Anxiolytic-Like Effects of NPS

Strong NPSR mRNA expression in amygdala, the dorsal endopiriform nucleus, and various hypothalamic nuclei suggest that the NPS system might influence emotional behaviors such as stress or anxiety responses. Behavioral studies using validated paradigms to measure anxiety, including open field, light–dark box and elevated plus maze, demonstrated that central administration of NPS produces anxiolytic-like effects (Xu et al. 2004). These tests are based on the natural aversion of rodents for open or unfamiliar spaces and anxiolytic drugs increase exploration of these exposed areas. Therefore, these tests are also sensitive to confounding interference from agents that increase locomotor activity. The marble-burying test was used to control for such potentially confounding effects of NPS. In this paradigm, anxiolytic drugs reduce a naturally defensive behavior, i.e., burying of marbles in bedding material. Central administration of NPS reduced burying of marbles in a dose-dependent pattern, indicating that NPS produces potent anxiolytic-like effects in addition to its arousing effects (Xu et al. 2004). The anxiolytic-like properties of NPS have been confirmed independently in the four-plate test (Leonard et al. 2005). Together, these data show that NPS appears to produce robust anxiolytic effects across five different tests of anxiety-like behavior and might therefore be involved in the modulation of emotional responses to stress, such as fear and anxiety. The observation that specific NPSR alleles appear to be associated with panic disorder, which is considered a spe-

cific form of anxiety disorder, adds further notion to the hypothesis that the NPS system might also modulate emotional behaviors in humans (Okamura et al. 2007).

6 Modulation of Feeding Behavior by NPS

Studies from two independent groups indicate that NPS can transiently inhibit food intake in a dose-dependent manner (Beck et al. 2005; Smith et al. 2006). However, another paper challenged this hypothesis recently and reported orexigenic effects after NPS administration (Niimi 2006). Interpretation of these conflicting results is difficult, but should not hinder further studies in this direction. The NPS doses that were found to produce anorectic effects in the study by Beck et al. (2005) also promote significant hyperlocomotion. They reported that NPS injected intracerebroventricularly (ICV) at 1 and 10 μg (corresponding to 0.45 and 4.5 nmol NPS, respectively), attenuated food intake in fasted rats during the first hour, but cumulative 24-h food intake was not affected (Beck et al. 2005). In contrast, Smith et al. (2006) found no effect on food intake in fasted rats after ICV administration of NPS doses up to 10 nmol. According to their study, only local injections of NPS (0.1–1 nmol) into the paraventricular hypothalamic nucleus (PVN) attenuated food intake in fasted rats during the first hour. They also reported that intra-PVN administration of NPS did not increase locomotion but instead promoted rearing behavior accompanied by decreased grooming activity. From these data it is difficult to conclude whether NPS has a direct anorectic effect or whether the suppression of food intake occurs secondary to the behavioral arousal induced by the peptide.

Anatomical substrates for NPS-mediated effects on feeding behavior might be the arcuate nucleus or lateral hypothalamic areas that express high levels of NPS receptors (Xu et al. 2007). Both brain structures have well-established roles in feeding and satiety circuits (Vettor et al. 2002). Data presented in the study by Niimi (2006) indicate NPS-mediated activation of c-fos expression in orexin/hypocretin-positive neurons of the lateral hypothalamus. Although a direct activation of these neurons by NPS still needs to be established, the observation might indicate a functional link between the two systems. The orexin/hypocretin system is critically important for the maintenance of awake states (Chemelli et al. 1999; Lin et al. 1999) and has also been implicated in the coordination of arousal with food intake (Saper 2006). Under certain conditions, orexin/hypocretin can produce orexigenic effects (Sakurai 2006) and it has been shown that orexin/hypocretin-synthesizing neurons are activated by ghrelin but inhibited by glucose and leptin (Yamanaka et al. 2003). Interaction between NPS and orexin/hypocretin systems might also explain a functional link between the arousal effects that have been reported for both systems.

Detailed anatomical and physiological studies are required to explore this hypothesis.

7
The NPS System as Part of Neural Networks

The expression of NPSR transcripts in various parts of the hypothalamus indicates that NPS might also influence endocrine functions. Indeed, it was found that central administration of nanomolar doses of NPS can modulate the hypothalamus–pituitary–adrenal (HPA) axis (Smith et al. 2006). NPS increased plasma levels of corticosterone and adrenocorticotropine (ACTH) after central administration in rats in a time- and dose-dependent manner. NPS was also shown to stimulate release of CRF and vasopressin, but not neuropeptide Y, from hypothalamic explants, indicating that NPS might have a direct effect on these systems. Since elevated plasma corticosterone and ACTH levels or release of CRF and vasopressin are commonly associated with increased levels of stress, these observations are in apparent contrast with the anxiolytic-like behavioral effects produced by NPS. It is, however, possible that increased physical activity and elevated behavioral arousal produced by NPS administration could subsequently trigger HPA activation and might thus not be related to the primary effects of NPSR activation. On the other hand, data showing NPS-stimulated CRF release from hypothalamic slices indicate a rather direct effect on the HPA system. Further experiments to investigate interactions of the NPS system with the anatomical and neurochemical components of the HPA axis in the context of its behavioral effects are obviously needed.

Two recent reports demonstrated regulation of NPS precursor and NPS receptor mRNA expression by caffeine or nicotine treatment, respectively (Lage et al. 2006, 2007). Two hours after treatment with a single dose of the adenosine A_1 antagonist caffeine, a decrease of NPS precursor transcripts in the brainstem was detected while no change was observed after chronic caffeine treatment for 48 h. Conversely, no acute effect on hypothalamic NPSR mRNA levels was observed after caffeine treatment with a single dose while chronic treatment for 48 h induced NPSR expression in the hypothalamus. These observations indicate that adenosine neurotransmission might provide differential input to the NPS system, depending on the duration of the stimulus and the location of its anatomical substrate. Caffeine has well-known stimulating and wakefulness-enhancing effects and is also used clinically to provoke panic attacks in susceptible patients (Bourin et al. 1995). It is therefore intriguing to hypothesize that caffeine might exert at least part of its stimulating effects by influencing gene expression of NPS and NPSR.

Nicotine is another exogenous substance with well-known effects on arousal, wakefulness, and anxiety in both humans and animal models. In add-

ition, nicotine administration is known to reduce food intake. The parallels between the behavioral effects of nicotine and NPS had been outlined already in an editorial that accompanied the original report on NPS (Koob and Greenwell 2004). A recent study by Lage et al. (2007) found that acute nicotine treatment only increased NPSR mRNA levels in the brainstem but did not affect NPS precursor mRNA expression. In contrast, chronic nicotine treatment for 48 h increased both NPS and NPSR transcript levels in the brainstem and also induced increased NPSR expression in the hypothalamus. Although the study did not address mechanistic questions of direct versus indirect interactions between the two systems, these data suggest that nicotine might produce at least some of its effects on wakefulness, emotional behavior, and feeding by regulating components of the NPS system. Further anatomical and functional studies of these proposed interactions are certainly necessary to substantiate the two hypotheses.

Studies on the neurochemical properties of NPS-synthesizing neurons have shown that NPS is abundantly co-localized with other excitatory transmitters such as glutamate, acetylcholine, and CRF (Xu et al. 2007). Based on contemporary models of neural function, the data indicate that NPS might be co-released together with these excitatory transmitters and could exert modulatory or cooperative functions at the postsynaptic level. For example, ionotropic glutamate receptors have been shown to interact with GPCRs with profound consequences for postsynaptic responses (Lin et al. 2001; Lee et al. 2002). Electrophysiological and biochemical studies may help to investigate such a role for NPSR.

8 Conclusions

Due to its novelty, characterization of the NPS system and its physiological functions is still in its infancy. The many brain structures that express NPSR transcripts suggest that additional functions will be discovered. In addition, genetic mouse models that have been targeted for either over-expression or absence of NPS or NPSR, respectively, will certainly become useful tools to study this system further. For pharmacological studies, the availability of selective small molecule agonists and antagonists will be critically important.

Substantial progress has already been made to establish NPS as a potent modulator of arousal, sleep–wakefulness, and anxiety behaviors. New results are pointing at additional roles for NPS in energy homeostasis and endocrine regulation. The combination of arousal-enhancing and anxiolytic effects describes a unique pharmacological spectrum for NPS: Psychostimulants such as amphetamine or cocaine are known to produce anxiogenic responses in the behavioral anxiety models (Hascoet and Bourin 1998; Paine et al. 2002) whereas traditional anxiolytic drugs such as benzodiazepines induce sedation

and hypolocomotion (Haefely 1989; Chaouloff et al. 1997). The pharmacological spectrum of NPS also appears to be different from other endogenous neurotransmitters or neuropeptides, where arousal appears to be generally associated with increased anxiety (Okamura and Reinscheid 2007). The similarity between NPS and nicotine with respect to their effects on arousal, sleep, anxiety, and feeding deserves further attention and might yield clues about functional interactions at the systems level. Finally, these early studies imply that the NPS system might be an interesting candidate for development of new therapeutics to target sleep, anxiety, and metabolic disorders.

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The Melanin-Concentrating Hormone System and Its Physiological Functions

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Abstract Melanin-concentrating hormone (MCH) is a neuropeptide that was originally isolated from salmon pituitary where it causes pigment aggregation. MCH is also abundantly present in mammalian neurons and expressed in the lateral hypothalamus and zona incerta, brain regions that are known to be at the center of feeding behavior. MCH binds to and activates two G protein-coupled receptors, MCH1R and MCH2R. Although MCH2R is non-functional in rodents, genetic and pharmacological studies have demonstrated that rodent MCH1R is involved in the regulation of feeding behavior and energy balance. Unexpectedly, some antagonists have provided evidence that MCH signaling participates in the regulation of other processes, such as emotion and stress. The discovery of MCH receptors has extensively promoted the progress of MCH studies and may represent an ideal example of how deorphanized receptors can open new directions toward more detailed physiological studies.

Abbreviations

AGRP	Agouti-related peptide
NPY	Neuropeptide Y
POMC	Pro-opiomelanocortin
CART	Cocaine-amphetamine-regulated transcript

1 Introduction: MCH from Fish Scales

Melanin-concentrating hormone (MCH) was originally isolated from salmon pituitaries where it induces the aggregation of melanin granules in melanophores, thereby resulting in a pale skin color (Kawauchi et al. 1983). This effect is opposite to the pigment-dispersing effects of alpha-melanotropin (α-MSH) found in lower vertebrates. Similar to α-MSH, MCH was found to be a neurohypophysial hormone produced by neurons in the hypothalamus and released from the neurohypophysis of teleosts as a circulating factor. Fish MCH is a cyclic 17-amino acid peptide with a dicysteine bridge at positions 5 and 14 forming a ring structure. Rat MCH was purified from 60 000

hypothalamic fragments using antibodies directed against salmon MCH, and its primary structure was determined (Vaughan et al. 1989). Rat MCH consists of 19 amino acids, and is therefore two amino acids longer than its salmon counterpart (Fig. 1A). The ring structure, which is essential for the biological function in teleost fish, is highly conserved in rat MCH. Furthermore, MCH is identical at the amino acid level in all mammals analyzed to date, including mice, rats, rabbits, and humans. Rat MCH is prominently expressed in neurons in the lateral hypothalamus (LHA) and zona incerta, brain regions that are known to be involved in feeding behavior. Unlike teleost MCH-expressing neurons, mammalian MCH-expressing neurons do not extend abundantly to the neurohypophysis but project broadly throughout the central nervous system (CNS) from the olfactory bulb to the spinal cord (Bittencourt et al. 1992). This extensive terminal distribution suggests that the peptide may be involved in many brain functions by acting as a neurotransmitter/neuromodulator. The most active area of research on the MCH system has focused on its role in the regulation of food intake and energy homeostasis, while the characterization of MCH receptors and identification of small-molecule antagonists for MCH receptors has exclusively enhanced our

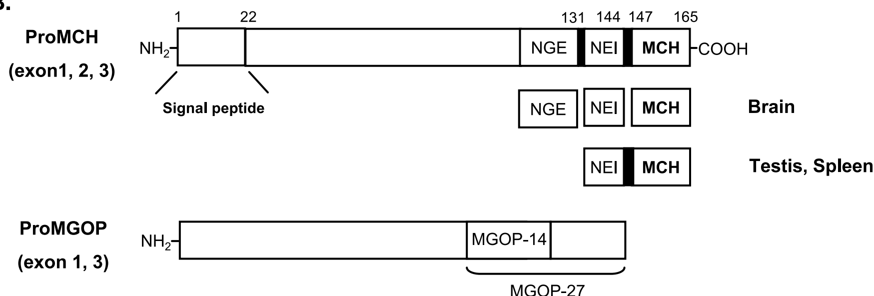

Fig. 1 Structure of MCH and its related peptides. **A** Alignment of rat, mouse and human MCH peptides with fish MCH. Fish MCH lacks two amino acids at the N-terminus compared to mammalian MCH. **B** Schematic diagram of the possible peptides derived from the MCH gene detected either in the brain or in other organs. The prepro-MCH precursor is composed of three exons and includes additional peptide sequences designated NGE and NEI (*above*). An alternative splicing variant, which contains exons I and II, encodes two putative peptides designated MGOP-14 and -27 (*below*). In the rat and human brain, mature peptides, cyclic MCH and amidated NEI, were found, while in mature MCH and NEI were not found in peripheral organs but a large MCH-immunoreactive form was identified in human and mouse

understanding of its pharmacology and physiology. This review will introduce recent data concerning genetic and physiological studies that have revealed how MCH and its receptors are involved in the regulation of signaling and various biological functions.

2
MCH Gene and Its Primary Functions in Mammals

A rodent MCH cDNA was first identified in 1989 (Nahon et al. 1989), and revealed that MCH is generated by cleavage at a dibasic amino acid site in the C-terminus of a 165-amino acid precursor. Subsequently, the rat, mouse, and human MCH mRNA sequences were found to show high degrees of homology, with 90% overall nucleotide identity. Analysis of rat mRNA indicated that the MCH transcript encodes a preprohormone containing other neuropeptides, designated neuropeptide E-I (NEI) and neuropeptide G-E (NGE) (Fig. 1B). NEI is indeed present with MCH in hypothalamic neurons, and has been proposed to affect grooming and locomotion (Sanchez et al. 1997) and be involved in regulating stress responses (Blue-Pajot et al. 1995) and suppressing thyrotropin-releasing hormone release (Kennedy et al. 2001). However, it remains unclear whether NGE is liberated from pro-MCH and exists as a functional peptide. Furthermore, an alternative splicing variant of the prepro-MCH mRNA encodes two other potentially bioactive peptides, designated MCH gene-overprinted peptide (MGOP)-14 and -17 (Fig. 1B). No processed MGOP peptides were detected in the rat hypothalamus by Western blot analyses, and MGOP mRNA expression was restricted to MCH-expressing neurons (Toumaniantz et al. 2000; Allaeys et al. 2004). In addition, a large MCH gene-related transcript, designated antisense RNA-overlapping MCH gene (AROM), has been isolated from PC12 rat pheochromocytoma cells (Borsu et al. 2000). AROM appears to be encoded by the opposite strand at the same locus as the MCH gene, and to generate multiple transcripts by alternative splicing. However, the coding sequence of these peptides does not overlap with the MCH cDNA. It is speculated that AROM may be crucial for RNA-binding or protein–protein interaction selectivity.

Humans have two related, but distinct, MCH gene systems involving authentic and variant MCH genes, although only a single MCH gene has been found in rodents. The authentic human MCH gene is mapped on chromosome 12q23, while the variant genes, PMCHL1 and PMCHL2, are localized on chromosomes 5p14 and 5q13, respectively. PMCHL2 does not yield an mRNA, whereas the sense unspliced RNA of PMCHL1 is transcribed in the developing human brain into an 8-kDa putative protein named VMCH-p8 (Viale et al. 2000). Although their putative functions are still puzzling, these human genes offer us the opportunity to examine the molecular mechanisms of gene re-

modelling and selection of functions in the human lineage (Courseaux and Nahon 2001).

MCH has been implicated in the regulation of several behaviors in rodents. Alpha-MSH increases auditory gating by depth recordings in the dorsal hippocampus, whereas MCH has the opposite effect. When MCH was administered prior to α-MSH, the ability of α-MSH to increase auditory gating was blocked (Miller et al. 1993). Regarding grooming, locomotor activity and rearing, MCH did not influence any of these behaviors, and had the opposite effects to α-MSH and NEI (Sanchez et al. 1997). MCH itself has been shown to modulate learning and memory processes. For example, infusion of MCH into the hippocampus, amygdala and entorhinal cortex increased the response latency in a one-trial step-down inhibitory avoidance test in rats (Monzon et al. 1999). Recently, MCH has been implicated in the control of the sleep-wake cycle, since intracerebroventricular (icv) administration of MCH induced a dose-dependent increase in rapid eye movement sleep and slow-wave sleep quantities (Verret et al. 2003). However, the central effect of MCH that has attracted the most attention is its involvement in the regulation of feeding behavior and energy homeostasis in mammals (Fig. 2). A substantial amount of literature involving genetic studies and administration of selective MCH receptor antagonists has been published and reviewed (Pissios and Maratos-Frier 2006; Handlon and Zhou 2006).

Acute icv injections of MCH transiently stimulated food intake in rats (Rossi et al. 1997), while chronic infusion of MCH into the lateral ventricle significantly increased the food intake, body weight, white adipose tissue mass, and liver mass in mice fed a moderately high-fat diet ad libitum (Qu et al. 1996; Della-Zuana et al. 2002; Ito et al. 2003). The observed reduction in brown adipose tissue functions and increased plasma glucose, insulin and leptin levels in these mice indicate that MCH-induced obesity is caused by not only hyperphagia but also regulation of metabolism (Ito et al. 2003). The relevance of the MCH system to the modulation of energy metabolism is also supported by studies on leptin-deficient obese (*ob/ob*) mice. Briefly, RT-PCR differential display analyses revealed that prepro-MCH mRNA was upregulated in *ob/ob* mice (Qu et al. 1996), while MCH mRNA expression was increased by three-fold in fasted *ob/ob* mice compared to four-fold in fasted wild-type mice. Further characterization via genetic approaches indicated the importance of the MCH system as a potential candidate in obesity treatment. MCH-knockout mice revealed an important physical role for MCH (Shimada et al. 1998), since these mice were 24–28% leaner than their control littermates as a result of hypophagia and exhibited a reduction in body fat and low circulating leptin levels. The MCH-knockout mice were resistant to obesity development obesity on a high-fat diet and consumed more oxygen (Kokkotou et al. 2005). In addition, the lean phenotypes of MCH-null mice persisted for up to 90 weeks due to both increased locomotor activity and a higher basal metabolic rate. Furthermore, these mice were resistant to aging-associated

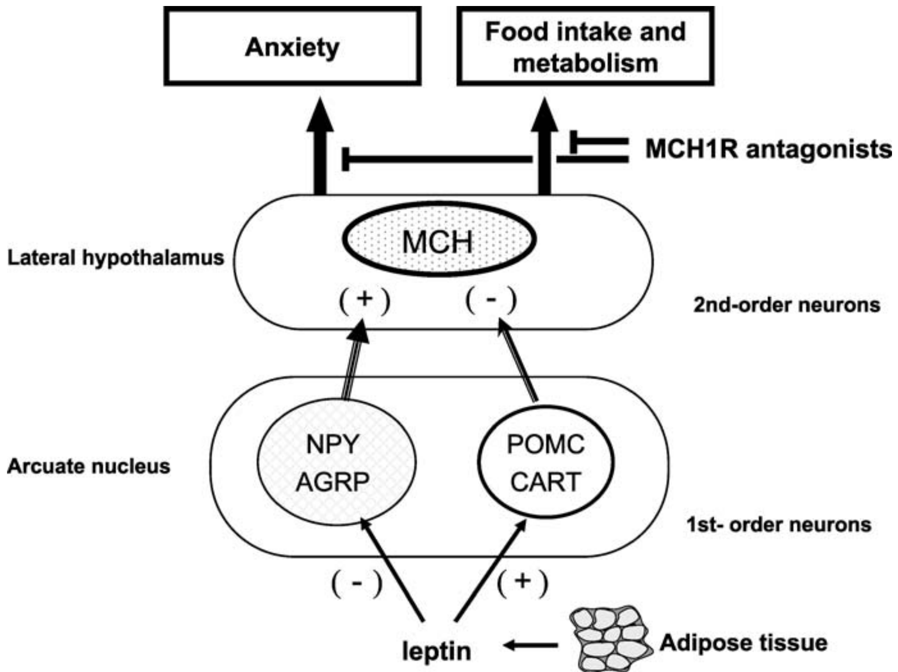

Fig. 2 Schematic representation of MCH circuitry in the hypothalamus. NPY/AGRP and POMC/CART neurons in the arcuate nucleus in the hypothalamus are the first-order neurons that responses to the circulating adiposity signal, leptin. Leptin activates anorectic POMC/CART neurons while inhibits orexigenic NPY/AGRP neurons. These neurons project to the lateral hypothalamus, center of the second-order neurons in the regulation of food intake and energy homeostasis. MCH neurons in the lateral hypothalamus are inhibited by the input from POMC/CART cells, whereas NPY/AGRP neurons exhibit the opposite effect. This anabolic pathway via MCH system can be disrupted by various MCH receptor antagonists. MCH system is also involved in HPA axis, regulating stress and anxiety. The anxiolytic effect of MCH is attenuated by MCH receptor antagonists

glucose intolerance (Jean et al. 2006). Very recently, a toxin-mediated genetic cell ablation strategy using a truncated ataxin-3 has been used to induce apoptosis of MCH-expressing neurons in vivo (Alon and Friedman 2006). MCH/ataxin-3 mice developed a late onset syndrome characterized by leanness, hypophagia and, in males, increased energy expenditure without any obvious changes in the gross histologic appearance of the hypothalamus. These phenotypes are remarkably similar to those of mice with induced mutations of the MCH gene, suggesting that MCH itself is a key molecule that regulates energy balance (Fig. 2).

In contrast to the absence of MCH, overexpression of the MCH gene leads to increased susceptibility to obesity. Transgenic mice overexpressing MCH in the LHA at approximately two-fold higher levels than normal mice were generated. On the original FVB background, the mice were not obese on

a standard diet. However, when the gene was bred to homozygosity, the resulting mice became obese on a high-fat diet. The mice were hyperphagic, hyperleptinemic and had higher blood glucose levels. Furthermore, the mice were also significantly hyperinsulinemic and failed to respond to an insulin challenge (Ludwig et al. 2001).

Leptin treatment can blunt the rapidly induced increases in MCH mRNA in both wild-type and *ob/ob* mice. This implies that the MCH system is targeted by leptin and required for the obesity observed with leptin deficiency. Furthermore, double null animals generated by crossing MCH-knockout mice with *ob/ob* mice revealed attenuated phenotypic manifestations of leptin deficiency (Segal-Lieberman et al. 2003). The marked reduction in weight in these double null mice was secondary to decreased total fat body fat rather than decrease food intake. These mice displayed increased locomotor activity and thermoregulation compared to *ob/ob* mice, but were more hyperphagic than *ob/ob* mice. These observations further indicate that the weight loss induced by the absence of MCH results from increased energy expenditure.

However, pharmacological approaches to MCH research have been hampered due to the lack of suitable selective antagonists for MCH receptors. The discovery of a relevant receptor for MCH in 1999 dramatically changed this situation and offered a new feature for understanding the more diverse physiological roles of MCH (see Sect. 4.2).

3 MCH Receptors and Receptor Signaling

3.1 Discovery of MCH Receptor Through Orphan Receptor Strategies

Since MCH was originally discovered on the basis of its regulation of skin melanocyte aggregation, initial efforts of identify MCH receptor were performed by binding assays using cell lines such as keratinocytes and melanoma cells (Drozdz et al. 1995). Although various cell lines were found to posses a specific binding site for an MCH analogue, [Phe^{13}, Tyr^{19}]-MCH, the pharmacological profiles and signaling associated with this binding rendered the existence of a functional MCH receptor in these cells questionable (Audinot et al. 2002). However, this aspect may deserve further investigation using other approaches (Eberle et al. 2004).

The first MCH receptor was identified by analyzing orphan G-protein coupled receptors (GPCRs), which are cloned GPCRs that recognize undiscovered natural ligands (Civelli et al. 2001). One of the orphan GPCRs, SLC-1, was originally discovered as an expressed sequence tag exhibiting about 40% homology in its hydrophobic domains to the five human somatostatin receptors (Kolakowski et al. 1996). A subsequently identified rat ortholog was

found to share 91% overall sequence identity to the human SLC-1 receptor, and be 49 amino acids shorter in its N-terminal segment (Lakaye et al. 1998). The existence of a shorter form was later reported in humans (Mori et al. 2001). In 1999, five independent groups, including ours, almost simultaneously reported the identity of the cognate ligand of SLC-1 using orphan receptor strategies (Table 1). Three groups used brain extracts as the starting material and monitored SLC-1 activity via three different second messenger responses, namely increases in intracellular-free Ca^{2+} levels with a chimeric Gα protein in transiently transfected CHO cells (Conklin et al. 1983; Saito et al. 1999), cyclic AMP inhibition assays in stable CHO cells (Shimomura et al. 1999) and G protein-gated potassium channels in *Xenopus* oocytes (Bachner et al. 1999). Two other groups screened large libraries of known bioactive substances as potential activators of SLC-1 (Chambers et al. 1999; Lembo et al. 1999), and monitored SLC-1 reactivity by measuring the intracellular-free Ca^{2+} levels. Finally, each group arrived at the same conclusion, namely that the cognate ligand for SLC-1 was the known peptide MCH. Following this deorphanization, the MCH peptide could be studied from the aspect of the MCH-MCH receptor system. The SLC-1 receptor is hereafter referred to as the MCH-1 receptor, MCH1R.

The highest expression of MCH1R is detected in the brain where high levels of its mRNA expression are observed in most anatomical areas implicated in the control of olfaction, such as the olfactory nerve layer, olfactory

Table 1 Characterization of MCH1R. Orphan receptor strategies have been successful in identifying MCH as the cognate ligand for the orphan GPCR SLC-1. SLC-1 is referred to as MCH1R in this review

Cell system	Transfected cDNA	Assay system	Source	Purification steps	Refs. (1999)
HEK (stable)	Human SLC-1	Calcium influx	Compound library (over 500)		Chamber et al.
HEK (stable)	Rat SLC-1	Calcium influx	Compound library		Lembo et al.
CHO (transient)	Rat SLC-1 + Gq/i3 chimera (1 : 1)	Calcium influx	Rat brain extract (whole)	400 g 6 steps	Saito et al.
CHO (stable)	Human SLC-1	Inhibition of cyclic AMP accumulation	Rat brain extract (whole)	70 brains 6 steps	Shimomura et al.
Xenopus oocytes	Rat SLC-1 + GIRK	GIRK-mediated current	Rat brain extract (whole)	67 g 7 steps	Bachner et al.

GIRK: G-protein-gated inwardly rectifying potassium channel

nucleus and tubercle (Hervieu et al. 2000; Saito et al. 2001a). Strong labeling is also detected in the hippocampal formation, subiculum, basolateral amygdala and nucleus accumbens shell, which are substrates for learning, memory, addiction and motivated behavior. Moderate MCH1R mRNA expression is particularly found in regions that are involved in the neuronal circuitry of feeding, such as the arcuate nucleus, ventromedial hypothalamic nucleus and ZI. These localizations imply a role for the MCH system in the integration of taste and olfaction, as well as in positive reward aspects of feeding and satiety (Saito et al. 2001a).

Studies of MCH1R-deficient mice have provided additional evidence that the MCH system is involved in the regulation of metabolism and activity levels. These mice were lean with decreased fat mass and increased energy metabolism (Marsh et al. 2002; Chen et al. 2002). Consistent with their hyperactive phenotype, the mice showed increased resistance to diet-induced obesity. MCH1R-deficient mice were also resistant to the orexigenic actions of MCH, demonstrating that MCH1R is a physiologically relevant MCH receptor. It has been reported that the hyperactivity of MCH1R-deficient mice may be mediated by the mesolimbic dopamine system (Smith et al. 2005). These mice were also hyper-responsive to dopamine stimulation and showed significant upregulation of dopamine D1 and D2 receptors in the nucleus accumbens shell, olfactory tubercle and ventral tegmental area. Since mesolimbic dopamine signaling has been suggested to underlie the reward system stimuli, MCH signaling may have a role in reinforcement in addition to energy homeostasis.

A second high-affinity receptor for MCH was characterized based on its low homology to human MCH1R (Mori et al. 2001; Sailer et al. 2001; Rodriguez et al. 2001). This receptor, referred to as MCH2R in this review, is positively coupled to the Gαq signaling pathway (Sailer et al. 2001; Rodriguez et al. 2001), while MCH1R is coupled to Gαi, Gαo and Gαq. Notably, MCH2R was found to be a pseudogene in rodent species, but is functional in dogs, ferrets, rhesus monkeys, and humans (Tan et al. 2002). The distribution of MCH2R in brain nearly overlaps with that of MCH1R, but the latter shows much higher relative levels and a wider distribution pattern (Mori et al. 2001). MCH2R is expressed in several human brain areas, including the hippocampus and amygdala, although its distribution in the hypothalamus remains controversial. Specifically, it was reported to be mainly expressed in the arcuate nucleus and ventromedial hypothalamic nucleus in African green monkeys by in situ hybridization (Sailer et al. 2001), while three other reports did not detect its expression in the human hypothalamus by RT-PCR (Mori et al. 2001; Hill et al. 2001) or Northern blot analysis (Rodriguez et al. 2001). The functional importance of MCH2R in obesity remains unknown due to the lack of available animal models. Interestingly, three MCH receptor sequences from zebrafish and two receptor sequences from fugu have been identified in whole genome shotgun datasets (Logan et al. 2003). Zebrafish and fugu have

clear MCH1R and MCH2R orthologues. Phylogenetic analyses of these receptors have suggested that an initial duplication of the MCH receptor occurred early in evolution, giving rise to MCH1R and MCH2R. Further characterization of fish MCH receptors may provide further insights into MCH functions in fish, rodents, and humans.

3.2 Characterization of the MCH1R-Signaling Pathway

In MCH1R-overexpressing CHO or HEK293T cells, the receptor was found to couple with various second messenger systems, including elevation of intracellular Ca^{2+} levels, inhibition of forskolin-stimulated cyclic AMP production and activation of extracellular-signal-regulated kinase 1/2 (ERK1/2) (Chambers et al. 1999; Saito et al. 1999; Lembo et al. 1999; Hawes et al. 2000). The observed EC_{50} values for cyclic AMP inhibition and calcium influx suggested that the coupling to $G\alpha i$ was stronger than that to $G\alpha q$ in an exogenous receptor-expression system. A number of mutations have been identified in MCH1R that affect its activity, including its signaling. MCH1R contains three consensus *N*-glycosylation sites and several potential phosphorylation sites in its intracellular loops. Biochemical analyses have shown that an aspartic acid residue (Asp^{123}) in the third transmembrane domain is crucial for ligand binding (MacDonald et al. 2000) and that an asparagine residue (Asn^{23}) in the extracellular N-terminal region is the most important site for *N*-linked glycosylation of MCH1R and cell surface expression (Saito et al. 2003). Thr^{255}, which is located at the junction of intracellular loop 3 and transmembrane domain 6, is also necessary for cell surface expression. A single point mutation, T255A, dramatically reduced the cell surface expression of MCH1R, and resulted in the receptor being retained in the endoplasmic reticulum (Fan et al. 2005). Arg^{155} in the second intracellular loop of MCH1R also has a critical role, since mutation of this basic residue to glutamine or lysine produced 75- and 50-fold higher EC_{50} values for elevation of the intracellular Ca^{2+} levels (Saito et al. 2005). The membrane proximal region of MCH1R is predicted to form an amphiphilic cytoplasmic helix, and two dibasic amino acids (Arg^{319} and Lys^{320}) in this helix are also important for receptor signaling (Tetsuka et al. 2004). On the other hand, the distal portion of the C-tail is necessary for the receptor internalization process (Saito et al. 2004).

The actin- and intermediate filament-binding protein periplakin appears to be coexpressed with MCH1R in the mouse brain and may interact with the intracellular C-terminal of MCH1R to impede MCH1R-initiated signal transduction (Murdoch et al. 2005). Calcium mobilization is inhibited by periplakin, although ERK1/2 phosphorylation is induced normally. Recently, the neurite outgrowth-related factor neurochondrin was identified to interact with the C-terminus of MCH1R (Francker et al. 2006). Neurochondrin interacts with the proximal C-terminus of the receptor and inhibits MCH-

induced signal transduction in a similar manner to periplakin. The physiological significance of these interactions with periplakin and neurochondrin is presently unknown.

Although exogenous receptor-expression cellular systems have provided useful information regarding the function and pharmacology of GPCRs, it is still possible that such systems do not reflect the physiological situation in intact cells. In fact, endogenous MCH1R in human melanoma SK-MEL37 cells and neuroblastoma Kelly cells is associated with a signaling pathway that inhibits forskolin-induced cyclic AMP production and induces ERK1/2 activation in a pertussis toxin (PTX)-sensitive manner, but not a calcium influx (Saito et al. 2001b; Schlumberger et al. 2002). An MCH-signaling pathway that activates ERK1/2 and pp70 S6 kinase is also present on 3T3-L1 adipocytes expressing endogenous MCH1R (Bradley et al. 2002). Treatment of 3T3-L1 adipocytes with MCH acutely downregulates MCH1R, indicating a mechanism for ligand-induced receptor downregulation.

Since both MCH-expressing neurons and MCH receptors are found in the LHA, the cellular actions of MCH-expressing neurons in the LHA have been examined using whole-cell recording in current and voltage clamps (Gao and Van Den Pol 2001, 2002). MCH was found to play a dramatic inhibitory role in the regulation of glutamatergic and GABAergic synaptic transmission in LHA neurons, and this effect is based on a reduction of voltage-dependent calcium currents via PTX-sensitive G-protein pathways, probably the Gαi/o pathway. MCH attenuates L-, N- and P/Q-type calcium channels, with the greatest inhibition found for N-type currents. MCH actions in LHA neurons differ from those in non-neuronal cells that express exogenous MCH1R, since the non-neuronal cells show an MCH-mediated increase in calcium, while the reverse occurs in neurons. Previous reports have also suggested that MCH activates G protein-coupled inwardly rectifying potassium channels in non-neuronal cells (Bächner et al. 1999), but no effects of MCH on voltage-dependent potassium channels were observed in LHA neurons. In the hippocampus, where MCH1R mRNA is highly expressed and MCH fibers are projected, exogenously applied MCH lowers the long-term potentiation thresholds by increasing hippocampal synaptic transmission through an *N*-methyl D-aspartate receptor-dependent pathway (Varas et al. 2003). Although the ventral tegmental area receives dense projections from the LHA, MCH does not affect the firing of dopaminergic or fast-firing GABAergic cells in the area (Korotkova et al. 2003). Further electrophysiological characterization is necessary in other regions that express high levels of MCH1R, such as the nucleus accumbens shell or amygdala.

4 Effects of MCH1R Antagonism on Physiological Responses

4.1 Efficacy of Feeding Behavior and Energy Balance

The strong association of the MCH-MCH1R system with obesity has accelerated the development MCH1R agonist/antagonists and their use in behavioral studies. The effects of more than 50 MCH analogues on MCH1R-expressing cells have been investigated and extensive structure-activity relationships have been clarified (Audinot et al. 2001). Acute central administration of these MCH analogues led to a rapid and significant increase in food intake with a potency that was correlated with the affinity of the agonist for MCH1R (Suply et al. 2001). This study clearly indicated that MCH1R is the mediator of the orexigenic effects of MCH. Furthermore, chronic icv infusion of synthetic MCH1R agonists induced obesity in rodents (Della-Zuana et al. 2002; Ito et al. 2003), and their weight gain was accompanied by hyperphagia, a reduced core temperature, and stimulated lipogenic activity in the liver and white adipose tissue. These observations again suggest that MCH plays an essential role in the development of obesity by modulating energy homeostasis.

T-226296 was the first reported non-peptide MCH1R-selective antagonist. This orally active antagonist effectively blocked the food intake stimulated by icv administration of MCH in rats (Takekawa et al. 2002). T-226296 was reported to suppress spontaneous food intake in diet-induced obese rats by selectively decreasing the sizes of the meals consumed rather than by a generalized behavioral malaise (Kowalski et al. 2004). A second non-peptide antagonist, SNAP7941, has provided the first evidence that chronic oral administration of an MCH1R antagonist can effect sustained reductions in body weight (26% weight loss relative to vehicle-treated rat and food intake, that were greater than the effects elicited by D-fenfluamine, an effective anorectic agent (Borowsly et al. 2002). The third reported antagonist was an MCH-modified peptide, designated compound B, and its chronic icv administration to rats resulted in reductions in appetite, caloric efficiency, body weight gain and body fat gain without any effect on lean mass (Shearman et al. 2003). These findings are consistent with the sustained feeding and body weight effects of SNAP7941 in diet-induced obese rats. Although chronic compound B treatment significantly attenuated body weight in wild-type mice, no effects were seen in MCH1R-knockout mice, indicating that compound B specifically acts by interacting with MCH1R (Georgescu et al. 2005). Moreover, other small-molecule antagonists of MCH1R exhibited efficacy in animal feeding and weight loss in chronic rodent models with no toxicity or adverse behavioral effects (Handlon and Zhou 2006). These consistent findings all support the proposal that MCH1R antagonists will provide promising target strategies for obesity treatment (Fig. 2).

4.2 Efficacy in Anxiety, Depression, and Stress

Since MCH1R is localized in several limbic areas and the nucleus accumbens shell, an area involved in the regulation of emotion, stress, motivation and reward (Hervieu et al. 2000; Saito et al. 2001b), the MCH system appears to be important for the regulation of stress and anxiety-related responses in addition to the crucial roles of MCH in feeding behavior.

The administration of MCH into the medial preoptic area induced anxiety in female rats (Gonzalez et al. 1996), while injection of MCH into the nucleus accumbens shell increased depressive behavior (Georgescu et al. 2005). Regarding its role in stress, the direct injection of MCH into the paraventricular nucleus increased the plasma adrenocorticotropic hormone (ACTH) level (Kennedy et al. 2003). MCH also induced corticotropin-releasing factor (CRF) release from hypothalamic explants, an effect that was sensitive to blockade by an MCH1R antagonist (Kennedy et al. 2003), while increases in plasma ACTH following icv injection of MCH were prevented by an anti-CRF antibody (Jezova et al. 1992). Thus, stimulation of MCH1R seems to cause activation of the hypothalamus-pituitary-adrenal (HPA) axis through increases in CRF excretion. On the other hand, several contrasting studies have been reported. Briefly, icv, intra-amygdaline or intra-hippocampal MCH administration was reported to exert dose-response anxiolytic effects (Monzon et al. 2001) consistent with experiments showing anti-anxiety properties for MCH in a test called Vogel's punished drinking test (Kela et al. 2003). However, another study reported that exogenous MCH induced a moderate decrease in ACTH secretion under resting conditions when injected during the light phase (Bluet-Pajet et al. 1995). These divergent results regarding the role of MCH may be attributed to its wide circadian variation or negative feedback in the basal HPA axis. It is also likely that the different routes of MCH administration and/or the various rodent models used to score the behavior may produce such differences in the function of MCH in the regulation of anxiety.

The most recent studies using genetic and pharmacological approaches have provided support for the anxiogenic effects of the MCH-MCH1R system (Fig. 2). For example, chronic administration of the MCH1R antagonist SNAP7941 showed efficacy for reducing anxiety, and mimicked antidepressant effects in modified forced swim tests (Borowsly et al. 2002). Other nonpeptide MCH1R antagonists, ATC0065 and ATC0175, were synthesized and their oral administration produced anxiolytic and antidepressant activities in a series of behavioral models (Chaki et al. 2005). Similarly, the MCH1R antagonist GW3430 produced anxiolytic-like effects in animal models of anxiety (Smith et al. 2006). Furthermore, direct delivery of the MCH1R peptide antagonist compound B to the nucleus accumbens shell blocked feeding and further produced an antidepressant-like effect in forced swim tests, while

injection of MCH into the nucleus accumbens shell increased depressive behavior as described above (Georgescu et al. 2005). Given these reports, the MCH-MCH1R system is involved in not only regulation of feeding and energy balance but also regulation of mood and emotion via the hypothalamic-nucleus accumbens neural association. Characterization of the phenotypes of MCH1R-deficient mice revealed anxiolytic-like behavior when tested by a number of behavioral paradigms commonly used to assess fear and anxiety responses in rodents (Smith et al. 2006; Roy et al. 2006), and further revealed antidepressant-like behavior in female mice, but not male mice (Roy et al. 2007). It is noteworthy that MCH1R-selective antagonists had anxiolytic-like effects in wild-type mice, but not in MCH1R-deficient mice (Smith et al. 2006).

Overall, although a consistent link between the MCH-MCH1R system and mood has not yet been established, the effects of MCH1R antagonists in animal models suggest that these compounds deserve further investigation as potential etiologic treatments for affective disorders.

5
Peripheral Roles of the MCH–MCH Receptor System

MCH was initially isolated as a pituitary peptide in teleost fish in which the activity of the hormone decreased skin pigmentation (Kawauchi et al. 1982). Although the expressions of both MCH and MCH1R have been identified in human melanocytes and melanoma cell lines (Saito et al. 2001b; Hoogduijin et al. 2002), their physiological roles in the skin have not yet been fully elucidated. Pathologically, MCH1R on melanocytes was reported to be one of the targets of autoantibody responses in vitiligo, which is a common depigmentation disorder resulting from the loss of melanocytes in the skin (Kemp et al. 2002).

In the process of evolution from fish to mammals, it is likely that MCH and its counteracting hormone MSH have changed their primary functions from melanocyte regulation to energy metabolism. In the periphery, MCH or MCH1R expression in some of the digestive systems or adipose tissues is associated with energy and lipid metabolism. Hervieu and coworkers detected MCH immunoreactivity and mRNA expression in the lamina propria of the duodenum and colon in both humans and rats (Hervieu et al. 1996). The end product of the MCH gene in the digestive tract is an immature form of the MCH precursor, NEI-MCH, which consists of the 17-amino acid NEI attached to the N-terminal of MCH (Fig. 1B). Feeding was more potently boosted by icv administration of NEI-MCH than MCH, and this effect may arise via reduced susceptibility to proteases (Maulon-Feraille et al. 2002). The authors of the latter study also suggested the possibility that NEI-MCH may act as superagonist in vivo.

Since both white and brown adipose tissues express MCH1R, it is also possible that adipose tissues are directly regulated by MCH in the circulation. In mouse 3T3-L1 adipocytes, MCH induced rapid and transient increases in ERK1/2 and pp70 S6 kinase, which activated the transcriptional activity of leptin (Bradley et al. 2002). MCH may not directly regulate triglyceride metabolism in adipocytes, since it had no effect on lipogenesis or lipolysis in 3T3-L1 adipocytes and primary cultures of murine white adipose tissue (Bradley et al. 2002). Combined with the fact that central administration of MCH increased fat mass (Ito et al. 2003), it is anticipated that humoral or neuronal factors mediate MCH-expressing neurons in the CNS and adipose tissues.

In contrast to the possible endocrine actions in adipose tissues, autocrine cells expressing both MCH and MCH1R were recently found in vagus nerve system (Burdyga et al. 2006) and pancreatic islet (Pissios et al. 2007), both associate with energy metabolism. In the nodose ganglion of vagus neurons, which transmit chemical and physical inputs from digestive systems to the CNS, 10% of the neural soma coexpressed MCH and MCH1R. MCH and MCH1R are simultaneously increased by fasting, and then both decreased upon refeeding. Interestingly, cholecystokinin (CCK) is responsible for the suppression of the MCH system in MCH-expressing autocrine neurons through CCK1R. A further important fact is that the anorectic CART peptide is colocalized with MCH in MCH-expressing neurons. CCK reciprocally regulates the expressions of CART and MCH, and the orexigenic ghrelin counteracted CCK (Lartige et al. 2007). These findings suggest that modulation of gut-brain signaling is involved in the control of food intake.

The autocrine system of MCH and MCH1R is also found in beta cells in both human and mouse pancreatic islets. Genetic interventions of the MCH gene modify the size of the pancreatic islets, since mice overexpressing MCH exhibited islet hyperplasia (Shimada et al. 1998; Ludwig et al. 2001), while MCH-knockout mice had a significantly reduced beta cell mass (Pissios et al. 2007). MCH also increased insulin secretion, and altered the expressions of islet-enriched genes, such as glucagon, forkhead homeobox A2, hepatocyte nuclear factor (HNF) 4 and HNF1. These data illustrate that the autocrine system of MCH partially regulates beta-cell mass dynamics and islet secretory functions.

MCH immunoreactivity is also measurable in the human circulation. Plasma MCH levels were positively correlated with fat mass and increased by 25% after fasting (Gavrilla et al. 2004), consistent with the orexigenic and fat-increasing nature of MCH. However, many issues regarding circulating MCH, including its processing, source and dynamics, remain unsolved.

Taken together, the MCH system may play significant roles in peripheral tissues and be involved in energy metabolism. Studies of the MCH system in peripheral tissues will provide important findings for lipid and glucose metabolism that may provide direct links to the clinical implications of drugs targeting MCH receptors.

6 Conclusions

In recent years, obesity therapy has become a major focus of pharmaceutical research. The worldwide market for obesity therapeutics has increased dramatically over the past decade, and obesity has been linked with numerous risk factors. Combined with the knowledge that the hypothalamic area is one of the critical sites for the control of energy expenditure, the discovery of the different roles of MCH has attracted the interest of many research groups. In conjunction with energy balance, recent progress has suggested that the MCH-MCH1R system is involved in the regulation of certain types of complex behavior, such as stress, anxiety, and depression. Since many research groups have MCH receptor antagonist programs, it is likely that several compounds will succeed in advancing highly selective antagonists with pharmacokinetic properties into the clinical setting for obesity and mood disorders. Questions still largely remain as to how the signals from MCH are integrated into intracellular mechanisms that change neuronal activity, and how MCH neurons interact with other neuronal populations and finally control satiety, mood and emotion. For this purpose, identification of live MCH neuron by a viral approach has a substantial advantage (van den Pol et al. 2004). Full understanding of such complex brain circuitry will lead to deep insights into the clinical associations between anxiety, depression and eating disorders.

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Biological Function of Prokineticins

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Abstract Secreted peptides have been implicated in diverse physiological functions. Prokineticins are a pair of regulatory peptides that signal through two highly homologous G protein-coupled receptors. Prokineticins possess a unique structural motif of five disulfide bonds and conserved N-terminal stretches. Diverse biological functions, ranging from development to adult physiology, have been attributed to prokineticins. Herein we provide an overview of current knowledge of this interesting pair of regulatory peptides.

Keywords Angiogenesis · Basic helix-loop-helix transcriptional factors · Circadian rhythm · G protein-coupled receptor · Neurogenesis · Neuropeptides · Prokineticin

1 Introduction

Secreted peptides play critical roles in the integration of body physiology and brain functions. Snake venom and skin secretion from frogs have been rich sources for identification of biologically active secreted peptides. Before the molecular identification of mammalian prokineticins, peptides with a unique structural motif of five disulfide bonds had been isolated from snake venom and frog skin secretions (Joubert and Strydom 1980; Schweitz et al. 1990, 1999; Mollay et al. 1999). Mammalian prokineticins (prokineticin 1, PK1 and prokineticin 2, PK2) consist of two secreted proteins of about 80 residues (Wechselburger et al. 1999; Li et al. 2001; LeCouter et al. 2001; Chen et al. 2005). PK1 and PK2 have about 45% amino acid identity between them (Li et al. 2001). In addition to the five disulfide bond motif (Boisbouvier et al. 1998; Li et al. 2001), sequence alignment clearly shows that PK1 or PK2 and their vertebrate homologs exhibit a complete conservation of the first six amino acids (AVITGA) (Bullock et al. 2004). Mutagenesis and protease digestion experiments have revealed that the disulfide bonds and the conserved N-terminal stretch are critical for the bioactivities of prokineticins (Bullock et al. 2004; Negri et al. 2005). Bioassays indicate that the receptors that mediate the contracticle effect of prokineticins on smooth muscle cells belong to the G protein-coupled receptors (Li et al. 2001), and this was subsequently confirmed by three independent groups (Lin et al. 2002a; Masuda et al. 2002; Soga et al. 2002). The current knowledge of biological functions of proki-

neticins has been the subject of a number of recent reviews (Kaser et al. 2003; Ferrara et al. 2004; Zhou and Cheng 2005; Zhou 2006; Maldonado-Perez et al. 2007; Negri et al. 2007)

2
Distribution of Prokineticin Receptors

Two G-protein-coupled receptors, prokineticin receptor 1 (PKR1) and prokineticin receptor 2, with an unusually high degree of sequence identity (>85%) have been identified as receptors for prokineticins (Lin et al. 2002a; Masuda et al. 2002; Soga et al. 2002). This high degree of receptor sequence conservation suggests the critical functions of prokineticin receptors for essential biological processes. Intriguingly, even with the high degree of conservation, PKR1 and PKR2 reside in different human and mouse chromosomes (Lin et al. 2002a). In essentially all functional assays, PK1 or PK2 exhibit little selectivity over PKR1 or PKR2 (Lin et al. 2002a). This apparent non-selectivity of ligand/receptor activation implies that the availability of ligands (PK1 or PK2) and receptors (PKR1 or PKR2), i.e., the gene expression regulation, likely determines which possible signaling pair is involved for a particular biological process. Distribution studies have revealed that PKR1 is widely distributed in the peripheral organs, including the gastrointestinal system, lungs, blood system, and various endocrine organs (Lin et al. 2002a; Masuda et al. 2002; Soga et al. 2002). PKR2 may also be expressed in various endocrine tissues, including thyroid, pituitary, adrenal gland, testis and ovary (Lin et al. 2002a; Masuda et al. 2002; Soga et al. 2002). In situ analysis indicates that PKR2 is the dominant receptor in the adult brain, with particularly high expression of PKR2 in the hypothalamus, the olfactory ventricular regions and the limbic system (Cheng et al. 2002, 2006; Ng et al. 2005), which has been confirmed by a series of functional studies.

3
Regulatory Function of PK2 in Circadian Rhythms

The suprachiasmatic nucleus (SCN) in the anterior hypothalamus is the primary mammalian circadian clock that drives daily rhythms of diverse physiology and behavior (Reppert and Weaver 2002). While an understanding of the molecular mechanism for the circadian clockwork has emerged (Reppert and Weaver 2002; Lowrey and Takahashi 2004), much less is known about how the timing information from the SCN clock is transmitted to control physiology and behavior. The initial observation that PK2 mRNA in the SCN displays dramatic daily oscillation has implicated the potential regulatory function of PK2 in the circadian rhythm (Cheng et al. 2002). Multiple lines of evidence

have emerged to support that PK2 function as a prominent communicating signal from the SCN to its targets in the generation of circadian rhythms. In vitro studies have revealed that PK2 is a first-order clock-controlled gene with its expression controlled by CLOCK and BMAL1 acting on the E-box elements in its promoter (Cheng et al. 2002). CLOCK and BMAL1 are critical positive components of the circadian clock (Reppert and Weaver 2002). Correlative in vivo experiments have confirmed that PK2 is a clock-controlled gene that lies downstream of CLOCK and BMAL1. In mutant mice lacking functional clockwork, including Clock mutant and $Cry1^{-/-}Cry2^{-/-}$ mice, the PK2 mRNA oscillation in the SCN is abolished (Cheng et al. 2002). Moreover, the molecular rhythm of PK2 in the SCN was shown to adapt faster to the delay of light cycles than to the advance of light cycles (Cheng et al. 2005). It is well known that the circadian clock is asymmetrically built. The differential rates of adaptation of the PK2 rhythm to the delay and advance of light cycles were also consistent with the respective rates of behavioral and physiological adaptation observed in animals and humans (Yamazaki et al. 2000). Furthermore, intracerebroventricular delivery of recombinant PK2 suppressed the nocturnal wheel-running activity (Cheng et al. 2002) and feeding behavior (Negri et al. 2004), when endogenous PK2 level is minimal. Receptor distribution studies also revealed the capability of SCN targets to respond to an oscillatory PK2 signal from the SCN. The receptor for PK2 (PKR2) is expressed in virtually all known primary SCN targets, the paraventricular nucleus of the hypothalamus (PVN), the dorsal medial nucleus of the hypothalamus (DMH), paraventricular and paratenial nuclei of the thalamus (PVT/PT), and lateral septal nucleus (LS) (Cheng et al. 2002)

Recently, direct genetic evidence for the role of PK2 in the control of the circadian rhythm has been reported. The generation and characterization of mice lacking the PK2 or PKR2 gene has confirmed the critical role of PK2 signaling for the maintenance of robust circadian rhythms (Li et al. 2006; Prosser et al. 2007). Under both normal light/dark and constant dark housing conditions, the reduction of circadian locomotor rhythmicity in PK2-null mice was apparent (Li et al. 2006). PK2-null mice also displayed significantly reduced rhythmicity for a variety of other circadian indices, including sleep-wake cycle, body temperature, circulating glucocorticoid and glucose levels as well as the expression of peripheral clock genes. The fact that PK2-null mice have essentially normal clockwork genes oscillation in the SCN is consistent with the postulated role of PK2 as an output molecule. The circadian phenotypes of PKR2-mutant mice are almost identical with that of PK2-null mice (Prosser et al. 2007). The targeted null mutation of PKR2 disrupts circadian coordination of the activity cycle and thermoregulation. Specifically, mice lacking PKR2 lost precision in timing the onset of nocturnal locomotor activity; and under both a light/dark cycle and continuous darkness, there was a pronounced temporal redistribution of activity away from early to late circadian night. Moreover, the coherence of circadian locomotor behavior

was significantly reduced, and nocturnal body temperature was depressed. As with PK2-null mutants, entrainment by light is not dependent on PKR2, and bioluminescence real-time imaging of organotypical SCN slices showed that the mutant SCN is fully competent as a circadian oscillator, consistent with the notion that PKR2 only functions in the output pathway. In a transgenic model of Huntington's disease, a correlation between increased daytime locomotor activity and reduced SCN expression of PK2 molecular rhythm was also observed (Morton et al. 2005). Studies from these mutant mice strongly supported the notion that PK2 signaling is an essential link for coordination of circadian behavior and physiology controlled by the SCN.

The daily cycle of sleep/wakefulness is probably the most important physiological process regulated by the SCN circadian clock. The sleep characterization of PK2-null mice has recently been reported (Hu et al. 2007). Sleep regulation has been postulated to consist of homeostatic and circadian processes (Borbely 1982). The homeostatic process determines the duration and intensity of sleep, which builds up in the absence of sleep and dissipates during sleep. The circadian process, controlled by SCN, determines the timing of sleep (Klein et al. 1991). However, the relationship of these two processes is still controversial. Earlier results implied that the homeostatic process is independent of the circadian process, since sleep amount and recovery sleep after sleep deprivation are unchanged in SCN-lesioned rats that lack normal circadian expression of sleep (Mouret et al. 1978; Mistlberger et al. 1983; Tobler et al. 1983; Eastman et al. 1984; Borbely et al. 1989; Trachsel et al. 1992). However, in more recent studies on SCN-lesioned rats it has been interpreted that SCN is also involved in the homeostatic regulation of sleep (Wurts and Edgar 2000). Furthermore, SCN lesion in the monkeys indicated that the circadian process is an intimate component of the homeostatic process. Complete SCN lesion of the squirrel monkey led to a loss of circadian timing as well as a 4 h increase in daily sleep time (Edgar et al. 1993). Studies with circadian gene mutant mice have revealed that these circadian genes affect not only circadian sleep distribution, but the homeostatic regulation of sleep as well. Clock gene mutant mice sleep 2 h less than wild-type mice daily (Naylor et al. 2000), whereas the Bmall/Mop3 mutant mice (Laposky et al. 2005), and double Cry1 and Cry2 mutant mice (Wisor et al. 2002) displayed 1.5 h and 1.8 h increases in total sleep amount under baseline conditions, respectively. In wild-type mice, sleep deprivation (SD) is followed by a compensatory sleep increase. Mice lacking both Cry1 and Cry2 (Wisor et al. 2002) had a reduced non-rapid eye movement (NREM) sleep rebound, while mice lacking Clock (Naylor et al. 2000), the Bmall/Mop3 (Laposky et al. 2005) had a reduced rapid eye movement (REM) sleep rebound in response to SD. In PK2-null mice, the total sleep time under entrained light-dark and constant darkness conditions was reduced (Hu et al. 2007). Furthermore, the reduced sleep time occurred predominantly during the light period, consistent with the expression of PK2 during this period. Intriguingly, PK2-null mutant mice displayed

an impaired response to sleep deprivation (Hu et al. 2007). These studies indicate that PK2 plays roles in both circadian and homeostatic regulation of sleep.

Daytime restricted feeding (RF), is known to compete with SCN, to control locomotor rhythm (Damiola et al. 2000; Stokkan et al. 2001). In response to a daytime RF, rodents will feed at unusual periods for the sake of survival and gradually become active before the food is made available, a phenomenon called food anticipatory activity (FAA). FAA is a rhythmic event and has been associated with the food entrained oscillators (FEO) in the brain (Gooley et al. 2006). Thus, during RF, the FEO competes with the light-entrained oscillator (LEO, i.e. SCN) for the control of activity and physiological events. The increased FAA has been interpreted as a relatively stronger FEO control (Dudley et al. 2003; Pitts et al. 2003). When the FAAs of PK2-null and control mice were monitored in response to RF, PK2-null mice displayed significantly higher FAA (Li et al. 2006). Thus, in the absence of the PK2 signal, the control of locomotor rhythm was weakened, and their response to RF was enhanced. This study supports the notion that PK2 functions as a signal from the SCN that suppresses inappropriate feeding activity.

The molecular rhythm of PK2 in the SCN of a diurnal rodent has also been investigated (Lambert et al. 2005). Similar to the oscillation pattern observed in nocturnal mouse and rat (Cheng et al. 2002; Masumoto et al. 2006), PK2 mRNA in the SCN of diurnal *Arvicanthis niloticus* was rhythmically expressed, with peak levels in the morning hours and essentially absent during the night phase (Lambert et al. 2005). Thus, the phase of PK2 expression in the SCN of diurnal rodents is the same as that of nocturnal rodents, consistent with a growing body of evidence suggesting that the key to diurnality lies downstream of the SCN circadian clock. It will be very interesting to demonstrate whether nocturnal and diurnal animals respond differentially to the same-phase PK2 signaling from the SCN clock.

Recent studies have identified a possible neurophysiological mechanism of PK2 in mediating SCN output in controlling diverse circadian rhythms. PK2 was shown to excite neurons that express the PK2 receptor (Cottrell et al. 2004). This first physiological study of PK2 revealed a possible link between this oscillating PK2 messenger and the neuronal firing rates. The neurophysiological effect of SCN-derived PK2 on the PVN, one critical SCN target, has recently been reported (Yuill et al. 2007). PVN, a primary SCN efferent nucleus, is involved in the regulation of endocrine rhythms or oscillation of the autonomic nervous system. PK2 was able to excite PVN neurons of both parvocellular and magnocellular branches (Yuill et al. 2007). Importantly, a peptide-based PK2 receptor antagonist was able to decrease the basal activity of parvocellular neurons in the hypothalamus slice (including SCN and PVN) only during the light phase, when PK2 is highly expressed in the SCN (Yuill et al. 2007). Thus, endogenous SCN-derived PK2 excites the parvocellular branch of PVN neurons in a phase-dependent manner.

4 Function of PK2 in Neurogenesis

In mammals, neurogenesis occurs mainly during embryonic to early postnatal stages. However, neurogenesis persists in certain regions of adult mammalian brains, including the olfactory bulb (OB) and the dentate gyrus (DG) of the hippocampus (Altman 1969; Gage 2000). New neurons are continuously generated in the OB from progenitor cells of the subventricular zone (SVZ) of the lateral ventricle. These progenitor cells proliferate and migrate through the rostral migratory stream (RMS) to the OB to form mature granular and periglomerular cells through the adult life of mammals (Kaplan and Hinds 1977; Luskin 1993; Lois and Alvarez-Buylla 1994). Recently, it was demonstrated that PK2 functions as a chemoattractant for SVZ-derived neuronal progenitors, and this PK2 signal is essential for the normal development of OB architecture (Ng et al. 2005). The expression of PK2 and its receptor was complementary in the OB, with PK2 expressed in the mature granular and periglomerular layers of the OB whereas its receptors (PKR1 and PKR2) are expressed in the immature ependyma and subependymal layers of the olfactory ventricle (Ng et al. 2005; Cheng et al. 2006). In vitro migration assays indicated that PK2 stimulates migration of neuronal progenitors from the SVZ in both adult and postnatal rats, and this migration is directional and could be inhibited by the PK antagonist (Ng et al. 2005). The critical role of PK2 in OB development was confirmed by studies with PK2-null mice. PK2-deficient mice have abnormal development of OB, including the dramatic reduction in OB volume, and the loss of normal OB layer architecture (Ng et al. 2005). Although both PKR1 and PKR2 are expressed in the immature ependyma and subependymal layers of the olfactory ventricle, genetic analysis indicated that PKR2, but not PKR1, is a critical receptor for OB development (Matsumoto et al. 2006). Whereas the OB development in PKR1-deficient mice is essentially normal, PKR2-deficient mutant mice exhibited similar abnormal development of the OB, as that of PK2-null mice. These genetic studies reveal that PK2-PKR2 signaling is essential for normal neurogenesis in the OB.

It has been demonstrated that CLOCK and BMAL1 are the positive upstream regulators of PK2 in mediating circadian rhythms (Cheng et al. 2002). The normal OB development in Clock and Bmal1 mutant mice (Vitaterna et al. 1994; Bunger et al. 2000) indicates that PK2 is likely under the control of different transcriptional factors in regulating neurogenesis of the OB. Recent studies have elucidated the identities of these upstream regulators of the PK2 gene for OB neurogenesis (Zhang et al. 2007). In the OB, the PK2 gene is found to be a functional target of proneural basic Helix-Loop-Helix (bHLH) factors NGN1 and MASH1. bHLH transcription factors have been shown to be crucial regulators of neurogenesis (Bertrand et al. 2002). During development, NGN1 and MASH1 regulate several important steps of neurogenesis,

including the commitment of stem cells to neuronal and glial lineages, the specification of neuronal subtype identities, and neuronal migration. The evidence that NGN1 and MASH1 are the upstream regulators of the PK2 gene in OB neurogenesis includes: (1) NGN1 and MASH1 activate PK2 transcription by binding to E-boxes on the PK2 promoter; (2) NGN1 and MASH1 are co-expressed with PK2 in OB neurons; (3) Chromatin immunoprecipitation has demonstrated the association of NGN1 and MASH1 with the PK2 promoter in vivo; (4) Similar defects in OB neurogenesis have been identified in the mutant mice that lack Ngn1, Mash1 and PK2. These results indicate that PK2 is a critical downstream target gene of NGN1 and MASH1 in regulating OB neurogenesis.

Thus, it is interesting to learn that the same PK2/PKR2 signaling pair plays a critical role in apparently completely different biological processes: OB neurogenesis during development and circadian clock output of adult physiology. For both processes, the PK2 gene is the common functional target of different families of bHLH transcriptional factor: Ngn1 and MASH1 for OB neurogenesis and Clock and Bmal1 for circadian clock output. It is truly intriguing to learn that the same set of E-boxes in the promoter of the PK2 gene is utilized by Ngn1/MASH1 and CLOCK/BMAL1 (Zhang et al. 2007). Thus, PK2 has been established as a common functional target gene for different bHLH transcriptional factors in regulating their respective functions (Fig. 1). It is clear that NGN1/MASH1 and ClOCK/BMAL1 also have other target genes. This gene network of transcriptional control of PK2 and its upstream regulators has provided an elegant example showing that the complexity of the mammalian genome is probably not due to a sheer increase in the quantity of genes, but more likely to complicated gene transcriptional control.

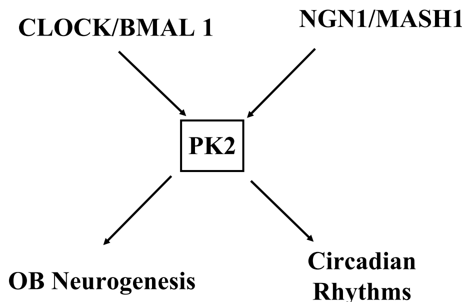

Fig. 1 Transcriptional regulation of prokineticin 2 (PK2) by basic helix-loop-helix (bHLH) transcription factors. The promoter of the PK2 gene contains multiple E-box sequences (CACGTG) that are targeted by bHLH transcription factors CLOCK/BMAL1 and NGN1/MASH1. PK2 is a common functional target gene for both sets of transcriptional factors in regulating their respective biological processes: circadian rhythm and olfactory bulb (OB) neurogenesis

5 Functions in Angiogenesis

Angiogenesis is crucial for diverse biological processes including development, tumorigenesis, reproduction, and wound healing. When screening a library of secreted molecules, LeCouter et al. identified PK1 as a molecule that was capable of inducing proliferation of primary bovine adrenal-cortex-derived capillary endothelial (ACE) cells (LeCouter et al. 2001). It was later found that, similarly to adrenal gland-derived endothelial cells (EC), PKs enhanced the proliferation and inhibited apoptosis in EC derived from another endocrine gland, the corpus luteum (Kisliouk et al. 2005; Podlovni et al. 2006). In fact, PKs also induced proliferation of bovine aortic EC (BAEC), a well-characterized model for a macrovessel, indicating that PKs are mitogens and serve as survival factors for microvascular (AEC and luteal EC) and macrovascular (BAEC) cells (Kisliouk et al. 2005; Podlovni et al. 2006). However, these different EC types each have a distinct pattern of PKR expression (Fig. 2). In contrast to ACE or luteal EC, which expressed both PKR1 and PKR2, BAEC mainly expressed PKR1. BAEC and luteal EC differed not only in their receptor repertoire, but also in their regulation in response to apoptotic cues such as TNFa and serum starvation (Fig. 2) (Podlovni et al. 2006), suggesting cell-specific biological effects. Under conditions of serum starvation, PK1 maintains its antiapoptotic effects only in luteal EC (and not in BAEC) (Podlovni et al. 2006). These findings highlight the importance of PK1 action on stressed EC, suggesting that the presence of PK-R2 provides luteal EC with

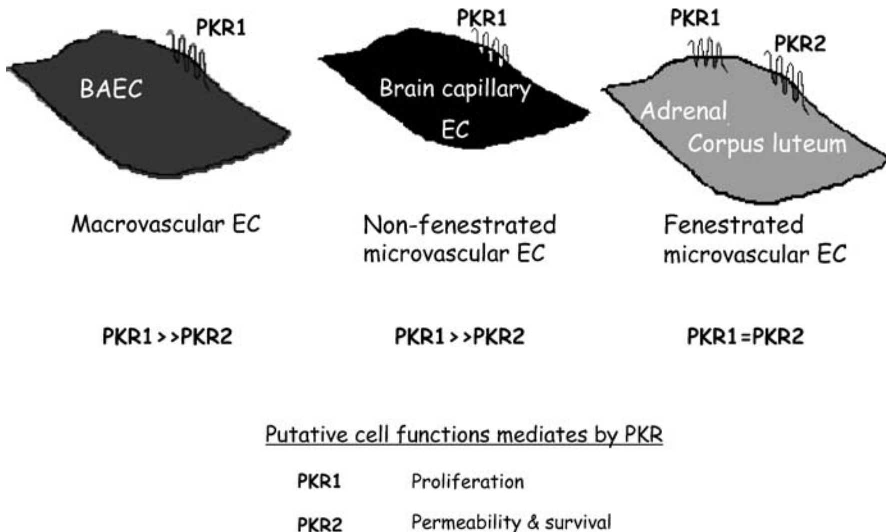

Fig. 2 Prokineticins receptor distribution in different endothelial cell types

an additional tool to resist stress-induced apoptosis. Indeed, due to their tissue microenvironment, luteal EC are more prone than BAEC to experience extreme conditions such as low oxygen tension and a shortage of nutrients.

Besides mitogenesis, endothelial permeability plays an important role in angiogenesis. In addition to the functions mentioned above, the presence of PKR2 appears to be indispensable for PK1-induced endothelial cell permeability. These effects were only observed in PKR2 expressing LEC and not in BAEC or another cell examined: brain capillary EC (BCEC; Fig. 2) (Podlovni et al. 2006). The case of BCEC is particularly interesting: in contrast to other microvascular EC, the brain capillary endothelium serves as a protective shield or barrier between blood and the underlying tissue (the central nervous system) (Abbott 2000; Kniesel and Wolburg 2000). To date, PKR2 has only been identified in fenestrated EC such as those found in the adrenal cortex, CL, kidney, and liver, in agreement with the notion that PKR2 plays a role in EC permeability (Kisliouk et al. 2005; LeCouter et al. 2003c; Lin et al. 2002) (Fig. 2). Notably, in aorta and corpus luteum, PK1 was immunolocalized to the smooth muscle layer in blood vessel walls, and could not be detected in EC (Podlovni et al. 2006). Hence, PK1, which is necessary for activating EC types via PK-Rs, is produced in neighboring cells, suggesting that PK1 has a paracrine mode of action.

The promoter of PK1 possesses putative HIF-1 (hypoxia-inducing factor) binding sites, and the expression of PK1 is induced by hypoxia. Delivery of recombinant virus, which expresses PK1 in ovaries, elicited potent angiogenesis, but the angiogenic effect was absent when delivered to cornea or skeletal muscles. PK1 was hypothesized as the first example of a tissue-specific angiogenic factor (LeCouter et al. 2003a), although its angiogenic effect is most likely broader than originally claimed (Tanaka et al. 2006; Podlovni et al. 2006). Interestingly, PK1 is up-regulated under certain diseased conditions, including malignancy (Ferrara et al. 2003; Zhang et al. 2003; Pasquali et al. 2006; Ngan et al. 2007). In essentially all in vitro angiogenic assays, including the proliferation, survival, and migration of different EC types (BAEC, luteal EC, and ACE), PK2 behaved similarly to PK1 (LeCouter et al. 2003b; Kisliouk et al. 2005; Podlovni et al. 2006). The expression of PK2 is also hypoxia-inducible (LeCouter et al. 2003b), and the PK2 promoter possesses more probable consensus HIF1 binding sites than that of PK1. To date, however, the expression studies of PK2 in tumors are rather limited.

6
Functions in the Reproductive System

Since angiogenesis is crucial for normal development and function of the corpus luteum and the placenta, several groups have examined the possible roles of PKs in reproductive organs. Subsequently, it was reported that PK1

and its receptors are expressed in the ovary, uterus, placenta, testis, and prostate, with functions extending beyond the vascular network. A possible paracrine role for PKs in the function of the endometrium and placenta has been suggested by functional studies and expression analyses. Battersby et al. demonstrated that PK1 expression, but not PK2, was elevated in the endometrium during the secretary phase of the menstrual cycle, and this PK1 elevation was induced by treatment with progesterone (Battersby et al. 2004). The presence of PKR1 and PKR2 in the glandular epithelial cells and smooth muscle cells, along with endothelial cells, suggests that PKs have angiogenic as well as nonangiogenic functions such as myometrial contraction.

Hoffmann and colleagues (Hoffmann et al. 2006) have shown that in the human placenta, expression of PK1 and PKR1 peaks during the first trimester of pregnancy, corresponding to the hypoxic period of placental development. Moreover, in cultured trophoblast cells, PK1 and its type 1 receptors were up-regulated by hypoxia. Interestingly, although both PK1 and VEGF were induced by hypoxia, these two peptides exhibited distinct spatiotemporal patterns of expression, with PK1 mainly localized to the syncytiotrophoblast layer and VEGF to the cytotrophoblast and extravillous trophoblast cells (Hoffmann et al. 2006). Similar findings were reported for the mouse placenta, where PK1 was elevated during early gestation (Hoffmann et al. 2007).

It has recently been shown that PK1 is also localized to the ovary (Frazer et al. 2005; Kisliouk et al. 2005b). In the corpus luteum its expression increased throughout the luteal phase, with the highest levels found during the late luteal phase including in regressed glands undergoing apoptosis (Frazer et al. 2005; Kisliouk et al. 2005b, 2007). As in the placenta, the relative abundance of PK2 mRNA in human corpus luteum was low (Kisliouk et al. 2003; Frazer et al. 2005). Elevated PK1 mRNA levels were also detected in another ovarian apoptotic tissue: the granulose cell layer of atretic follicles. Immunostaining and FACS analysis eventually showed that PK1 is expressed by macrophages infiltrating the regressed corpus luteum (Kisliouk et al. 2007). Functional studies demonstrated the involvement of PK1 in enhancing the recruitment and subsequent activation of leukocytes in atretic follicles and regressing CL (Kisliouk et al. 2007). Therefore during different reproductive stages in the ovary as in placenta and uterus, PK1 undertakes different roles, vascular as well as non-vascular functions.

The PK system has also been indirectly implicated in reproductive organ development (Matsumoto et al. 2006; Pettiloud et al. 2007). For example, PK2-deficient mice exhibit hypogonadotropic hypogonadism, and are essentially infertile (Pettiloud et al. 2007). The hypogonadotropic hypogonadism in PK2-deficient mice is due to a dramatic decrease in the GnRH neuron population in the hypothalamus. The fact that PK2-deficient mice responded normally to exogenous human chorionic gonadotropin indicates that the reproductive phenotype is most likely due to a migration defect in GnRH neurons in

these mice (Pettiloud et al. 2007). Earlier studies have revealed that OB is required for the proper migration of GnRH neurons from the nasal cavity to the hypothalamus during development (Wierman et al. 2004). A similar reproductive defect was also observed in PKR2-deficient mice (Matsumoto et al. 2006). Thus, the PK2/PKR2 signaling pathway is essential for OB ontogenesis and therefore critical for the normal migration of GnRH neurons (Ng et al. 2005; Matsumoto et al. 2006; Pettiloud et al. 2007). Recently, homozygous deletion mutations in the PK2 gene have been reported to account for two patients with Kallman Syndrome and one patient with normosmic idiopathic hypogonadotropic hypogonadism (Pettiloud et al. 2007). The identified deletion results in a truncated PK2 protein of only 27 amino acids (rather than 81 in its mature form), which completely lacks bioactivity. Thus, homozygous loss-of-function PK2 mutations cause both Kallman Syndrome and normosmic idiopathic hypogonadotropic hypogonadism. Heterozygous mutations in PK2 or PKR2, including compound heterozygotes, have also been associated with Kallman syndrome (Dode et al. 2006). This indicates that insufficient PK2 signaling through PKR2 leads to abnormal development of the olfactory system and reproductive axis in humans. It will be very interesting to examine the OB and reproductive phenotype of PK2 and PKR2 compound heterozygous mice.

7
Prokineticins as Regulators of Gastrointestinal Motility

The contractile activity of prokineticins on gastrointestinal smooth muscle was the first demonstrated biological activity for this family of regulatory peptides (Mollay et al. 1999; Schweitz et al. 1999; Li et al. 2001). The ligand-binding studies and functional studies with gastrointestinal preparations had revealed the presence of high-affinity prokineticin receptors in the smooth muscle cells of small intestines (Li et al. 2001), and these observations have helped the initial characterization of prokineticin receptors and eventual molecular identification (Lin et al. 2002a). Whereas it is still unclear whether the effects of prokineticins on gastrointestinal smooth muscle cells are pharmacological or physiological, in vivo studies have indicated that exogenous PK1 and PK2 stimulate the gastrointestinal transit (US patent filing US2004/0162238A1; Owyang C, personal communication), and thus the contractile effects of prokineticins on gastrointestinal smooth muscle might be propulsive. The role of prokineticins in gastric and colonic contractility has also been investigated (Bassil et al. 2005; Hoogerwerf 2006). PK2 was found to increase the emptying of a liquid meal from rat stomach (Lewis 2004), although this observation was not confirmed by a different study (Bassil et al. 2005). In colons, PK1 was found to suppress giant contractions of the circular muscle via the release of nitric oxide indicating an indirect effect of proki-

neticins on gastrointestinal motility is also likely (Hoogerwerf 2006). This study further revealed that PKR1 is expressed on myenteric plexus neurons and it co-localizes with a small subset of NOS-expressing neurons. Thus, PK1 or PK2 may regulate gastrointestinal motility directly via activating smooth muscle cells, and indirectly via modulating the activities of enteric neurons. Additional studies, including those with mutant mice as well as small molecule receptor antagonists, need to be carried out to increase our understanding of the roles of prokineticins in regulating gastrointestinal motility.

8
Prokineticins and Pain Perception

A series of studies have indicated the role of prokineticins or their non-mammalian homologues in sensitivity of pain perception. Intraplantar injection of PK2 and Bv8 causes a strong and localized hyperalgesia by reducing the nociceptive thresholds to thermal and mechanical stimuli (Mollay et al. 1999; Negri et al. 2002; Hu et al. 2006). Systemic injection of Bv8 into rats also induces hyperalgesia to tactile and thermal stimuli (Negri et al. 2002). The hyperalgesia caused by PK2 or Bv8 is likely due to activation of dorsal root ganglia (DRG) neurons, which express both PKR1 and PKR2 mRNAs (Negri et al. 2002; Vellani et al. 2006). Functional assays indicate that PK2 and Bv8 were able to mobilize calcium in cultured rat DRG neurons (Negri et al. 2002; Hu et al. 2006). Transient receptor potential vanilloid 1 (TRPV1) has recently been identified as a possible molecular link between PKR1/R2 and DRG neuron activation. TRPV1 is an excitatory ion channel that is critical for the detection and integration of pain-producing chemical and thermal stimuli by DRG neurons (Caterina and Julius 2001). Colocalization experiments have revealed that the majority of PKR-positive DRG neurons also express TRPV1 (Vellani et al. 2006), and calcium image studies have revealed that the majority of DRG neurons that respond to PK2 were also activated by capsaicin (Hu et al. 2006). Mice lacking the PKR1 gene exhibited impaired pain perception to various stimuli, including noxious heat, mechanical, capsaicin, and protons (Negri et al. 2006), indicating that PKR1 is probably the dominant receptor that exerts a tonic activation of TRPV1 in DRG neurons. The reduced response of TRPV1-null mice to Bv8 (Negri et al. 2006) further indicates that TRPV1 is a critical downstream signaling component of PKR1 in pain perception.

The expression pattern indicates that PK2 might be the ligand for PKR1 in regulation of pain perception, especially in inflammatory pain. PK2 is highly expressed in peripheral blood cells, notably in monocytes, neutrophils, and dendritic cells (LeCouter et al. 2004; Dorsch et al. 2005). Thus, PK2 could be released into the sites of inflammation by neutrophils, activate macrophages and subsequently induce the release of other proinflammatory cytokines such

as interleukin 1 and interleukin 12 (Martucci et al. 2006). Genetic studies with PK2-deficient mice confirm the critical involvement of PK2 in acute and inflammatory pain (Hu et al. 2006). PK2-deficient mice displayed significant reduction in nociception induced by thermal and chemical stimuli. Thus, PK2-PKR1 appears to be the dominant ligand/receptor pair in the regulation of pain sensation.

9
Role of Prokineticins in the Development and Function of Blood Cells

The detection of the mRNAs of prokineticins and their receptors in bone marrows and other hematopoietic organs implicates a possible role in hematopoiesis (Li et al. 2001). Detailed expression analyses indicate that both PKR1 and PKR2 are expressed in the hematopoietic stem cells (LeCouter et al. 2004). Dorsch et al. (2005) first investigated the differentiative effect of PK1 on bone marrow cells and demonstrated that PK1 drastically promoted the differentiation of mouse and human bone marrow cells into the monocyte/macrophage lineage. LeCouter et al. (2004) showed the similar effect of PK2 on monocyte lineage, and further demonstrated that PK2 promoted the survival and differentiation of granulocytic lineages in cultures of human or mouse hematopoietic stem cells. Taken together, these studies imply the possible role of prokineticins, particularly PK2, in hematopoiesis, which may be conserved during evolution (Soderhall et al. 2005).

As both PKR1 and PKR2 are expressed in mature blood cells, prokineticins may also regulate the functionality of specific mature blood cells by altering their behaviors. PK2 and Bv8 induced chemotaxis of macrophages with very high potency (LeCouter et al. 2004; Martucci et al. 2006). PK1 treatment altered the morphology of human peripheral monocytes and expression of several cytokines or cytokine receptors, such as interleukin-10, interleukin-12, and tumor necrosis factor α (Dosrch et al. 2005; Kisliouk et al. 2007). These observations are interesting in the context of the sites of PK1 and PK2 expression. PK1 is expressed constitutively in B cells, T cells, and also in inflamed tissues (LeCouter et al. 2001; Dosrch et al. 2005). PK2 is detected in dendritic cells, neutrophils, and macrophages (LeCouter et al. 2004; Martucci et al. 2006). PK2 is also highly expressed in infiltrating cells at sites of inflammation, predominantly in neutrophils, and the expression of PK2 and both PK receptors are induced in monocytes upon exposure to lipopolysaccharide (LeCouter et al. 2004). These results indicate that PK1 or PK2 may regulate an immune response by altering monocyte activation, migration of granulocytes and monocytes. Thus, it is likely that prokineticins are hematopoietic cytokines that modulate the innate and the adaptive immune systems.

10 Summary and Perspectives

Over the last few years, prokineticins have evolved as a pair of signaling peptides that regulate diverse functions ranging from embryonic development to adult physiology. These diverse functions could be generally classified into two categories: cell excitability and cell differentiation/migration. The regulatory function of prokineticins in circadian rhythm, pain perception, and gastrointestinal motility relates to the enhanced cell excitability, whereas the functions in angiogenesis, neurogenesis, and hematopoiesis are linked to the effects of prokineticins on cell differentiation and migration. Another emerging theme is that PK2 has been shown as a common functional target gene for different sets of upstream transcriptional factors in regulating separate biological processes. It is obvious that the majority of the biological functions of prokineticins are yet to be discovered.

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Neuromedin S: Discovery and Functions

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Abstract Neuromedin S, a novel neuropeptide of 36 amino acids, was isolated from rat brain as an endogenous ligand for the orphan G protein-coupled receptors FM-3/GPR66 and FM-4/TGR-1, identified to date as type-1 and type-2 neuromedin U (NMU) receptors, respectively. The peptide was designated neuromedin S (NMS) because it is specifically expressed in the suprachiasmatic nucleus of the hypothalamus. NMS is structurally related to NMU; these peptides share a C-terminal core structure. In this review, we will outline the recent discoveries regarding the structure, cognate receptors, distribution, and possible physiological functions of NMS.

1
Discovery of Neuromedin S

Neuropeptides have been implicated in a wide range of physiological processes. Previous identifications of novel neuropeptides have revealed novel regulatory mechanisms in physiological processes; thus, researchers have continued to search for novel neuropeptides. Although a variety of neuropeptides have been isolated based on their functionality, a powerful survey method to isolate novel neuropeptide with unknown functions was developed in the 1990s (Civelli 1998). Human genomic sequencing revealed the existence of several hundred orphan G protein-coupled receptors (GPCRs), for which ligands have remained unidentified (Vassilatis et al. 2003). As dozens of these orphan GPCRs exhibit sequence similarity to GPCRs with known neuropeptide ligands, these orphan GPCRs have been used as tools to identify novel neuropeptides. This survey method has the advantage of identifying both the neuropeptide and its cognate receptor simultaneously. The rate of neuropeptide discovery increased considerably with this strategy. Over the course of a decade, use of a reverse-pharmacological technique has led to the discovery of approximately ten novel neuropeptides, which have been discovered as endogenous ligands of orphan GPCRs (Civelli et al. 2006).

Neuromedin U (NMU), originally isolated from porcine spinal cord, is a brain–gut peptide with a potent activity to cause uterine smooth muscle contraction (Minamino et al. 1985). As the receptor had not been identified, however, the physiological roles of NMU were poorly understood. In 2000, NMU was found to be an endogenous ligand for two orphan GPCRs, FM-

3/GPR66 and FM-4/TGR-1, which were renamed the NMU receptor type-1 and type-2, respectively (Fujii et al. 2000; Hedrick et al. 2000; Hosoya et al. 2000; Howard et al. 2000; Kojima et al. 2000; Raddatz et al. 2000). FM-3/GPR66 mRNA is widely distributed throughout peripheral tissues. In contrast, FM-4/TGR-1 mRNA is predominantly expressed in the central nervous system. The unique distributions of NMU receptors have provided novel insights into the function of NMU. Peripherally, NMU induces smooth muscle contraction (Minamino et al. 1985), elevates blood pressure (Minamino et al. 1985), modifies intestinal ion transport (Brown and Quito 1988), and promotes inflammation (Moriyama et al. 2005). Centrally, NMU functions in the regulation of feeding behaviors (Howard et al. 2000; Kojima et al. 2000), energy homeostasis (Nakazato et al. 2000; Hanada et al. 2003), stress responses (Hanada et al. 2001), circadian rhythms (Nakahara et al. 2004a), and nociceptive responses (Yu et al. 2003; Nakahara et al. 2004b).

In cases apart from ours, NMU was identified as an endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 from a library of bioactive molecules, including synthetic peptides. In contrast, we purified natural ligands from tissue extracts. Thus, we were able to succeed in the isolation of neuromedin S (NMS) (Mori et al. 2005). Gel filtration of rat small intestine extracts revealed a single agonist activity capable of increasing intracellular calcium ion con-

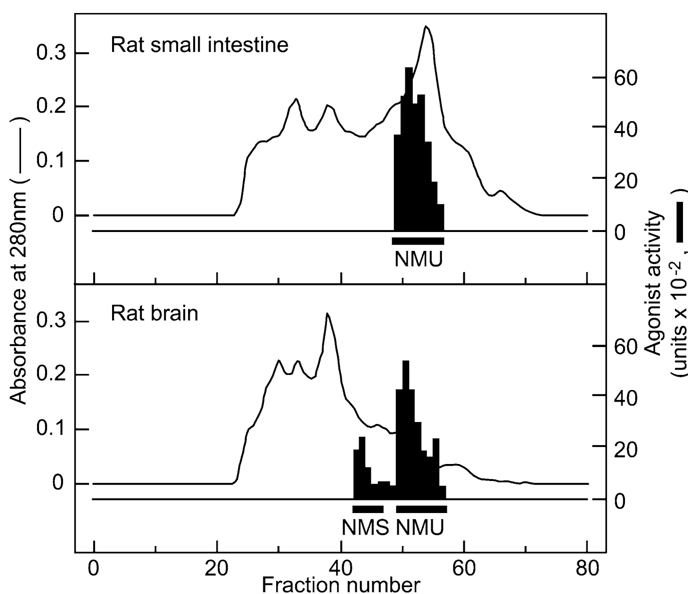

Fig. 1 Discovery of neuromedin S. Gel filtration on a Sephadex-G50 column of tissue extracts from rat small intestine (*upper panel*) and rat brain (*lower panel*). The *black bars* indicate agonist activity for FM-4/TGR-1 expressed recombinantly in CHO cells. Active fractions containing NMS and NMU are indicated

centrations in CHO cells expressing FM-4/TGR-1 (Fig. 1). In contrast, two agonist activities were found following gel filtration of rat brain extracts, indicating the presence of two endogenous ligands. The activity that eluted in the fractions at a smaller molecular mass corresponded to the activity found in the small intestine. NMU had previously been purified from rat small intestine as an endogenous ligand for FM-3/GPR66 (Kojima et al. 2000). Therefore, the second isolated activity likely corresponded to a novel neuropeptide. This ligand was designated neuromedin S (NMS) because of its specific expression in the suprachiasmatic nucleus (SCN) of the hypothalamus (Mori et al. 2005).

2 Structure of Neuromedin S

Rat NMS is a C-terminal amidated neuropeptide of 36 amino acid residues. NMS homologs have been identified to date in humans, rats, mice, and frogs (Fig. 2) (Mori et al. 2005; Chen et al. 2006). NMS is structurally related to NMU. The seven-residue C-terminal amidated sequence of NMS is identical to that of NMU; this structure is essential for NMU receptor binding (Minamino et al. 1985). The N-terminal portion of NMS, however, has no sequence homology to any known peptides or proteins. NMS is not a splice variant of NMU, because the human *NMS* and *NMU* genes map to chromosomes 2q11.2 and 4q12, respectively.

The NMS pro-protein and gene are structurally similar to those of NMU (Mori et al. 2005). The NMS pre-pro-protein contains four potential processing sites cleavable by subtilisin-like pro-protein convertases. These four sites are conserved in the NMU pre-pro-protein, indicating similar domain structures. NMS and NMU are produced from precursor proteins by proteolytic processing at the third and fourth of these sites. The amino acid sequences between the first and second processing sites exhibit homology to each other. As with the *NMU* gene, the *NMS* gene is composed of ten exons; the exon–intron boundaries in the NMS and NMU pre-pro-proteins are comparably conserved.

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LPRLLHTDSRMATIDFPKKDPTTSLGRPFFLFRPRN-NH2 rat NMS
LPRLLRLDSRMATVDFPKKDPTTSLGRPFFLFRPRN-NH2 mouse NMS
   ILQRGSGTAAVDFTKKDHTATWGRPFFLFRPRN-NH2 human NMS
   FLFQFSRAKDPSLKIGDSSGIVGRPFFLFRPRN-NH2 frog NMS
           YKV-NEYQGP-VAPSGGFFLFRPRN-NH2 rat NMU
           FKA--EYQSPSVGQSKGYFLFRPRN-NH2 mouse NMU
           FRVDEEFQSPFASQSRGYFLFRPRN-NH2 human NMU
             SDEEVQVPGGVISNGYFLFRPRN-NH2 frog NMU
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Fig. 2 Structure of NMS. The amino acid sequences of NMS are compared to those of NMU. Residues that are identical between the peptides are *shaded*

NMS was also isolated from the dermal venom of Eurasian bombinid toads, indicating that the *NMS* and *NMU* genes had diverged at the level of the Amphibia during evolution (Chen et al. 2006). A high degree of splice variations were observed in the NMS transcripts of toads. Differential splicing was highly conserved throughout tetrapod vertebrates. Alternative splice variants of NMS mRNA have also been cloned from mammals (unpublished data from the author's laboratory).

3 Receptors for Neuromedin S

NMS and NMU have the same core structure that is required for binding to their cognate receptors (see Sect. 2), suggesting that NMS shares at least two receptors, FM-3/GPR66 and FM-4/TGR-1, with NMU. In 1998, FM-3/GPR66 was cloned as an orphan GPCR similar to the neurotensin and growth hormone secretagogue receptor families (Tan et al. 1998). Subsequent homology search identified FM-4/TGR-1, which is similar to FM-3/GPR66 (Hosoya et al. 2000; Howard et al. 2000). Human FM-3/GPR66 exhibits 52% amino acid identity with human FM-4/TGR-1. Expression of FM-3/GPR66 mRNA is widely distributed throughout various tissues; high levels of expression are found in peripheral tissues (Fujii et al. 2000; Raddatz et al. 2000). In contrast, FM-4/TGR-1 mRNA is primarily expressed in the central nervous system (Hosoya et al. 2000; Raddatz et al. 2000). In rat brain, FM-4/TGR-1 mRNA expression is clearly detected in the paraventricular nucleus (PVN), the wall of the third ventricle in the hypothalamus, and the CA1 region of the hippocampus (Howard et al. 2000).

Pharmacological characteristics of NMS were examined using recombinant receptors exogenously expressed in CHO cells (Mori et al. 2005). Both NMS and NMU induce robust increases in intracellular calcium ion concentrations in CHO cells expressing either FM-3/GPR66 or FM-4/TGR-1. NMS and NMU possess similar efficacy and potency at these receptors. Competitive radioligand binding analysis demonstrated high-affinity binding of NMS to these receptors. NMS and NMU display similar inhibition constants for FM-3/GPR66. Interestingly, NMS has a higher binding affinity for FM-4/TGR-1 than NMU.

4 Distribution of Neuromedin S

The tissue distribution of NMS mRNA in rats was investigated by quantitative reverse transcription–polymerase chain reaction (Mori et al. 2005). NMS mRNA is primarily expressed in the central nervous system, spleen, and

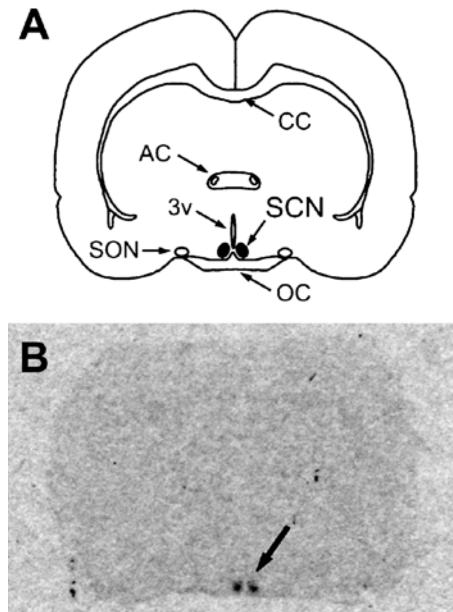

Fig. 3 Specific expression of NMS mRNA in the SCN of the hypothalamus. **A** Schematic representation of a coronal rat brain section containing the SCN. *3v* third ventricle, *AC* anterior commissure, *CC* corpus callosum, *OC* optic chiasm, *SCN* suprachiasmatic nucleus, *SON* supraoptic nucleus. **B** In-situ hybridization analysis of NMS mRNA expression in a coronal section of rat brain. Specific expression in the SCN is indicated by the *arrow*

testis. The highest levels of NMS mRNA are detected in the hypothalamus. In rat brain, NMS mRNA is predominantly expressed in the SCN of the hypothalamus; only minimal expression is found in other areas of the brain. The SCN, which is comprised of a pair of structures that contain about 20 000 neurons, is located on either side of the third ventricle, superior to the optic chiasma (Fig. 3a) (Reppert and Weaver 2001). In-situ hybridization histochemistry revealed that NMS mRNA expression is restricted to the SCN in rat brain (Fig. 3b) (Mori et al. 2005). The SCN is divided into ventrolateral and dorsomedial portions, in which the neuropeptides vasoactive intestinal polypeptide (VIP) and arginine vasopressin (AVP) are expressed, respectively. NMS mRNA is expressed in the ventrolateral SCN in a similar distribution to VIP mRNA.

5 Functions of Neuromedin S

Intracerebroventricular (ICV) administration of NMS to rats has primarily been used to investigate its central functions, as one of the NMS receptors,

Table 1 The central effects of NMS

Biological function	Comment
Circadian oscillator system	Induce phase shift of circadian rhythm
Feeding regulation	Decrease food intake
Gonadotropic axis	Increase LH release
Urinary output	Increase AVP release

AVP arginine vasopressin, *LH* luteinizing hormone

FM-4/TGR-1, is expressed within the central nervous system (see Sect. 3). These functions, summarized in Table 1, are outlined in this section.

5.1 Circadian Oscillator System

The SCN of the hypothalamus contains the master circadian pacemaker that governs the circadian rhythms underlying behavioral and physiological processes in mammals. Therefore, specific expression of NMS mRNA within the SCN strongly suggests a role for NMS in the circadian oscillator.

Examination of the time-dependent profile of NMS expression (Mori et al. 2005) revealed that NMS mRNA levels fluctuate rhythmically within the SCN in rats under 12-hour light/dark cycles; expression is high during the daytime and low at night. In contrast, NMS mRNA expression is stable when animals were maintained under conditions of constant darkness, indicating that the rhythmic expression of NMS within the SCN is not generated spontaneously. The intrinsic circadian rhythmicity of gene expression within the SCN is generated by clock-gene families of transcription factors that act at CACGTG E-box elements (Reppert and Weaver 2001). No CACGTG E-box element is present in the promoter region of the *NMS* gene (Mori et al. 2005), which is consistent with the observation that NMS mRNA expression levels do not oscillate under conditions of constant darkness. These data indicate that the expression of NMS is not under the control of clock-gene family proteins.

ICV administration of NMS affects the circadian rhythms in rats maintained under constant darkness (Mori et al. 2005). ICV injection of NMS during the subjective day elicits phase advance of the circadian rhythm of locomotor activity, while administration at the end of the subjective night induces a phase delay. The phase-response curve for NMS, the interaction between the circadian time of treatment and the magnitude of the phase advance/delay, is very similar to that for nonphotic stimuli. In the SCN, two receptors for NMS are expressed; FM-4/TGR-1 is expressed at higher levels than FM-3/GPR66 (Nakahara et al. 2004a; Mori et al. 2005). These data strongly

suggest that NMS functions to regulate the circadian pacemaker in an autocrine and/or paracrine manner within the SCN.

The pacemaker located in the SCN independently generates a near-24-hour circadian rhythm via an autoregulatory transcription/translation feedback loop composed of clock-gene families. This rhythm is entrained to the 24-hour daily cycle by periodic environmental cues, such as light and temperature, typical photic and nonphotic signals, respectively (Lowrey and Takahashi 2000; Reppert and Weaver 2001, 2002). The circadian rhythm can also be phase-shifted by photic and nonphotic stimuli (Mrosovsky 1996; Lowrey and Takahashi 2000). The ventrolateral SCN receives and integrates photic and nonphotic signals from the retina and other brain areas to entrain the circadian rhythm (Reppert and Weaver 2001). Several neuropeptides are implicated in circadian entrainment. VIP, which is expressed in the ventrolateral SCN, plays a role in photic entrainment of the circadian rhythm (Piggins and Cutler 2003). No SCN-intrinsic neuropeptide involved in the nonphotic circadian entrainment, however, has been identified. NMS mRNA is expressed in the ventrolateral portion of the SCN. As ICV administration of NMS induces a nonphotic-type phase shift in the circadian rhythm, NMS is a candidate for a nonphotic entrainment factor intrinsic to the SCN.

ICV administration of NMU also induces a nonphotic-type phase shift of the circadian rhythm (Nakahara et al. 2004a). NMS and NMU, however, appear to play distinct roles in the regulation of the circadian oscillator system. In contrast to NMS mRNA, NMU mRNA is expressed in the dorsomedial SCN (Graham et al. 2003), which is involved in the spontaneous generation of a strong rhythm. Moreover, its expression shows a circadian rhythm in rats maintained in constant darkness (Nakahara et al. 2004a), indicating that NMU, unlike NMS, is controlled by the circadian pacemaker. Therefore, NMU may act either as a part of the central clock mechanism or as an output signal of the SCN.

5.2 Feeding Regulation

NMU, an anorexigenic neuropeptide, functions in the central regulation of feeding behaviors (Howard et al. 2000; Kojima et al. 2000). Deficiency of NMU in mice leads to hyperphagia and obesity (Hanada et al. 2004). The *NMS* gene locus in humans is consistent with the location of a quantitative trait locus implicated in obesity (Mori et al. 2005).

Central NMS injection was used to investigate the role of NMS in feeding regulation (Ida et al. 2005). ICV administration of NMS decreases 12-hour food intake during the dark (night-equivalent) period in a dose-dependent manner. This anorexigenic effect is more potent than that induced by similar doses of NMU. The amount of food intake induced by ghrelin, neuropep-

tide Y, and agouti-related protein is reduced by co-administration of NMS. The PVN and arcuate nucleus (Arc) of the hypothalamus regulate feeding through a complex neuronal network of orexigenic and anorexigenic neuropeptides. Two anorexigenic neuropeptides, α-melanocyte-stimulating hormone (α-MSH) and corticotropin-releasing hormone (CRH), are necessary for NMS actions on feeding mediated by these nuclei. ICV administration of NMS increases proopiomelanocortin, precursor of α-MSH, and CRH mRNA levels in the Arc and PVN, respectively. The suppression of food intake by NMS can be attenuated by pretreatment with both SHU9119 and α-hCRF, antagonists of α-MSH and CRH, respectively.

When rat NMS and NMU were administered to avian species, an interesting phenomenon is observed (Shousha et al. 2005). ICV administration of rat NMS into adult Japanese quail results in the expected suppression of feeding behavior. In contrast, ICV-administered rat NMU increases food intake. Although both peptides decrease food intake in the rat, rat NMU exhibits an opposing effect to rat NMS on feeding regulation in Japanese quail, despite the fact that both peptides share the C-terminal core structure required for receptor binding (see Sect. 2).

While exogenously administered NMS strongly suppresses food intake, the physiological role of NMS in feeding regulation is not completely elucidated. Further investigation of the physiological significance of NMS is required. It is interesting how satiety signals affect NMS expression in the PVN and Arc of the hypothalamus, key centers of feeding regulation, as NMS mRNA is usually expressed at only low levels in these nuclei (Mori et al. 2005). NMS projections from the SCN to the PVN and/or Arc may be important in the regulation of feeding behavior. Multiple reports have linked the development of obesity to disruption of the circadian rhythm; mice deficient in *Clock*, a transcription factor that is an essential component of master circadian pacemaker in the SCN (Reppert and Weaver 2001), are hyperphagic and obese (Turek et al. 2005). The interaction between molecular control of the circadian rhythm and energy homeostasis remains unclear.

5.3 Gonadotropic Axis

NMS has also been implicated in the central regulation of the female gonadotropic axis (Vigo et al. 2007). In female rats, hypothalamic NMS mRNA is only minimally expressed during the neonatal period, but increases during the late-infantile and juvenile stages of postnatal development. NMS mRNA expression decreases again around puberty and is then restored upon reaching adulthood. In the hypothalamus of adult females, NMS expression fluctuates significantly throughout the estrous cycle; maximum expression is detected at proestrus. NMS mRNA levels in the hypothalamus are de-

creased after ovariectomy and rescued by progesterone, but not estradiol, supplementation.

ICV administration of NMS into pubertal female rats results in significant increases in serum luteinizing hormone (LH) levels. In adult females, the magnitude of the NMS-induced stimulatory effect on LH release is affected by the stage of the estrous cycle. When administered at estrus, NMS induces a potent increase in circulating LH level, whereas only modest LH secretion is induced by ICV-administered NMS at diestrus. Robust increases in LH secretion are also elicited by NMS in female rats during a short-term fast. In contrast, central administration of NMS reduces the elevated serum LH concentrations of ovariectomized rats.

5.4 Antidiuretic Action

ICV administration of NMS increases the plasma concentrations of AVP and decreases nocturnal urine volume (Sakamoto et al. 2007). NMS induces a more rapid release of AVP than NMU. A tenfold higher dose of NMU is necessary to exert the same effect as NMS. AVP is synthesized in the PVN and supraoptic nucleus (SON); FM-4/TGR-1 is expressed in these nuclei. Activation of a subset of AVP-producing neurons in the PVN and SON are indicated by the enhanced expression of c-Fos following ICV administration of NMS. These data suggest that NMS may function in the regulation of urinary output by altering AVP release from the PVN and SON.

6 Summary

Identification and examination of NMS has implicated several novel regulatory mechanisms functioning in physiologic processes. Current evidence suggests potential roles for NMS in the central regulation of circadian rhythms, feeding behaviors, LH secretion, and urinary output. The specific expression pattern of NMS in peripheral tissues, however, suggests tissue-specific physiological functions. High levels of NMS mRNA are observed in the spleen; the NMS receptor, FM-3/GPR66, is also expressed in the spleen and on immune cells (Hedrick et al. 2000), suggesting that NMS may play a role in immune responses.

NMS is a new bioactive peptide identified as a ligand for orphan GPCR, whose physiologic and pathologic roles are not fully understood. Engineering mice deficient in NMS and further detailed anatomical localization analysis will greatly improve our understanding of the functions of this peptide. Further characterization of NMS will hopefully provide novel insight into the regulatory mechanisms of physiological phenomena.

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Relaxin-3, INSL5, and Their Receptors

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Abstract Relaxin-3 (R3) is the most recently identified member of the insulin superfamily, which is composed of peptides with diverse sequences held together by characteristic disulfide links connecting A and B peptide chains. R3 has nearly exclusive expression in the brainstem. It was demonstrated to be an additional ligand for the relaxin receptor LGR7, which is a class-C hormone receptor type G-protein coupled receptor (GPCR). We recently identified R3 as a ligand for two orphan G-protein coupled receptors, GPCR135 (aka SALPR) and GPCR142 (aka GPR100), which are class-A GPCRs and typical neuropeptide receptors. The predominant brain expression for both R3 and GPCR135, coupled with their high affinity interaction, strongly suggests that R3 is the endogenous ligand for GPCR135. Both R3 and GPCR135 from different species are highly conserved from genetic sequences to in vitro pharmacology. In contrast, GPCR142 is a pseudogene in rats, and the mouse gene is less conserved with human GPCR142, suggesting that GPCR142 may have a diminished role as a receptor for R3 in rodents. Further studies of GPCR142 in monkeys, cows, and pigs demonstrate that GPCR142 in those species shares high homology to the human GPCR142, and that it behaves similarly to the human receptor in vitro. This suggests that GPCR142 has conserved functions in these non-rodent species, including humans. In addition, the tissue expression pattern of GPCR142, primarily in peripheral tissue, is drastically different from R3, suggesting that GPCR142 may have an endogenous ligand other than R3. Sequence analysis among insulin/relaxin family members shows that insulin-like peptide 5 (INSL5) is the closest member to R3. Pharmacological characterization shows that INSL5 is a specific agonist for GPCR142, but not for GPCR135. Specifically, INSL5 binds to and activates GPCR142 at high affinity. Although INSL5 binds to GPCR135 at low affinity, it does not activate GPCR135. INSL5 mRNA is primarily expressed in the periphery, and its expression pattern overlaps with that of GPCR142, consistent with INSL5 being the endogenous ligand for GPCR142. Endogenous ligands and receptors tend to co-evolve. Consequently, INSL5, like GPCR142, is a pseudogene in rats, which further implies that INSL5/GPCR142 is an endogenous ligand/receptor pair. R3 can activate GPCR135, GPCR142, and LGR7. Therefore, in vivo administration of R3 could potentially activate all three receptors, which complicates the functional studies of GPCR135. By substituting the A chain of R3 with the A chain of INSL5, we devised a chimeric peptide (R3/I5), which is about 1000-fold more selective for GPCR135 and GPCR142, than for LGR7. C-terminal truncation of this chimeric peptide resulted in a potent antagonist [R3(BΔ23-27)R/I5] for GPCR135 and GPCR142, with no affinity for LGR7. The selective agonist and antagonist pair is particularly helpful for in vivo studies of GPCR135 in rats lacking GPCR142. R3 is highly expressed in the nucleus incertus, a region of the brain stem, which has been known to send afferent connections to different brain regions. [^{125}I]R3/I5 is a radioligand that has an improved signal/noise ratio compared to [^{125}I]R3. Autoradiographic distribution of GPCR135 binding sites using [^{125}I]R3/I5 in rat brain shows that GPCR135 receptor is

prominent in many regions, including olfactory bulb, amygdala, thalamus, somatosensory cortex, and superior colliculus, which have been reported to have connections to the nucleus incertus. Different brain regions serve different functions. The expression pattern of R3 and GPCR135 in the brain suggests multiple functions of R3 and GPCR135. The high level expression of R3 in the brainstem co-localizes with the expression of corticotrophin releasing factor receptor 1 (CRF1), suggesting a potential role of R3/GPCR135 in stress response. Water-restraint stress-induced R3 mRNA expression in the brain stem seems to support this hypothesis. In addition, recent studies have shown that acute and chronic intracerebroventricular (i.c.v.) administration of R3 induces feeding in rats. More specifically, i.c.v. injection of R3/I5 (GPCR135 selective agonist) stimulates feeding in rats, an effect that can be blocked by the GPCR135-selective antagonist R3(BΔ23-27)/I5, thus confirming the involvement of R3 and GPCR135 in feeding. The availability of those pharmacological tools should greatly facilitate future studies of the physiology of GPCR135 and GPCR142.

1 Insulin Peptide Superfamily

The insulin family of peptides (Fig. 1) include insulin (Bell et al. 1980), IGF1 (Rinderknecht and Humbel 1978), IGF2 (Bell et al. 1984), relaxin (Hudson et al. 1983, 1984), insulin-like peptide 3 (INSL3) (Adham et al. 1993), INSL4 (Koman et al. 1996), INSL5 (Conlin et al. 1999), INSL6 (Lok et al. 2000), and Relaxin-3 (R3) (aka INSL7) (Bathgate et al. 2002). With the exception of IGF1 and IGF2, each member of the family is initially synthesized as a propeptide, and subsequentially processed into a mature peptide, which consists of two peptide subunits (an A-chain and a B-chain formed by the proteolytic

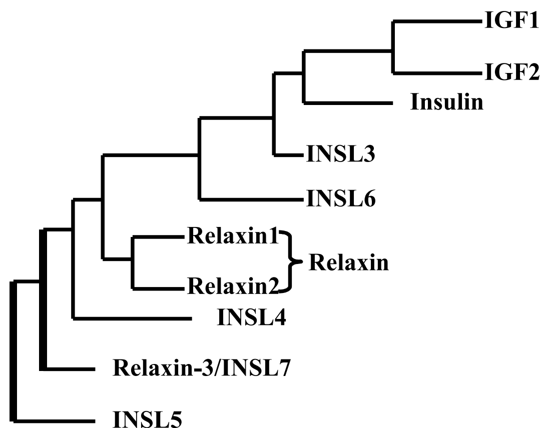

Fig. 1 Peptides in insulin superfamily. There are two relaxin genes (relaxin-1 & relaxin-3) in humans and primates and only one relaxin gene identified from other mammalian species. INSL4 is only identified in humans and primates. INSL5 is a pseudogene in rats. Insulin and relaxin-3 are the only members found in the chicken genome

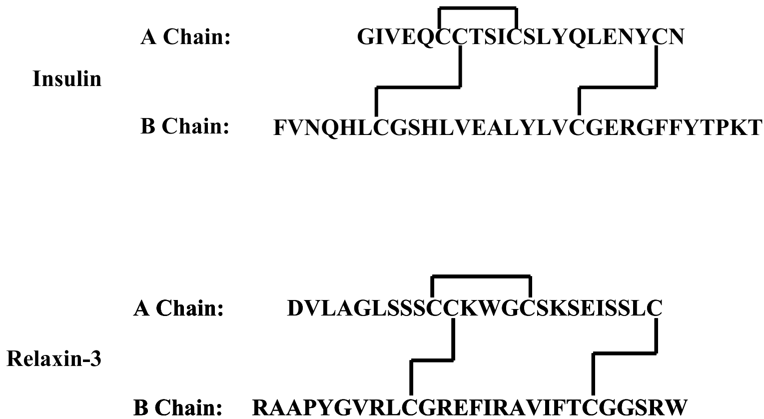

Fig. 2 Structure of insulin family of peptides. Sequences and structures of insulin and relaxin-3 are shown as examples. The characteristic disulfide bonds in the peptide shown above are conserved in all family members

removal of the C-peptide linking the A-chain and B-chain) that are linked by three conserved disulfide bonds (Fig. 2). For IGF1 and IGF2, there is no C-chain removal for the peptide maturation, and the A-chain and B-chain are fused into a continuous peptide. Insulin, IGF1, and IGF2 are known to be involved in the regulation of glucose metabolism. They signal through tyrosine kinase/growth factor receptors, which are single transmembrane receptors (Ullrich et al. 1985, 1986). Relaxin (for a review, see Sherwood 2004), a hormone highly expressed in ovary, plays multi-functional roles including uterus relaxation, reproductive tissue growth, and remodeling in females. In addition, relaxin has been reported to play important roles in non-reproductive functions, including wound healing (Yamaguchi and Yoshikawa 2001), cardiac protection (Casten et al. 1960), and allergic responses (Bani et al. 1995). The recent identification of two leucine-rich repeat-containing G-protein-coupled-receptors for relaxin, LGR7 and LGR8 (Hsu et al. 2002), opened a new era for the study of these insulin-like peptides. Activation of LGR7 and LGR8 by their ligands leads to increases in intracellular cAMP concentration. However, although relaxin activates LGR8 in vitro, recent studies have shown that LGR8 is likely the endogenous receptor for INSL3, and it may be involved in descent of testes (Kumagai et al. 2002; Bogatcheva et al. 2003).

The complexity has been increased by the discovery that R3 is capable of activating LGR7 (the relaxin receptor), but not LGR8 (the INSL3 receptor) (Sudo et al. 2003). Aside from the clear structural similarity to relaxin, the distribution of R3 mRNA in the brain stem (Burazin et al. 2002) signaled that the function of R3 would likely be distinct from the role of relaxin in reproductive biology. This article will summarize the recent findings in the R3 related field, including: the identification of R3 and INSL5 as ligands for two neuropeptide-like orphan GPCRs, GPCR135 and GPCR142; the creation

of selective pharmacological tools for GPCR135 and GPCR14; and the recent advancements in functional studies of the two receptors.

2 Is LGR7 the Physiological Receptor for R3?

R3 is the most recently identified member in the insulin/relaxin family (Bathgate et al. 2002). Its cDNA sequence was first identified from the Celera Genomics associated human databases. It was named relaxin-3 for the human gene, because of its structure/sequence homology (42%), its functional similarity to relaxin, and because in humans and primates there are already two known closely related relaxin genes (relaxin-1 (R1) and relaxin-2 (R2), collectively termed relaxin) that share 75% homology (Hudson et al. 1983, 1984). Following the recent finding that relaxin activates LGR7 (Hsu et al. 2002), synthetic R3 was also shown to be an active ligand for LGR7, albeit with significantly lower potency (Sudo et al. 2002). Since R3 mRNA is predominantly expressed in the brain (Bathgate et al. 2002; Burazin et al. 2002; Liu et al. 2003; Tanaka et al. 2005) it is unlikely that R3 will have the same physiological function as that of relaxin, which is primarily expressed in the periphery. In the periphery, it is evident that relaxin is the physiological ligand for LGR7, because relaxin is predominantly expressed in the ovary, and LGR7 is highly expressed in uterus. This is consistent with their functional roles in the female reproductive system, including uterus growth, remodeling during pregnancy, and relaxation during parturition (Hsu 2003; Sherwood 2004). Both relaxin and LGR7 female knockout mice (Zhao et al. 1999; Krajnc-Franken et al. 2004) show impaired nipple development and parturition, confirming the physiological roles of the relaxin/LGR7 pair in the reproductive system. In the brain, however, a physiological function for the R3 and LGR7 ligand-receptor pairing is less established, and it has been confounded by the lack of selective pharmacological tools. Nevertheless, it is reported that LGR7, relaxin, and R3 (Osheroff and Ho 1993; Liu et al. 2003) are all expressed in the brain with some overlap in anatomic distribution. Currently, although there is clear evidence to support the link between R3 and GPCR135 (an orphan GPCR recently identified as a receptor for R3, as described below), there is no evidence to exclude R3 as a physiological ligand for LGR7 in the central nervous system (CNS).

3 GPCR135 is Likely the Physiological Receptor for R3

The relationship between the various insulin-related peptides and their respective receptors was further complicated by the identification of an orphan GPCR, GPCR135, as the putative receptor for relaxin-3. Thus, in the rat

brain, we were able to identify a biological activity that activated GPCR135 (Fig. 3) using a GTPγS binding assay. This was achieved during the process of identifying the endogenous ligand for an orphan receptor GPCR135 (aka SALPR, Matsumoto et al. 2000), which is an attractive CNS target, due to its similarity to neuropeptide receptors and its unique distribution in key brain regions involved in the stress response system. Confirming this finding in a porcine brain extract (Fig. 3), we were able to do a large-scale purification of this unknown substance, resulting in a fraction that activated GPCR135 (Liu et al. 2003). The purified ligand turned out to be R3, the latest member of the insulin/relaxin family (Bathgate et al. 2002). To further characterize whether R3 is a ligand for GPCR135, human R3 was recombinantly expressed in mammalian cells, purified and radiolabeled with [^{125}I]. In a radioligand binding assay, [^{125}I]-R3 was found to bind to GPCR135 with high affinity (K_d = 0.3 nM) (Liu et al. 2003). Furthermore, in a functional assay, R3 potently inhibited cAMP accumulation in cells expressing GPCR135 (EC_{50} = 0.25 nM), indicating that GPCR135 is another receptor for R3 besides LGR7 (Liu et al. 2003). The mRNA expression profiles for both GPCR135 and R3 have been investigated by polymerase chain reactions (PCR) and in situ hybridization (Liu et al. 2003), showing that both mRNAs are predominantly expressed in the brain. More specifically, R3 mRNA is highly expressed in the nucleus incertus, a brainstem region that has projections to the hypothalamus and forebrain, where abundant GPCR135 mRNA expression is detected. The high affinity interaction between R3 and GPCR135, coupled with their

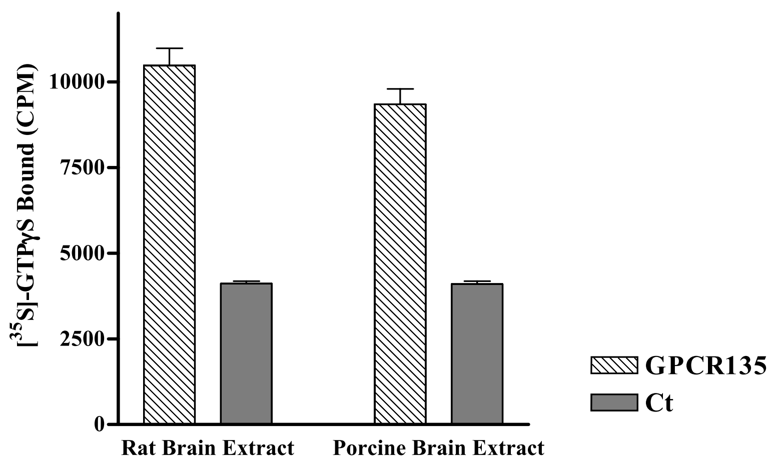

Fig. 3 Identification of ligand activity from rat and porcine brain extracts. GTPγS binding assay was used to characterize agonist stimulated GTP incorporation on GPCR135 expressing cell membranes. Mock transfected cell membranes were used as the negative control (Ct). [^{35}S]-GTPγS was added to the reaction at a final concentration of 1 nM. Rat or porcine brain extracts were added to cell membranes to stimulate [^{35}S]-GTPγS incorporation

predominant expression in the brain, strongly suggests that R3 is the endogenous ligand for GPCR135. Additional supporting evidence comes from the fact that R3 is the only known ligand that activates GPCR135. Members of the insulin/relaxin family (i.e., insulin, relaxin, INSL3, INSL4, INSL5, and INSL6) have been tested in GPCR135-expressing cell membranes. The results showed that R3 was the only ligand capable of stimulating GPCR135 (Liu et al. 2003).

4
GPCR135 and R3 are Highly Conserved Across Species

Between species, relaxin has low conservation at the amino acid levels. Humans have two relaxin genes [relaxin-1 (R1) and relaxin-2 (R2), collectively termed human relaxin] that share 75% homology due to gene duplication. Only one relaxin gene is reported for other non-primate species. Human R1 and R2 only share about 50% sequence identity to relaxins from other non-primate mammalian species (Table 1). R3 genes from humans, rats, mice, pigs, and Fugu fish have been recently reported (Chen et al. 2005), and, in contrast to relaxin, there is a high degree of conservation (>80%) across species (Table 1). Similar to R3, GPCR135 genes from humans, mice, and rats are also highly (≥85%) conserved (Chen et al. 2005). Searching the Fugu fish genome, five copies of GPCR135-like genes (Genbank Accession No: CAAB01000018, CAAB01001312, CAAB01001814, CAAB01003934; CAAB01004008) with reasonably high (57–61%) homology to mammalian GPCR135 have been found. Similarly, five copies of Fugu fish R3-like genes (Genbank Accession No: CAAB01000252, CAAB01001006, CAAB01001937, CAAB01001213, CAAB01004902) have also been identified (Hsu 2003). The homology of GPCR135 and R3 between different mammalian species and Fugu fish are listed in Table 1. Endogenous ligands and receptors are often linked during evolution. The high sequence conservation (82% for human and Fugu fish R3; 60% for human and Fugu fish GPCR135) between human and fish suggest that R3/GPCR135 is an endogenous ligand receptor pair that plays a conserved physiological role across different species.

5
Is There an Additional Receptor for R3?

Proteins with similar sequences tend to have similar functions. In an effort to find additional receptor(s) for R3, we searched the human genome for genes with similar sequences to GPCR135. Another orphan GPCR, which we call GPCR142 was identified. GPCR142 (aka GPR100, Fredriksson et al. 2003) shares about 40% sequence identity to GPCR135 at the amino acid level. To test whether R3 binds to GPCR142, [^{125}I]-R3 was used in a radioligand binding as-

Table 1 Percent of homology (amino acid sequence identity) of relaxin[a], R3, GPCR135, and GPCR142 between different species

Relaxin	Pig	Rat	Mouse	Dog
Human	52	51	50	46
Pig		50	50	52
Rat			70	44
Mouse				43

[a] Human Relaxin-2 sequence is used in comparison with relaxins from other species

Relaxin-3	Pig	Rat	Mouse	Fugu ruberipes*
Human	94	92	92	82
Pig		90	90	84
Rat			100	82
Mouse				82

* 5 Relaxin-3 like genes

GPCR135	Pig	Rat	Mouse	Fugu ruberipes*
Human	90	86	85	60
Pig		85	85	61
Rat			95	57
Mouse				58

* 5 GPCR135 like genes

GPCR142	Monkey	Cow	Pig	Mouse
Human	97	85	87	74
Monkey		86	86	74
Cow			91	71
Pig				72

Rat GPCR142 is a Pseudogene

say with membranes from GPCR142-expressing cells. The results showed that R3 binds GPCR142 with high affinity (K_d = 1.9 nM) (Liu et al. 2003), demonstrating that R3 is a ligand for GPCR142. In addition, in a GTPγS binding assay, R3 stimulated [^{35}S]-GTPγS incorporation in GPCR142 expressing cells (EC_{50} = 0.93 nM) (Liu et al. 2003). In a cAMP accumulation assay, R3 potently inhibits forskolin-induced cAMP accumulation in GPCR142 expressing cells (EC_{50} = 1.1 nM) (Liu et al. 2003), also indicating that GPCR142 is another receptor for R3. However, based on the tissue expression profile of GPCR142, it is questionable whether R3 is the physiological ligand for GPCR142. For example, the tissue expression profiles of R3 and GPCR142 did not substantially overlap (Fig. 4). While R3 is predominantly expressed in the brain, GPCR142 has abundant expression in peripheral tissues including the colon, thyroid, salivary gland, prostate, placenta, thymus, and kidney, which are tissues that do not express R3. Molecular and pharmacological analysis of GPCR142 from different species (Chen et al. 2005) also suggest that R3 is not the physiological ligand for GPCR142. Despite the high homology (92%) of R3 genes between humans, mice, and rats, the rodent GPCR142 is quite different from that of humans. The rat GPCR142 gene was found to be a pseudogene, and the mouse GPCR142 gene shows lower overall conservation (74% identity at the amino acid level) compared to human GPCR142 (the rate of conservation between the human GPCR135 and mouse GPCR135 is 85%). In addition, the mouse GPCR142 has a longer and different C-terminus (Chen et al. 2005). Furthermore, functionally, recombinant mouse GPCR142 behaves differently compared to human GPCR142. Specifically, in a Ca^{2+} mobilization assay, cells expressing human GPCR142 and $G_{\alpha 16}$ respond positively to R3 stimulation. In contrast, cells expressing mouse GPCR142 and $G_{\alpha 16}$ do not demonstrate detectable Ca^{2+} mobilization. Since GPCR135 and R3 in mice and rats are still

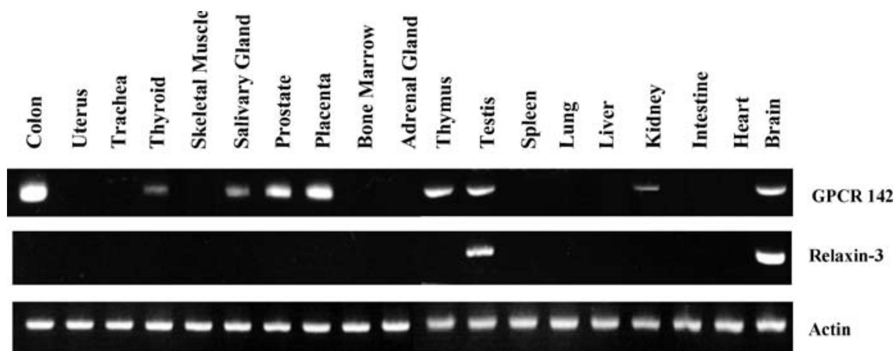

Fig. 4 Comparison of tissue expression patterns between relaxin-3 and GPCR142. cDNAs from different human tissues were PCR amplified using primers designed specifically to human relaxin-3 or GPCR142, respectively. The PCR products were run in agarose gel, stained with ethidium bromide, and visualized under UV. Human beta-actin was amplified in parallel as internal controls for PCR

highly conserved with humans and other species, these results also suggest that R3 may not be the physiological ligand for GPCR142.

6
Does GPCR142 Play a Significant Role in Higher Species?

Since GPCR142 demonstrates significant sequence variation in mice, and since it is a pseudogene in rats, it is questionable whether GPCR142 plays a functional role in humans. The analysis of GPCR142 from other mammalian species may help evaluate the significance of GPCR142 in human physiology. Monkey, cow, and pig GPCR142 genes have been cloned (Chen et al. 2005) and have been found to be highly conserved (Table 1). Human GPCR142 shares 97%, 85%, and 87% sequence identity to the monkey, cow, and pig GPCR142, respectively. In addition, pharmacological characterization of recombinant GPCR142 receptors from these species demonstrated that R3 binds to GPCR142 at high affinity (Chen et al. 2005). In GTPγS binding assays, human R3 stimulated monkey, bovine, and porcine GPCR142 with EC_{50} values around 1 nM. The high conservation of human, monkey, cow, and pig GPCR142 suggests that GPCR142 may still play a functional role in humans. A patent search shows a published patent application (WO 2005/124361 A2) describing that GPCR142 knockout mice have lower basal glucose levels and elevated insulin levels. This suggests that GPCR142 may play a role in metabolism. Because the GPCR142 receptor in mice and rats (two frequently used species for animal studies) demonstrate significant differences from GPCR142 receptor in humans, other mammalian species may have to be used for future functional studies. The cloning and characterization of monkey, cow and pig GPCR142 provide useful information for the future physiological study of GPCR142.

7
Is There an Additional Ligand for GPCR142?

Searching for ligands for GPCR142 other than R3, members of the insulin/relaxin family were considered as the top candidates. Relaxin, insulin, INSL3, INSL4, and INSL6 have been tested and found to neither bind to nor activate GPCR142 (Liu et al. 2003). INSL5 is the member in the insulin/relaxin family with the highest homology (53%) to R3, suggesting that INSL5 may be a potential ligand for GPCR142. Lacking a commercial source of INSL5 peptide, we expressed the human INSL5 peptide in mammalian cells and purified the mature peptide. The purified INSL5 was tested as a putative ligand for GPCR142, as well as for GPCR135, LGR7, and LGR8. The results showed that [^{125}I]-INSL5 does not have any affinity for LGR7 and LGR8, but has high

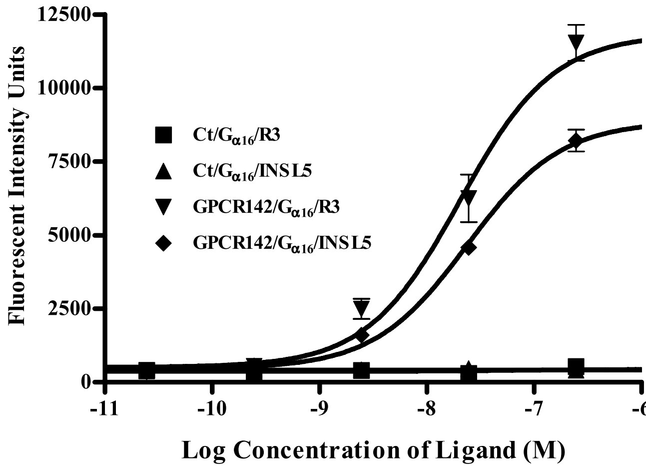

Fig. 5 INSL5 stimulates Ca^{2+} mobilization in HEK293 Cells, expressing GPCR142 and $G_{\alpha16}$. HEK293 cells were co-transfected with GPCR142 and $G_{\alpha16}$. The transfected cells were loaded with Ca^{2+} dye Fluo-3. R3 or INSL5 at different concentrations were used to stimulate intracellular Ca^{2+} mobilization (monitored by a FLIPR instrument). Cells transfected by $G_{\alpha16}$ alone were used as the controls in the same experiment

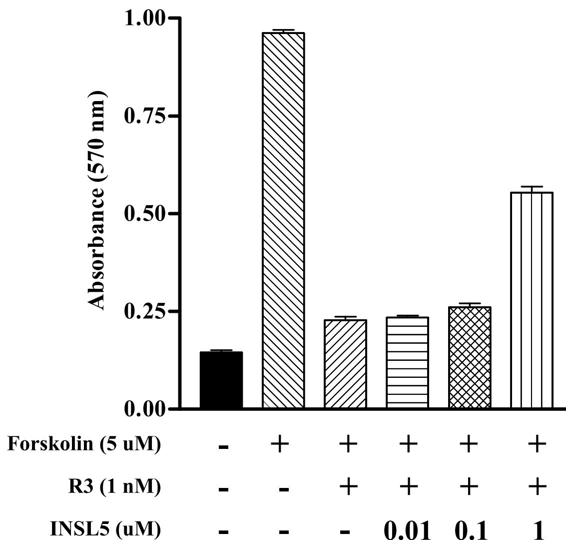

Fig. 6 INSL5 inhibits relaxin-3's activity in SK-N-MC/β-gal cells expressing GPCR135. SK-N-MC/β-gal cells harbor a β-galacosidase gene under control of CRE promoter. An increase in intracellular cAMP concentration is correlated with β-gal expression, whose activity is measured using Chlorophenol Red-β-D-Galactopyranoside as the substrate. The absorbance is read at wavelength of 570 nm. Forskolin (5 μM) was used to induce the intracellular cAMP accumulation. Relaxin-3 (R3) (1 nM) was used to inhibit forskolin induced cAMP accumulation. Different concentrations of INSL5 were used to suppress the activity of R3, and to restore forskolin induced cAMP accumulation

affinity for GPCR142 (K_d = 1.5 nM), and low affinity for GPCR135 (K_d = 1 μM) (Liu et al. 2005). INSL5 was also tested for its agonist activity for GPCR135, GPCR142, LGR7, and LGR8. The results demonstrated that INSL5 only activates GPCR142, but none of the other receptors. In addition to stimulating GTPγS binding and inhibiting cAMP accumulation in GPCR142-expressing cells, INSL5 stimulates Ca^{2+} in HEK293 cells co-expressing $G_{\alpha 16}$ (Fig. 5). Since GPCR135 demonstrates an affinity, albeit low, but does not respond to INSL5, we further investigated whether INSL5 has any antagonist activity for GPCR135. Therefore, INSL5 was tested for its ability to inhibit the agonist activity of R3 at the GPCR135 receptor. The results showed that INSL5 does inhibit R3's activity for GPCR135, although at low potency (IC_{50} = 1 μM) (Fig. 6).

8
INSL5 Is Likely the Endogenous Receptor for GPCR142

GPCR142 has a markedly different tissue expression profile from that of R3 (Fig. 4), suggesting that the endogenous ligand for GPCR142 may be another peptide other than R3. The observations that INSL5 can also bind to and activate GPCR142 shed light on the search for the endogenous ligand for GPCR142. We investigated the tissue expression profile of INSL5 mRNA in different human tissues. We found that INSL5 mRNA is expressed in the brain, kidney, prostate, ovary, thymus, bone marrow, placenta, spleen, heart, and colon (Liu et al. 2005). Among those tissues expressing INSL5 mRNA, it was found that the brain, kidney, prostate, thymus, placenta, and colon also express GPCR142 mRNA (Liu et al. 2003), suggesting that INSL5 may be the endogenous ligand for GPCR142. Since INSL5 only binds but does not activate GPCR135 at very low affinity, it is unlikely that INSL5 plays a physiological role for GPCR135. Knowing that GPCR142 is the only receptor for INSL5, coupled with their high affinity interaction and overlapping tissue expression patterns, we strongly believe that INSL5 is a physiological ligand for GPCR142. Rat GPCR142 is a pseudogene (see above). By searching and analyzing the rat genomic DNA database, we found that rat INSL5 is also a pseudogene (Liu et al. 2005). Given that endogenous ligand/receptor pairs tend to evolve together, these results further support, albeit indirectly, the hypothesis that INSL5 is the physiological ligand for GPCR142.

9
Insulin Family of Peptides Activate Three Different Classes of Receptors

With the identification of R3 and INSL5 as ligands for GPCR135 and GPCR142, respectively, three classes of receptors have now been identified for insulin family of peptides. It is well-understood that insulin and IGFs sig-

nal through insulin receptor (IR) and IGF receptor (IGFR), which are single transmembrane cytokine/growth factor type tyrosine kinase receptors (Ullrich et al. 1985, 1986) (Fig. 7). Relaxin and INSL3 receptors, LGR7 and LGR8 (Hsu et al. 2002), belong to the class-C hormone receptor family of GPCR, which are similar to thyroid stimulatory hormone receptor (Nagayama et al. 1989; Laugwitz et al.1996) and luteinizing hormone receptor (Gudermann et al. 1992). These hormone receptor GPCRs typically have long N-terminal extracellular domains (>300 amino acids, Fig. 7). Both LGR7 and LGR8 are linked to G_s protein. Activation of those receptors leads to intracellular accumulation of cAMP (Hsu et al. 2002). In contrast to LGR7 and LGR8, GPCR135 and GPCR142 are typical class-A neuropeptide-like GPCRs. Receptors in this subclass normally have short N-terminal extra-cellular domains (<100 amino acids) (Fig. 7). Both GPCR135 and GPCR142 are coupled to $G_{i/o}$ proteins. Consequently, the activation of these receptors leads to the inhibition of cAMP in cells (Liu et al. 2003). One might wonder how the insulin family of peptides which have a high structural similarity activates receptors in three different classes. While the unifying answer to this question remains uncertain, clear differences have been observed between insulin/IGF and other members of this ligand family. Sequence comparison (Fig. 8) of peptides in the family shows that two positively charged residues corresponding to Arg^8 and Arg^{16} of the R3 B-chain are conserved in R1, R2, R3, INSL3, INSL4, INSL5, and INSL6, but not in insulin and IGFs. Mutation studies have shown

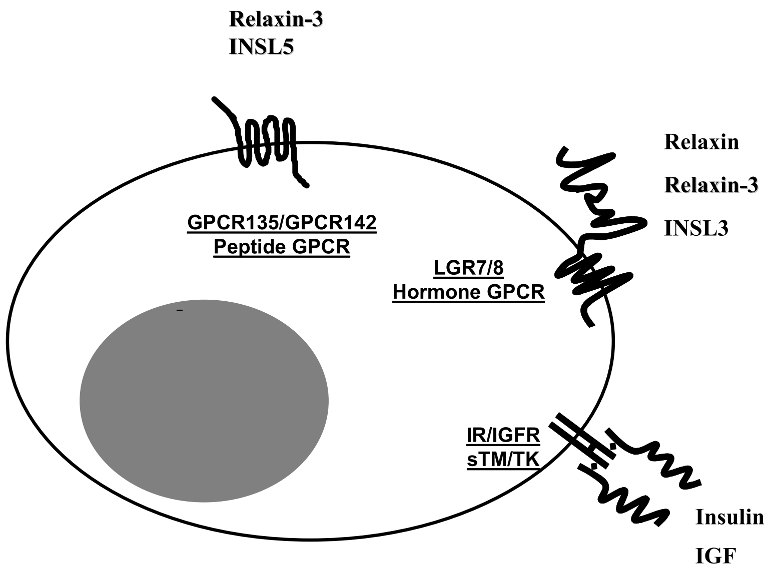

Fig. 7 Schematic diagram of the three classes of receptors activated by insulin family of peptides. IR = insulin receptor; IGFR = IGF receptor; sTM = single transmembrane; TK = tyrosine kinase

		B-chain	A-chain
Relaxin Subfamily	**Relaxin-1:**	KWKDDVI**K**LCGRELV**R**AQIAICGMSTWS	RPYVALFEKCCLIGCTKRSLAKYC
	Relaxin-2:	DSWMEEVI**K**LCGRELV**R**AQIAICGMSTWS	QLYSALANKCCHVGCTKRSLARFC
	Relaxin-3:	RAAPYGV**R**LCGREFI**R**AVIFTCGGSRW	DVLAGLSSSCCKWGCSKSEISSLC
	INSL3:	PTPEMRE**K**LCGHHFV**R**ALVRVCGGPRWSTEA	NPARYCCLSGCTQQDLLTLC
	***INSL4:**	ESLAAEL**R**GCGPRFG**K**HLLSYCPMPEK	SGRHRFDPFCCEVICDDGTSVKLCT
	INSL5:	KESV**R**LCGLEYI**R**TVIYICASSRW	<EDLQTLCCTDGCSMTDLSALC
	***INSL6:**	ISSAR**K**LCGRYLV**K**EIEKLCGHANWSQF	GYSEKCCLTGCTKEELSIACLPYIDFKRL
Insulin Subfamily	**Insulin:**	FVNQHLCGSHLVEALYLVCGERGFFYTPKT	GIVEQCCTSICSLYQLENYCN
	IGF1:	GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA	
	IGF2:	AYRPSETLCGGELVDTLQFVCGDRGFYFSRP—ASRVSRRSR---GIVEECCFRSCDLALLETYCATPAKSE	

Fig. 8 Amino acid sequence comparison of members in the insulin family. The two conserved positive charged amino acids in the B-chains of relaxin-1, relaxin-3, relaxin-3, INSL3, INSL4, INSL5, and INSL6 were highlighted in bold letters. * The B-chain and A-chain termini for INSL4 and INSL6 are based on predictions

that those two residues are important for R3 to bind to GPCR135, GPCR142, and LGR7 receptor binding (Liu et al. 2007). The fact that the receptors for R1, R2, R3, INSL3, and INSL5 are recently identified as GPCRs (LGRs and neuropeptide receptors) suggests that the receptors for INSL4 and INSL6 might be GPCRs. Therefore, it is appropriate to divide the insulin/relaxin family of peptides in two subfamilies. While R1, R2, R3, INSL3, INSL4, INSL5, and INSL6 that signal (or are likely to signal) through GPCRs can be classed into a relaxin subfamily, insulin and IGFs that signal through growth factor receptors/tyrosine kinase can be classed into an insulin subfamily.

10 Relaxin-3, the Ancestor Gene for the Relaxin Subfamily of Peptides

The high structural similarity among the relaxin subfamily of peptides strongly suggests that they evolved from a common ancestor gene. Seven R3-related genes, including R1, R2, INSL3, INSL4, INSL5, INSL6, and R3, exist in the human genome. While INSL4 and R1 are only found in the human and primate genomes, INSL5 has been found as a pseudogene in rats. Except for R3, which is highly conserved from different mammalian species, all other members are much less conserved. Searching the puffer fish genome revealed at least five R3-like genes, which exhibit high sequence identity to each other and to the human R3, but a much lower degree of identity to other mammalian R3-related peptides. Similarly, in zebra fish, multiple R3-like peptide genes have been identified, and no clear orthologs can be identified for other mammalian R3-related peptides (Hsu et al. 2005). In addition, searching the chicken protein databases shows a single protein (Genbank Accession No. XP_424810) that is highly homologous to the human R3, but none to other R3-related peptides. These data strongly indicate that R3 is the most conserved gene in the relaxin subfamily. The multiple copies of R3-like genes in fish suggest that there was a duplication of the R3 gene during evolution. The modern mammalian and vertebrate relaxin subfamily members are likely

evolved from a R3-like ancestor gene. The fact that R3 is conserved among different species at such a high level suggests that it plays a very conserved role in many different species.

11 Creation of Selective Pharmacological Tools for GPCR135 In Vivo Studies

11.1 R3/I5 Chimeric Peptides as a Selective Agonist for GPCR135 and GPCR142 over LGR7

Identification of R3 as the ligand for GPCR135 and GPCR142 has created new opportunities for physiological studies of relaxin-related peptides in the CNS and periphery. However, the interpretation of the in vivo effects of R3 administration is complicated by R3 agonism at GPCR142 and LGR7, as well as at GPCR135. Although GPCR142 is a pseudogene in rats, and the analysis in that species is simplified, an agonist that is specific to GPCR135, especially with respect to LGR7, would allow clearer delineation of the functions of the R3/GPCR135 system.

Modifications were made to the R3 molecule in an effort to find a specific GPCR135 agonist. Early analysis indicated the B-chain of R3 is sufficient to activate GPCR135 and GPCR142, although the potency (EC_{50}) is much lower than mature R3 (Liu et al. 2003). The B-chain of R3 is not an agonist for either LGR7 or LGR8 (Liu et al. 2005). Therefore, chimeras of the R3 B-chain with A-chains from the other relaxin subfamily members, such as relaxin 1(R1), relaxin 2 (R2), INSL 3 (I3), INSL 4 (I4), INSL 5 (I5), and INSL 6 (I6), were recombinantly produced to look for differential interactions with GPCR135, GPCR142, LGR7, and LGR8 (Liu et al. 2005).

Results of these chimeric peptides binding to human GPCR135, GPCR142, LGR7, and LGR8 are summarized in Table 2. All of the chimeras, except R3/I4, bind to both GPCR135 and GPCR142 with similar high affinities (R3/I4 demonstrated very low affinity for GPCR135 and GPCR142, and no affinity for LGR7 and LGR8). LGR7 binds R3/R1, R3/R2, and R3/I3 with high affinities (low nM IC_{50}) similar to R3, while R3/I6 is bound with a slightly lower affinity (13 nM). A chimera of the R3 B-chain bonded to the I5 A-chain (R3/I5) is exceptional, as it is selective for GPCR135 and GPCR142, compared to LGR7.

Corresponding functional data for agonist activity of the same peptide chimeras and receptors is provided in Table 3. All of the chimeras were agonists for both GPCR135 and GPCR142. Agonist potencies mirrored the trends seen in competitive binding assays, with R3/R1, R3/R2, R3/I3, and R3/I5 showing potencies similar to R3 with low nanomolar EC_{50} values. The R3/I6 chimera was slightly less potent, and R3/I4 was nearly impotent, which is also consistent with the binding assay data described above. Chimera's agonism at

Table 2 IC_{50} values (nM) required for different chimeric peptides to inhibit [^{125}I]-R3 binding to human GPCR135, GPCR142 and LGR7, or [^{125}I]-I3 to bind LGR8

	GPCR135	GPCR142	LGR7	LGR8
Relaxin-3	0.30 ± 0.04	1.1 ± 0.19	2.0 ± 0.24	Inactive
R3/R1	2.9 ± 0.35	2.6 ± 0.31	5.1 ± 0.78	>1000
R3/R2	1.2 ± 0.27	1.8 ± 0.36	1.5 ± 0.37	615 ± 55
R3/I3	2.3 ± 0.32	3.5 ± 0.47	2.3 ± 0.35	475 ± 32
R3/I4	>1000	>1000	Inactive	Inactive
R3/I5	0.68 ± 0.13	1.4 ± 0.35	724 ± 83	Inactive
R3/I6	9.5 ± 1.7	11.3 ± 2.3	12.9 ± 1.9	Inactive

IC_{50} values (mean ±SEM, $N = 3$) were defined as the ligand concentration inhibiting 50% of the maximum specific binding of [^{125}I]R3 (for GPCR135 and GPCR142) or [^{125}I]R2 (for LGR7 and LGR8). Binding assays used membrane homogenates of the recombinant receptor of interest expressed in COS-7 cells, 100 pM tracer and dose responses of the indicated chimeric ligands in a total volume of 200 μl

Table 3 EC_{50} values required for chimeric peptides to stimulate cells expressing human GPCR135, GPCR142, LGR7, or LGR8

	GPCR135	GPCR142	LGR7	LGR8
Relaxin-3	0.42 ± 0.05	0.91 ± 0.12	1.5 ± 0.17	Inactive
R3/R1	1.8 ± 0.27	2.2 ± 0.31	4.5 ± 0.51	>1000
R3/R2	1.1 ± 0.14	1.4 ± 0.26	1.1 ± 0.16	575 ± 65
R3/I3	2.2 ± 0.31	2.9 ± 0.28	2.5 ± 0.32	258 ± 42
R3/I4	>1000	>1000	Inactive	Inactive
R3/I5	0.45 ± 0.10	0.91 ± 0.21	412 ± 74	Inactive
R3/I6	8.4 ± 1.6	11.2 ± 2.5	13.7 ± 1.9	Inactive

Functional assays used SK-N-MC cells stably expressing both the receptor of interest and a β-galactosidase gene under the control of a cyclic AMP response element. Activation of G_s linked GPCRs (LGR7 and LGR8) leads to increased β-galactosidase expression in these cells. For G_i linked receptors (GPCR135 and GPCR142) the assays were set up to examine the chimeric compounds' ability to inhibit the β-galactosidase response to 5 μM forskolin. The EC_{50} was defined for LGR7 and LGR8 binding assays as the compound concentration yielding half maximal induction of the β-galactosidase reporter. Similarly, the IC_{50} value for GPCR135 and GPCR142 was defined as the compound concentration that inhibiting the β-galactosidase response to forskolin by 50%

the LGR7 receptor was also consistent with the ligand binding data. R3/R1, R3/R2, and R3/I3 are potent LGR7 agonists, R3/I6 is slightly less potent, and R3/I5 has a very low potency as a LGR7 agonist.

Since R3 is not a LGR8 agonist, one might expect chimeric receptors of the R3 B-chain with A-chain of other family members to be inactive at LGR8.

While R3/I4, R3/I5, and R3/I6 were inactive at LGR8, the R3/R1 chimera had marginal agonist activity, and the R3/R2 and R3/I3 chimeras were weak LGR8 agonists.

11.2
R3 Chimeric Peptide Studies Shed Light on the Mechanism of the Ligand/Receptor Interactions Between Relaxin-Related Peptides and Their Receptors

A comparison of the results of the binding and functional assays for the chimeras suggests that the ligand requirements of the LGR relaxin receptors differ from GPCR135 and GPCR142. For instance, the R3 B-chain is sufficient for receptor binding and activation of both GPCR135 and GPCR142, and for both these receptors the A-chain improves the potency (EC_{50} or IC_{50}), yet the exact nature of the A-chain (i.e., the A-chain of either R1, R2, R3, INSL5, or INSL6) is much less important (with the exception of the I4 A-chain that seems to prevent interaction with all 4 receptors). This suggests the A-chain may be most useful in binding to the free sulfhydryl groups and optimizing the B-chain's tertiary structure for receptor interaction at this class of GPCRs. The LGR7 and LGR8 are more selective with respect to the A chain that is present in the chimera. LGR7 binds R3/R1, R3/R2, R3/I3, and R3/I6 with high affinity, while retaining very low affinity for R3/I5. Similarly, LGR8 binds R3/R1, R3/R2, and R3/I3 with low affinity, but has no affinity for chimeras of the R3 B-chain with A-chains of other family members. The B-chains of INSL3 and relaxin have been shown to be important for interaction with LGR7 and LGR8, respectively (Bullesbach et al. 1992; Bullesbach and Schwabe 1999). Thus, it seems that both peptide chains are critical to binding and/or activation of the LGR7 and LGR8. R1, R2, and INSL3 are natural agonists for LGR8. The fact that R3 is not an agonist for LGR8, but chimeras R3/R1, R3/R2, and R3/I3 are agonists for LGR8, suggests that the A-chain of R1, R2, and INSL3 may possess the LGR8 activation domain. Supporting this hypothesis, Bullesbach and Schwabe (2005) showed that INSL3 with a truncated A-chain binds LGR8 at high affinity, but lacks receptor agonist activity. Taken together, data from chimeric peptide study suggest that LGR relaxin receptors recognize elements from both peptide chains, while GPCR135 and GPCR142 receptors interact primarily with the B-chain of R3, which is optimally presented by the disulfide bonded A-chain.

11.3
[^{125}I]R3/I5 Is a Valuable Tool for Mapping GPCR135 Binding Sites in the Brain

Different brain regions play different functional roles. Knowing the precise expression pattern of a receptor in the brain will help us to establish the links between the receptor and the brain functions. Therefore, receptor localization studies are very important for the functional study of the receptor in

vivo. However, since wild type R3 is a very hydrophobic peptide, [^{125}I]R3 produces a high degree of non-specific binding to brain slices. Therefore, attempts to use this radioligand to detect GPCR135 binding sites in the rat brain have not been successful. In contrast, the R3/I5 chimeric peptide is much more hydrophilic due to the replacement of the hydrophobic R3 A-chain with the hydrophilic INSL5 A-chain. As a result, [^{125}I]R3/I5 produces much lower levels of non-specific binding, allowing the detection of [^{125}I]R3/I5 binding sites in rat brain slices, with GPCR135 mRNA expression examined in adjacent sections (Liu et al. 2005). The distribution of GPCR135 mRNA is generally similar to binding sites visualized with [^{125}I]R3/I5 (Fig. 9), with GPCR135 mRNA expression and [^{125}I]R3/I5 binding sites being prominent in the olfactory bulb, amygdala, thalamus, somatosensory cortex, and superior colliculus. Specific hybridization and binding sites were also found in other areas, such as the septum, habenula, paraventricular nucleus (PVN) of hypothalamus, hippocampal formation, interpeduncular nucleus, and in brain stem regions. Both GPCR135 mRNA and [^{125}I]R3/I5 binding sites were also found in the nucleus incertus (Sutton et al. 2004, 2005), which is the predominant source of R3 in the central nervous system (Burazin et al. 2002; Liu et al. 2003; Tanaka et al. 2005).

GPCR135 mRNA and [^{125}I]R3/I5 binding sites in the central nervous system share a common feature of involvement in sensory perception. For example, the olfactory bulbs, which prominently express GPCR135 mRNA and have high levels of [^{125}I]R3/I5 binding, are involved in the transmission of olfactory signals. The superior colliculus, another nucleus with strong GPCR135 mRNA expression, which also shows [^{125}I]R3/I5 binding, is known for its involvement in visual attention and saccadic eye movements. Auditory signals also pass through the superior colliculus on their way to higher centers. The lateral septum, which is involved in processing tactile information, expresses GPCR135 mRNA and has [^{125}I]R3/I5 binding sites. Expression of GPCR135 mRNA and the observation of [^{125}I]R3/I5 binding sites in these areas is consistent with a role for GPCR135 in the processing of visual, auditory, and tactile senses.

In the central nervous system, R3 is most prominently expressed in the nucleus incertus (Burazin et al. 2002; Liu et al. 2003; Tanaka et al. 2005), and is found to a lesser extent in the dentate gyrus of the hippocampus (Burazin et al. 2002). Projections of the nucleus incertus, which have been described (Goto et al. 2001), are generally consistent with [^{125}I]R3/I5 binding sites and the expression of GPCR135 mRNA. The nucleus incertus and dentate gyrus also prominently express the type 1 CRF receptor (Potter et al. 1994). Indeed, a number of other brain areas expressing GPCR135, such as the olfactory bulb, amygdala, superior colliculus, and inferior colliculus, also express CRF1 receptor (Potter et al. 1994; Charmers et al. 1995).

Anatomical data describing the brain distribution of GPCR135 mRNA, [^{125}I]R3/I5 binding sites, and R3 mRNA are consistent with the role of the

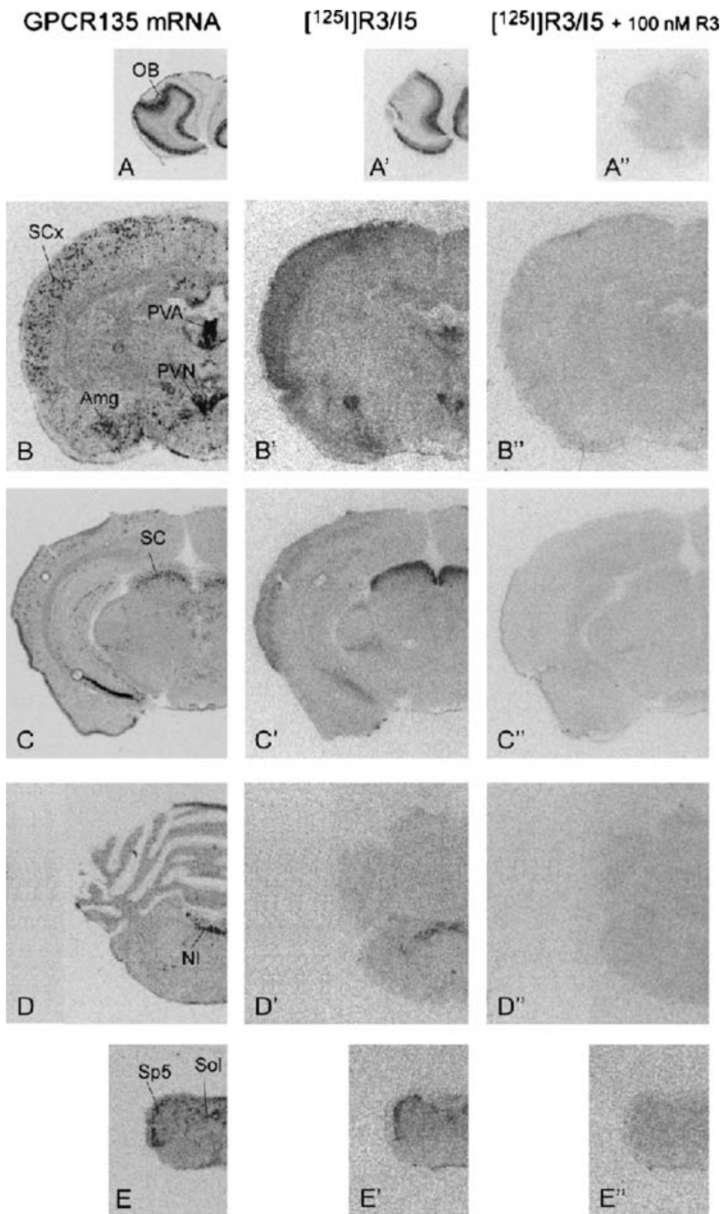

Fig. 9 Rostro-caudal distribution of GRPC135 mRNA (*A*, *B*, *C*, *D*, *E*) and [^{125}I]R3/I5 binding sites (*A′*, *B′*, *C′*, *D′*, *E′*) in autoradiograms from a series of coronal section through the rat brain (left hemisphere). Non-specific binding was determined in the presence of 100 nM R3 (*A″*, *B″*, *C″*, *D″*, *E″*). Amg, amygdala; NI, nucleus incertus; OB, olfactory bulb; PVA, paraventricular thalamic nucleus; PVN, paraventricular hypothalamic nucleus; SCx, somatosensory cortex; SC, superior colliculus; Sol, nucleus of solitary tract; Sp5, Spinal trigeminal tract

R3/GPCR135 signaling system in sensory processing. It has been suggested that nucleus incertus is involved in the extra-pituitary actions of CRF (Goto et al. 2001). Since GPCR135 mRNA and [^{125}I]R3/I5 binding sites are correlated with the expression of CRF1 receptor mRNA in areas involved in processing of sensory signals, the R3/GPCR135 signaling system might play a role in sensory processing, particularly under stressful circumstances.

11.4
R3(B△23-27)R/I5 Is a Selective Antagonist for GPCR135 and GPCR142

Mutation studies (Kuei et al. 2007) have shown that C-terminus of the R3 B-chain is important for R3 to activate GPCR135 and GPCR142. Point mutations at the C-terminal residues resulted in peptides with high affinity for GPCR135, but very little or no agonist activity (Kuei et al. 2007), sug-

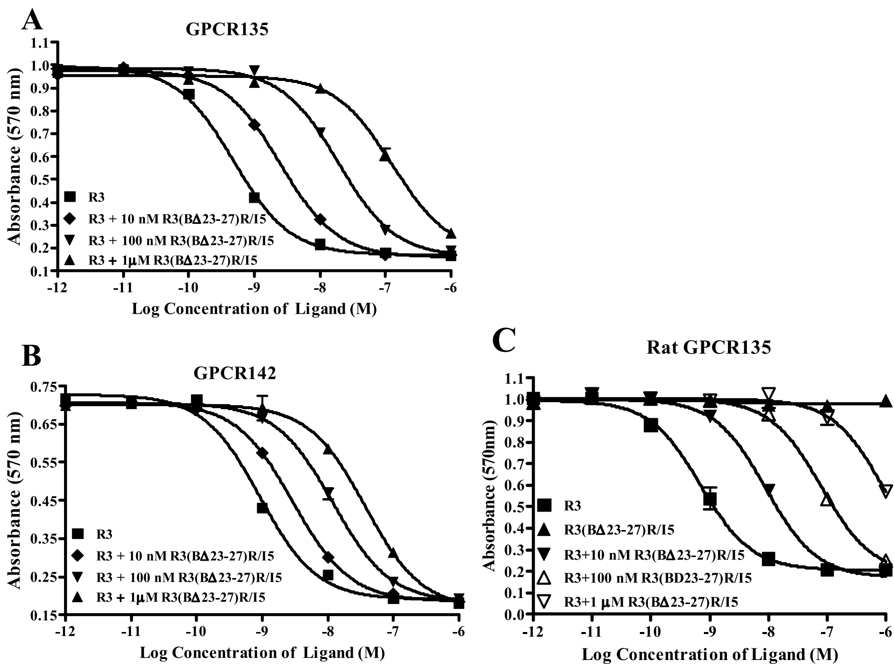

Fig. 10 Characterization of R3(B△23-27)R/I5 as an antagonist for human GPCR135, GPCR142, and rat GPCR135. In a functional assay using SK-N-MC/CRE-β-gal cells expressing human GPCR135 (**A**), GPCR142 (**B**), or rat GPCR135 (**C**), ascending concentrations of relaxin-3 (R3) were used to generate dose response curves either in the absence or presence of 10 nM, 100 nM, or 1 μM R3(B△23-27)R/I5. GPCR135 and GPCR142 are coupled to $G_{\alpha i}$ proteins. The assay was performed as inhibition of forskolin-induced β-gal expression. The antagonism of R3(B△23-27)R/I5 is indicated by the rightward-shift of the relaxin-3's dose-response curve. β-gal expression was measured by colorimetric assay using CPRG as the substrate. The absorbance was read at 570 nm

gesting the C-terminus of the R3 B-chain possesses the receptor activation domain. A series of truncations at the C-terminus of the R3 B-chain have been attempted. The resulting peptides have been evaluated for their ligand activity at GPCR135, GPCR142, and LGR7. The R3 B-chain consists of 27 amino acid residues. Truncation of C-terminal residues Gly23 to Trp27 of the R3 B-chain with the serendipitous replacement of Gly^{23} with Arg [R3(B△23-27)R] (Kuei et al. 2007) resulted in a peptide with high affinity for GPCR135 and GPCR142, but little affinity for LGR7. Further modification of the peptide, by replacing of the R3 A-chain with the INSL5 A-chain, a strategy that has been shown to reduce the affinity of R3 for LGR7 (Liu et al. 2005), resulted in a peptide, R3(B△23-27)R/I5, which has high affinity for human GPCR135 (IC_{50} = 0.67 nM) and GPCR142 (IC_{50} = 2.29 nM), but no affinity for human LGR7 (Kuei et al. 2007). Further characterization shows that R3(B△23-27)R/I5 is a potent functional human GPCR135 antagonist. In a functional assay, R3(B△23-27)R/I5 dose-dependently shifted agonism curves of R3 for GPCR135 (pA2 = 9.1) and GPCR142 (pA2 = 8.2) to the right (Fig. 10). Similarly, R3(B△23-27)R/I5 is bound by rat GPCR135 with high affinity (IC_{50} = 0.25 nM), and is a potent rat GPCR135 antagonist (pA2 = 9.6) (Fig. 10) that lacks interaction with rat LGR7 (Kuei et al. 2007). Currently, there are no known GPCR135 small molecule agonist and antagonists available. The creation of a GPCR135 selective antagonist provides very useful tool for GPCR135 in vivo studies.

12
GPCR135 Selective Agonist (R3/I5) Stimulates Feeding in the Rat, an Effect Blocked by Co-Administration of a GPCR135 Selective Antagonist [R3(B△23-27)R/I5]

McGowan et al. (2005) showed that acute i.c.v. administration of R3, but not relaxin, in rats induces feeding. Hida et al. (2006) and McGowan et al. (2006) also showed that chronic administration of R3 increases food intake in rats. These data suggest that GPCR135 may be the receptor responsible for R3-induced feeding. Since LGR7, the relaxin receptor, is also expressed in the brain and can be activated by R3, the possible LGR7 involvement in this R3 function cannot be ruled out. Using R3/I5, a selective GPCR135 agonist, we are able to show that R3/I5 induces feeding in satiated rat, confirming the previous findings (Fig. 11). Consistent with this hypothesis, co-administration of R3(B△23-27)R/I5, a selective GPCR135 antagonist with no affinity for LGR7, blocks R3/I5 induced feeding (Fig. 11). These results provide pharmacological evidence that the selective GPCR135 agonist and antagonist exert their functions in vivo.

The expression profile of GPCR135 in the rat brain (Fig. 9) suggests that R3/GPCR135 may be involved in multiple CNS functions. Tanaka et al. (2005)

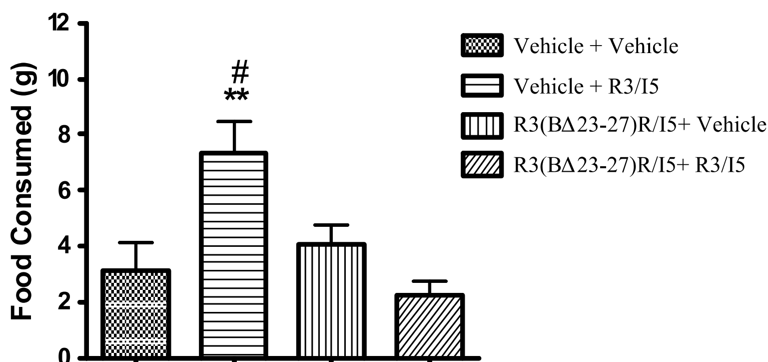

Fig. 11 Effects of GPCR135 agonist (R3/I5) and antagonist (R3(BΔ23-27)R/I5) on the feeding behavior in rat. Satiated Wistar rat was administered (i.c.v.) with vehicle, R3/I5, R3(BΔ23-27)R/I5, or in combination. Food consumption was monitored for the first hour following the administration of vehicle (5 μl) + vehicle (5 μl), vehicle (5 μl) + R3/I5 (10 μg), R3(BΔ23-27)R/I5 (10 μg) + vehicle (5 μl), and R3(BΔ23-27)R/I5 (10 μg) + R3/I5 (10 μg) (mean ± SEM; $n = 5$–6 per group)

have shown that R3 expression in the nucleus incertus co-localizes with that of CRF1, a receptor known to play a key role in the stress response, suggesting the possible involvement of R3/GPCR135 in the physiological response of stress. Water-restraint stress-induced R3 expression in CRF1 positive neurons further supports this hypothesis. Ma and colleagues (Ma et al. 2006) demonstrated that R3 immuno-positive neurons also express glutamic acid decarboxylase-65-immunoreactivity, an enzyme that catalyzes GABA synthesis, suggesting the potential involvement of R3/GPCR135 in the GABAnergic system. Whether and to what extent R3 and GPCR135 are involved in these proposed and other possible CNS functions is still to be established. Certainly, the availability of the GPCR135 selective agonist (R3/I5) and antagonist (R3(BΔ23-27)R/I5) for GPCR135 provides very useful tools to help answer these questions.

13 Summary

A new class of receptors, neuropeptide-like GPCRs, have now been added to the receptors of the insulin/relaxin peptide family. There are now three completely unrelated classes of receptors for insulin-like peptides. First, there are the single transmembrane domain tyrosine kinase receptors that respond to insulin and insulin-like growth factors (IGFs). Second, there are the seven transmembrane LGR7 and LGR8 with long N-terminal extracellular domains, which respond to relaxin, R3, and INSL3. Lastly, there are neuropeptide-like receptors, GPCR135 & GPCR142, that respond to R3 and/or INSL5. Our find-

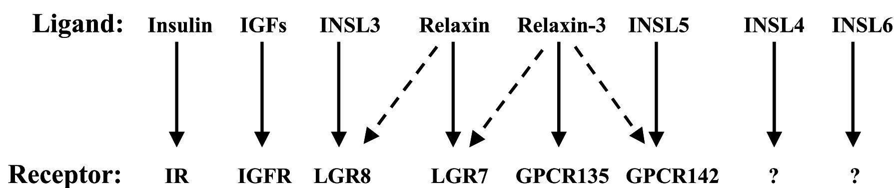

Fig. 12 Schematic diagram for INSL/relaxin related peptides and their relations to different receptors. *Solid lines* indicate the likely in vivo physiological ligand/receptor pairing. *Dashed lines* represent ligands that activate receptor in vitro but may not in vivo. IR = insulin receptor; IGFR = IGF receptor

ings add complexity to this system of ligand/receptor pairs. However, if we consider the expression profiles, receptor ligand affinity, and data from recent physiological studies, it is likely that one ligand is, in fact, stimulating one receptor under physiological conditions, i.e., relaxin/LGR7, INSL3/LGR8, relaxin-3/GPCR135, and INSL5/GPCR142 (Fig. 12). The receptors for INSL4 and INSL6 are still to be identified, but we predict that they are GPCRs and likely members of the LGR subfamily. With this improved understanding of the signaling mechanism of the insulin-like peptides, we should be able to increase the overall understanding of the physiological functions, as well as the therapeutic potential of these systems. To that end, we have devised a chimera of the B-chain of R3 bonded to the A-chain of INSL5 (R3/I5), which is a selective pharmacological tool to study the function of GPCR135 and GPCR142. In addition, we have also created a high affinity GPCR135- and GPCR142-selective antagonist (R3(BΔ23-27)R/I5), which has no affinity for LGR7. These GPCR135 agonist and antagonist have been tested in rats and proven to be active in vivo in the R3 stimulated feeding studies. The expression patterns of relaxin-3 and GPCR135 in the brain suggest that they may play multiple functional roles in vivo, some of which remain to be further studied, and some of which are to be discovered. For GPCR142, very little in vivo studies have been reported. Its physiological functions remain to be investigated. The availability of GPCR135 and GPCR142 selective agonist (R3/I5) and antagonist (R3(BΔ23-27)R/I5) will be very helpful to delineate the physiological functions of GPCR135 and GPCR142. However, to fully understand the physiological functions of R3, INSL5, GPCR135, and GPCR142, the knockout mice may be necessary for those peptides and receptors.

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The NPB/NPW Neuropeptide System and Its Role in Regulating Energy Homeostasis, Pain, and Emotion

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Abstract Neuropeptide B (NPB) and neuropeptide W (NPW) are neuropeptides that were recently identified as endogenous ligands for the previously orphan G-protein coupled receptors, GPR7 (NPBWR1) and GPR8 (NPBWR2). This neuropeptide system is thought to have a role in regulating feeding behavior, energy homeostasis, neuroendocrine function, and modulating inflammatory pain. Strong and discrete expression of their receptors in the extended amygdala suggests a potential role in regulating stress responses, emotion, anxiety and fear; however, there have been no functional studies to date to support this possibility. Future studies of NPB/NPW using both pharmacological and phenotypic analysis of genetically engineered mice will lead to further elucidation of the physiological role of this novel neuropeptide system.

1 Introduction

In 1995, O'Dowd et al. reported the existence of human genes encoding two structurally related orphan G protein-coupled receptors, GPR7 and GPR8, which share a 70% nucleotide and a 64% amino acid identity with each other. Among the other GPCR family members, they have high similarities with opioid and somatostatin receptors. Interestingly, GPR8 was not found in the rodent genome, while GPR7 was highly conserved in both human and rodents (O'Dowd et al. 1995).

Although the structures of both receptors suggest that their cognate ligands could be neuropeptides, the endogenous ligands were not identified for some time. Recently, two endogenous peptide ligands for these receptors were identified and named neuropeptide B (NPB) and neuropeptide W (NPW) (Brezillon et al. 2003; Fujii et al. 2002; Shimomura et al. 2002; Tanaka et al. 2003). Following the deorphaning of these receptors, GPR7 and GPR8 were reclassified by IUPHAR as Neuropeptide B/W receptor-1 (NPBWR1) and Neuropeptide B/W receptor-2 (NPBWR2) (Davenport and Singh 2005a,b). In this review, we discuss the discovery of these ligands and the recent findings concerning the pharmacology, histology, and the phenotypic analysis of genetically engi-

neered mice of the NPB/NPW system, and furthermore discuss other potential unexplored physiological roles of this neuropeptide/receptor system.

2
Identification of NPB and NPW

In 2002–2003, three groups independently identified endogenous peptide ligands for GPR7 and GPR8 by reverse pharmacology. To identify the cognate endogenous ligands for GPR7 (NPBWR1) and GPR8 (NPBWR2), Shimomura et al. expressed these receptors in Chinese Hamster Ovary (CHO) cells and used changes in forskolin-induced cAMP production in these cells as the read-out for receptor activation. HPLC fractions from bovine hypothalamic extracts were assayed using this system. While screening the hypothalamic extract fractions, they detected a decrease in forskolin-induced cAMP production, and subsequent purification and structural analysis of the ligands responsible for this inhibition of cAMP production led to the discovery of the novel neuropeptide, NPW. During their purification process, they identified two forms of NPW with different peptide lengths of 23 and 30 amino acid residues and named them NPW23 and NPW30, respectively.

In a similar manner, Fujii et al., Brezillon et al., and Tanaka et al. (Brezillon et al. 2003; Fujii et al. 2002; Tanaka et al. 2003) purified and identified NPB as an additional endogenous ligand for NPBW1 and NPBW2. Fujii et al. first screened the Celera database to identify novel secretory peptides and then expressed the cDNAs of the putative secretory peptides to find novel peptide ligands. Subsequent pharmacological studies and purification of the peptide from bovine hypothalamic extracts led to the identification of the second ligand for GPR7 and GPR8, which was named Neuropeptide B (NPB) due to the bromination of the first tryptophan residue. Brezillon et al. also identified human NPB mRNA with a bioinformatics approach by searching the EST database using the NPW sequence as a query (Brezillon et al. 2003). Tanaka et al. used the melanin-pigment aggregation assay in xenopus melanophore cells expressing GPR7 as the assay system to purify NPB from bovine hypothalamus. This system also detected a decrease in intracellular cAMP levels. Through EST database searches they also identified NPW as a putative paralogous peptide (Tanaka et al. 2003).

3
Structures of NPB and NPW

NPB and NPW belong to a distinct family of peptides that do not display any significant sequence similarity to other previously known peptides, while sharing a high degree of sequence similarity with each other (Fig. 1a)

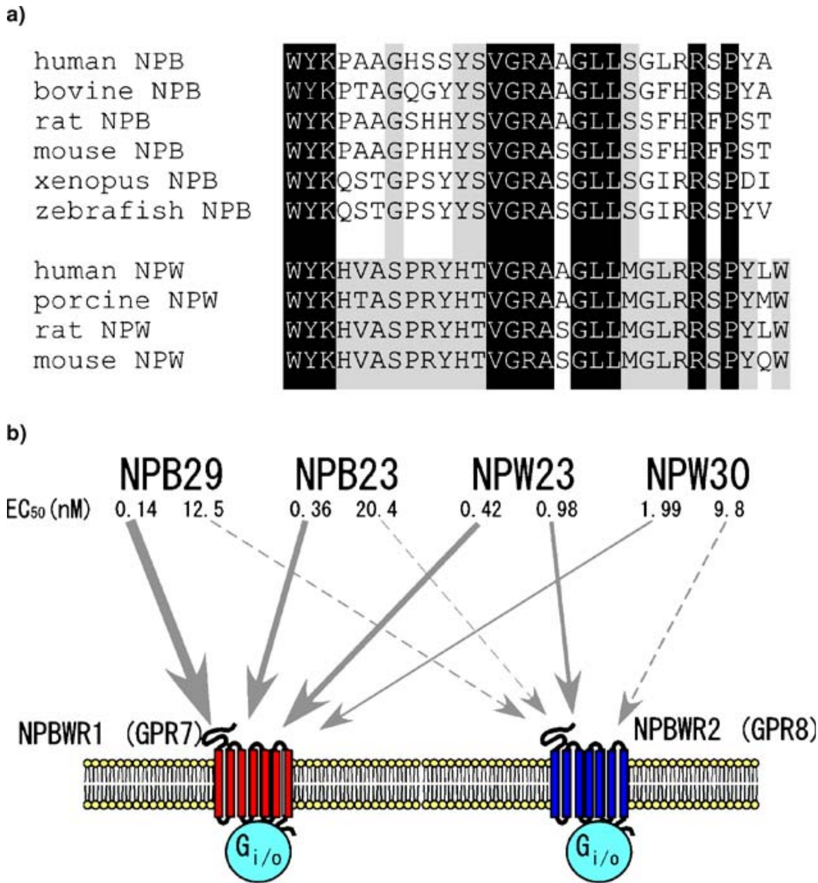

Fig. 1 The NPB/NPW neuropeptide system. **a** Amino acid sequences of NPB and NPW. *Dark shadow* shows amino acid identities between NPB and NPW. *Light shadow* shows conserved amino acids within NPB or NPW. **b** Interaction between NPB/NPW and NPBWR1/NPBWR2. EC_{50} values are determined by assays on inhibition of cAMP production in CHO cells expressing each receptor (Brezillon et al. 2003). These values are dependent on the assay systems, because expression levels of receptors and their abilities to evoke cellular responses vary in each system. However, potency rank orders of these peptides are consistent among the various reports (Brezillon et al. 2003; Fujii et al. 2002; Shimomura et al. 2002; Tanaka et al. 2003)

(Brezillon et al. 2003; Fujii et al. 2002; Shimomura et al. 2002; Tanaka et al. 2003).

NPB has a unique modification at the N-terminus tryptophane residue, C-6-bromination (Fujii et al. 2002; Tanaka et al. 2003). While this represents the first evidence of bromination in mammals, the biological significance of this bromination is unclear as it has been demonstrated that des-Bromo-NPB is equipotent to brominated NPB in in vitro cAMP inhibition assays (Tanaka

et al. 2003). Furthermore, bromination on Trp-1 has not been confirmed in any other mammalian species besides bovine. Further studies are needed to elucidate any possible role for this unique bromine modification in NPB.

By analogy with NPW, Brezillon et al. predicted that two isoforms of NPB, NPB23 and NPB29, could be produced from the processing of a 125-amino acid human precursor through the alternative usage of a dibasic amino acid pair. However, the dibasic motif, Arg24–Arg25, which is seen in human NPB, does not exist in NPB of other mammalian species including bovine, rat, and mouse. Furthermore, both Fujii et al. and Tanaka et al. were only able to isolate NPB29 from bovine hypothalamus extracts during their purification procedures. Therefore, it is unlikely that the NPB23 isoform exists as a mature peptide in mammalian species other than human. NPB (NPB29) binds and activates human NPBWR1 or NPBWR2 with median effective concentrations (EC_{50}) of 0.23 nM and 15.8 nM, respectively (Tanaka et al. 2003). This suggests that NPB is a relatively selective agonist for NPBWR1 (Fig. 1b).

As discussed earlier, peptide purification as well as sequence analysis showed that NPW has two isoforms with lengths of 23 and 30 amino acid residues. These are called neuropeptide W-23 (NPW23) and neuropeptide W-30 (NPW30) respectively, which are processed from the same precursor. NPW30 has a pair of arginine residues in the 24th and 25th positions. NPW23 is produced as a result of proteolytic processing at this site (Brezillon et al. 2003; Fujii et al. 2002; Tanaka et al. 2003). Therefore, the amino acid sequence of NPW23 is completely identical to that of the N-terminal 23 residues of NPW30. In vitro experiments using recombinantly expressed receptors showed that synthetic NPW23 activates and binds to both NPBWR1 and NPBWR2 at similar effective doses, while NPW30 shows slightly lower affinities to both receptors as compared with NPW23 (Fig. 1b) (Brezillon et al. 2003; Tanaka et al. 2003).

The preferred conformations of the desbromo-NPB and NPW have been determined by the combination of ^{1}H NMR, CD, and molecular modeling (Lucyk et al. 2005). NPB consists of a type II beta-turn involving residues Lys-3 to Ala-6. The C-terminal region of NPB exists in a conformational equilibrium between different secondary structures, including an alpha-helix from residues Arg-15 to Ser-21, and a 3-helix from residues Ser-12 to Ser-21s. The N-terminus of NPW exhibits a cation-pi interaction between the Lys-3 side chain and the quadrupole moment of the Trp-1 indole group. At the C-terminus of NPW, a well-defined alpha-helical conformation exists from Arg-15 to Met-21.

4
Structure-Activity Relationships of NPB and NPW

The rank order of potency has been determined in cell lines expressing NPBWR1 and NPBWR2. Pharmacologically, both NPW and NPB activate

both receptors, however, with varying degrees of affinity. NPBWR1 has a slightly higher affinity for NPB as compared with both forms of NPW, whereas NPBWR2 shows a potency rank order of NPW23 > NPW30 > NPB (Fig. 1b) (Brezillon et al. 2003; Fujii et al. 2002; Shimomura et al. 2002; Tanaka et al. 2003). NPB23 and NPB29 have similar affinities for both receptors. In brief, NPW23 and NPW30 bind to both receptors almost equally, while NPB is a relatively selective agonist for NPBWR1.

Tanaka et al. showed deletion of Trp-1 from NPB or NPW drastically decreased activity, suggesting that the N-terminus is involved in receptor binding (Tanaka et al. 2003). This is consistent with the fact that NPB and NPW have similarity in their sequences in N-terminal regions.

5 Structures and Functions of NPBWR1 and NPBWR2

From the chromosomal assignment by fluorescent in situ hybridization, human NPBWR1 was mapped to chromosome 10q11.2–121.1 and human NPBWR2 to chromosome 20q13.3.

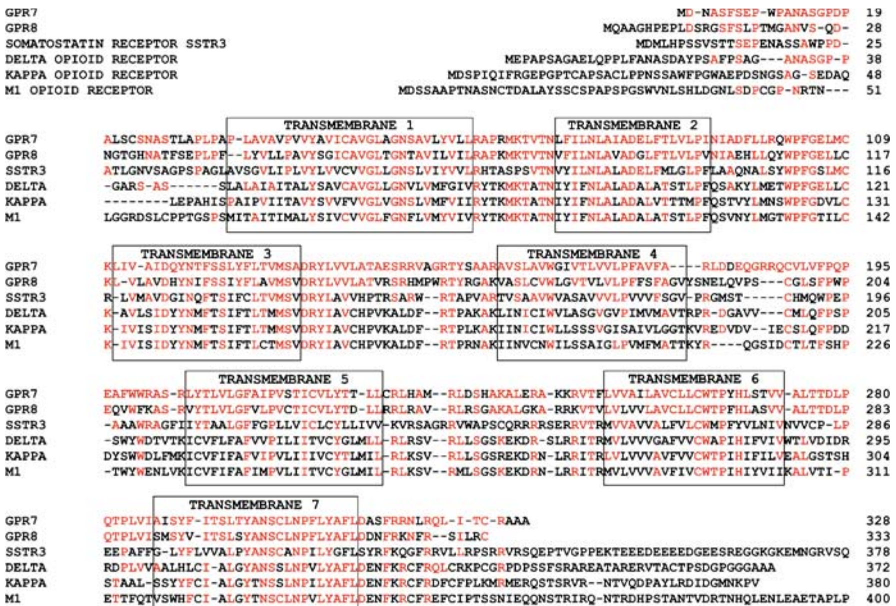

Fig. 2 Sequences of NPBWR1/NPBWR2 compared to somatostatin receptor and opioid receptors. Conserved amino acid residues are shown in *red letters*. Each of the transmembrane domains is boxed

Human NPBWR1 and NPBWR2 are predicted to have 328 and 333 amino acids, respectively and share 64% sequence homology with each other (Fig. 2). Among other family members of GPCRs, NPBW1 and NPBW2 are most closely related to opioid and somatostatin receptors (Fig. 2) (O'Dowd et al. 1995). Amino-acid analysis of NPBW1 orthologues in other mammalian species has revealed a high degree of conservation throughout evolution (Lee et al. 1999). In contrast, while the gene encoding NPBWR2 has been discovered in several mammalian species such as monkey, lemur, bat, shrew and rabbit, it has not been detected in rodents (Lee et al. 1999). This suggests that these two receptors were produced by a phylogenetically relatively recent gene duplication event (Lee et al. 1999).

As suggested by the assay systems used during their purification process, both NPBWR1 and NPBWR2 couple to the Gi-class of G-proteins (Tanaka et al. 2003). This suggests that these neuropeptides have inhibitory properties on neurons via activation of GIRK (Kir3) channels. NPB and NPW were also shown to stimulate Erk p42/p44 activities in human adrenocortical carcinoma-derived NCI-H295 cells (Andreis et al. 2005). These activations are probably mediated by beta/gamma subunits released from G_i-proteins (Tim van et al. 1995).

At present, no synthetic antagonists or agonists have been developed that are selective for either receptor.

6 Tissue Distributions of NPB/NPW and NPBWR1/NPBWR2

6.1 Neuropeptide B

In situ hybridization showed localizations of the prepro-NPB mRNA in several specific regions in the mouse brain such as the paraventricular hypothalamic nucleus (PVN), CA1-CA3 fields of the hippocampus, and several nuclei in the midbrain and brainstem, including the Edinger–Westphal nucleus (EW) as well as the sensory and motor nuclei of the trigeminal nerve, locus coeruleus (LC), inferior olive, and lateral parabrachial nucleus (Fig. 3) (Jackson et al. 2006; Tanaka et al. 2003).

Schulz et al. reported that NPB-immunoreactive cell bodies were observed in many regions within the hypothalamus which also contained high levels of NPBWR1 mRNA and NPB mRNA including the ventromedial hypothalamic nucleus, dorsomedial hypothalamic nucleus, arcuate nucleus, supraoptic retrochiasmatic nucleus, and in the area ventral to the zona incerta (Schulz et al. 2007). Although NPB mRNA was detected in several regions outside the hypothalamus, such as the hippocampus and brain stem, they did not report the existence of NPB-positive neurons in these regions (Jackson et al. 2006;

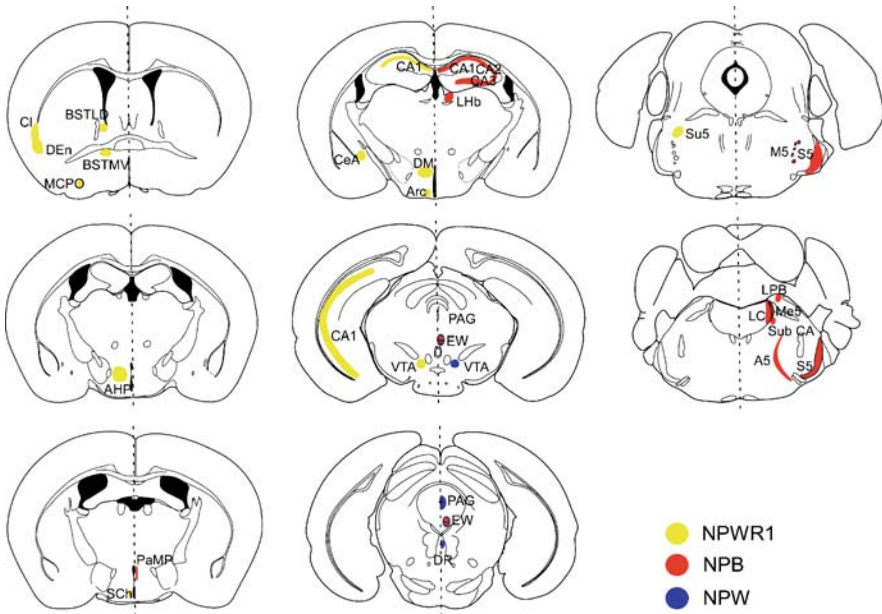

Fig. 3 Schematic representation of the NPB/W-GPR7 system in mouse brain. Distribution of NPB/NPW and NPBWR1 mRNA on mouse brain coronal sections are shown. Distribution of the receptor is shown in the *right* hemisphere, while the distributions of the ligands are shown in the *left*. The distribution of NPB mRNA (shown by *red regions*) included the hippocampus (CA1, CA2, CA3), lateral habenular nucleus (LHb), paraventricular hypothalamic nucleus, medial parvicellular part (PaMP), Edinger–Westphal (EW) nucleus, motor root of the trigeminal nerve (m5), sensory root of the teigeminal nerve (s5), lateral parabrachial nucleus alpha part (Sub CA), locus coeruleus (LC), noradrenergic cell group A5 (A5), and inferior olive subnucleus B (IOB) (Tanaka et al. 2003). The distribution of NPW mRNA (shown by *blue regions*) included the periaqueductal gray matter (PAG), EW nucleus (EW), ventral tegmental area (VTA), dorsal raphe nucleus (DR). The distribution of NPBWR1 mRNA (shown by *yellow regions*) included the claustrum (Cl), dorsal endopiriform nucleus (DEn), bed nucleus of the stria terminals, laterodorsal part (BSTLD), bed nucleus of the stria terminals, medioventrial part (BSTMV), suprachiasmatic nucleus (Sch), magnocellular preoptic nucleus (MCPO), paraventricular hypothalamic nucleus, posterior part (PaPo), dorsomedial hypothalamic nucleus (DM), central amygdala (CeA), CA1 field, hippocampus (CA1), ventral tegmental area (VTA), sensory root trigeminal nerve (Su5), subiculum (S), anterior hypothalamic area, posterior part (AHP), arcuate hypothalamic nucleus (Arc)

Schulz et al. 2007; Tanaka et al. 2003). In peripheral tissues, expression of human NPB mRNA was detected by RT-PCR in kidney, uterus, ovary, testis, and placenta, while murine NPB mRNA was detected by Northern blot at high levels in the stomach, spinal cord, testis and lower levels in the liver and kidney (Brezillon et al. 2003; Tanaka et al. 2003).

6.2 Neuropeptide W

Compared to the relatively widespread expression pattern of NPB mRNA, the expression of NPW mRNA in mouse brain is more confined to specific nuclei in midbrain and brainstem including the EW, ventral tegmental area (VTA), periaqueductal gray (PAG) and dorsal raphe nucleus (DR) (Fig. 3) (Kitamura et al. 2006; Tanaka et al. 2003). In humans, high levels of NPW mRNA were detected in the substantia nigra, and moderate expression levels were detected in the amygdala and hippocampus (Fujii et al. 2002). In peripheral tissues, expression of human NPW mRNA was confirmed by RT-PCR in the progenital system, comprising the kidney, testis, uterus, ovary, placenta, and also in the stomach and respiratory system, while murine NPW mRNA was detected by Northern blot at high levels in the lung and lower levels in the stomach (Brezillon et al. 2003; Tanaka et al. 2003).

Consistent with its mRNA distribution, NPW-immunoreactive (ir) cells were also exclusively detected in EW, VTA, PAG, and DR in rats (Kitamura et al. 2006). NPW-ir fibers were observed in several brain regions in rats including the lateral septum, bed nucleus of the stria terminalis (BNST), dorsomedial and posterior hypothalamus, CeA, CA1 field of hippocampus, interpeduncular nucleus, inferior colliculus, lateral parabrachial nucleus, facial nucleus, and hypoglossal nucleus. Among these regions, NPW-ir fibers were most abundantly observed in the CeA and the BNST, the output nuclei of the extended amygdala, which are regions implicated in fear and anxiety. These observations suggest that NPW-producing neurons are exclusively localized to the mid brain, and they project mainly to the limbic system, especially the CeA and BNST (Fig. 3).

Some reports showed the existence of NPW-ir cell bodies in the hypothalamic paraventricular nucleus (PVN) in rats and mice (Dun et al. 2003). However, a recent study suggested that the staining of NPW like immunoreactivity-positive cells in the PVN is probably due to non-specific staining for two main reasons (Kitamura et al. 2006). First, the PVN immunoreactivity is observed in the $NPW^{-/-}$ mice using many of the commercially available antibodies, and second the NPW mRNA is not expressed in the PVN in both mice and rats (Kitamura et al. 2006).

6.3 NPBWR1 (GPR7)

In situ hybridization and tissue binding studies showed that the CeA and BNST expresses the highest levels of NPBWR1 mRNA and binding signals (Fig. 3) (Jackson et al. 2006; Singh et al. 2004; Tanaka et al. 2003). Other nuclei with high levels of NPBWR1 expression and binding are the suprachiasmatic (SCN) and the ventral tuberomamillary nuclei of the hypothalamus.

Moderate levels are seen in the CA1-CA3 regions of the hippocampus, dorsal endopiriform, dorsal tenia tecta, bed nucleus, and the red nucleus. Low levels of expressions are seen in the olfactory bulb, parastrial nucleus, hypothalamus, laterodorsal tegmentum, superior colliculus, locus coeruleus, and the nucleus of the solitary tract.

Collectively, these observations suggest that NPBWR1 is most abundantly observed in the CeA and BNST. These results suggest that NPBWR1 might be involved in the regulation of stress and emotive responses, especially in fear and anxiety-related physiological and behavioral functions, which is discussed in detail later (Kitamura et al. 2006). Expression of NPBWR1 in the SCN suggests that they might be involved in the regulation of the circadian clock. However, as discussed later, *NPBWR1*$^{-/-}$ mice did not show any behavioral abnormalities in circadian behavioral pattern (our unpublished observation).

6.4 NPBWR2 (GPR8)

Only limited information about tissue distribution of NPBWR2 has been available. RT-PCR analysis showed that NPBWR2 mRNA is strongly expressed in human amygdala and hippocampus. Lower levels of expression were also detected in corpus callosum, cerebellum, substantia nigra, and caudate nucleus (Brezillon et al. 2003).

7 Pharmacological Activities of NPB as NPW

7.1 Feeding and Energy Homeostasis

From the distribution of NPBWR1, it was initially hypothesized that the NPB/W system may modulate feeding behavior (Shimomura et al. 2002; Tanaka et al. 2003). Therefore, many studies have focused on the roles of NPB/W in the regulation of feeding and energy homeostasis. The first physiological study on the action of NPW reported acute hyperphagia in male rats when NPW was administered intracerebroventricularly (i.c.v.) (Shimomura et al. 2002; Tanaka et al. 2003). However, Tanaka et al. showed that the effect of NPB in mice on feeding behavior is not simple (Tanaka et al. 2003). When NPB was i.c.v. injected during the light period, no significant effect of NPB on feeding was observed (Tanaka et al. 2003). In contrast, in the dark period, i.c.v. administration of 3 nmol of NPB increased feeding, but only within the first 2 h. A higher dose of NPB suppressed food intake in this interval. After 2 h, both doses of NPB decreased food intake. This bipha-

sic (early orexigenic followed by delayed anorexic) effect of NPB is different from the initially reported orexigenic action of NPW (Shimomura et al. 2002). Because rodents do not express NPBWR2 and only have NPBWR1, which accepts both peptides with relatively high affinities, these differences can not simply stem from the different potency rank orders of the two peptides on NPBWR1 and NPBWR2. In fact, we also observed a similar biphasic action of NPW in feeding behavior when administered i.c.v in mice or rats (our unpublished observation). Mondal et al. also reported anorexic effects of NPW (Mondal et al. 2003). These findings suggest a complex role for NPB and NPW in the regulation of food intake.

Interestingly, the anorexic effect of NPB was markedly enhanced when corticotrophin-releasing factor (CRF), a known anorexic peptide, was co-administered (Tanaka et al. 2003). The i.c.v. administration of these two peptides almost completely suppressed the food intake over 4 h. The biphasic effects of NPB/W on feeding behavior, and synergistic anorexic effects of NPB and CRF suggest the complex roles of these peptides in regulation of feeding behavior. The synergic effect of NPB with CRF in suppression of feeding suggests that this neuropeptide is implicated in inhibition of feeding under stressful conditions.

Continuous i.c.v. infusion of NPW using an osmotic minipump suppressed feeding and body weight gain over the infusion period (Mondal et al. 2003). Conversely, i.c.v. administration of anti-NPW IgG stimulated feeding suggesting that endogenous NPW play an inhibitory role in feeding behavior (Mondal et al. 2003). However, unlike the results from continuous i.c.v. infusion of NPW, bolus intra-PVN injection of NPW23 at doses ranging from 0.1 to 3 nmol increased feeding for up to 4 h, and bolus doses ranging from 0.3 to 3 nmol was reported to increase feeding for up to 24 h (Levine et al. 2005). This observation suggests that orexigenic versus anorectic effects of NPB/W could stem from different sites of action. When these peptides are injected into the lateral ventricles, they might be initially acting on the PVN, followed by acting on other regions implicated in the suppression of feeding. Alternatively, delayed inhibition of feeding by NPB/W might result from the production of other anorectic factors that are stimulated by NPB or NPW.

I.c.v. administration of NPW also increased body temperature and heat production (Mondal et al. 2003). These effects suggest that endogenous NPB/W might affect energy expenditure, which is consistent with the late onset obesity seen in male *NPBWR1*$^{-/-}$ mice and *NPB*$^{-/-}$ mice (Ishii et al. 2003; Kelly et al. 2005).

7.2 Effect on Inflammatory Pain

Initially, i.c.v. administration of NPB was reported to produce analgesia to subcutaneous formalin injection in rats (Tanaka et al. 2003). It was subse-

quently reported that intrathecal (i.t.) injection of either NPW23 or NPB decreased the number of agitation behaviors induced by paw formalin injection and attenuated the level of mechanical allodynia (Yamamoto et al. 2005). The effects were not antagonized by naloxone, suggesting that this effect is not mediated through the opioid receptor system. While i.t. injection of either NPW23 or NPB did not show any effect in the hot-plate test or mechanical nociceptive test, i.t. injection of either NPW23 or NPB significantly suppressed the expression of Fos-like immunoreactivity of the L4-5 spinal dorsal horn induced by paw formalin injection. These data suggest that both spinally applied NPW23 and NPB suppressed the input of nociceptive information to the spinal dorsal horn, and produced an analgesic effect in inflammatory pain, but not mechanical or thermal pain (Yamamoto et al. 2005). Consistent with the pharmacological studies, $NPB^{-/-}$ mice exhibited hyperalgesia to inflammatory pain, while they show normal responses for mechanical or thermal pain (Kelly et al. 2005). These observations suggest that NPB in the brain and/or spinal cord inhibits allodynia and modulates pain in a modality-specific manner.

Low levels of NPBWR1 were observed in Schwann cells in both normal human and rat nerves as well as in primary rat Schwann cell cultures. Peripheral nerve samples taken from patients exhibiting inflammatory/immune-mediated neuropathies showed a dramatic increase of NPBWR1 expression restricted to myelin-forming Schwann cells. Complementary animal models of immune-inflammatory and ligation-induced nerve injury and neuropathic pain similarly exhibited an increased myelin-associated expression of NPBWR1 (Zaratin et al. 2005). These observations suggest that NPBWR1 is involved in regulation of inflammatory pain in part by modulating Schwann cell function.

7.3 Neuroendocrine Regulation

When injected into the lateral cerebroventricle of conscious, unrestrained male rats, both NPB and NPW elevated corticosterone levels in circulation (Samson et al. 2004; Taylor et al. 2005). NPB was also reported to increase prolactin and decrease growth hormone levels (Samson et al. 2004). Pretreatment with a polyclonal anti-CRF antiserum or CRF antagonists completely blocked the ability of NPB or NPW to stimulate ACTH release and significantly inhibited the effect of NPB/W on plasma corticosterone levels (Samson et al. 2004; Taylor et al. 2005). These observations suggest that NPW and NPB may play a physiologically relevant role in the neuroendocrine response to stress via an activation of the hypothalamus-pituitary-adrenal (HPA) axis. Consistent with these observations, whole cell patch-clamp recording from hypothalamic slice preparation showed that bath application of NPW depolarized and increased the spike frequency of the majority of electrophysiologically

identified putative neuroendocrine PVN neurons. The effects on membrane potential were maintained in the presence of TTX suggesting that they are direct postsynaptic actions on these neuroendocrine cells (Taylor et al. 2005). These observations indicate that NPB/W may play an important role in the hypothalamic function in the endocrine response to stress by modulating the HPA axis.

7.4 Autonomic Regulation

I.c.v. administration of NPW30 was reported to increase the arterial blood pressure (ABP), heart rate (HR), and plasma catecholamine concentrations in conscious rats (Yu et al. 2007). The same report showed that most of the PVN neurons are excited, while a subset of smaller populations of PVN neurons are inhibited by NPW30; however, the chemical identities of these neurons was not shown. These observations suggest that NPB/W modulate PVN neuronal activities, which might be involved in the regulation of autonomic nervous system as well as the HPA axis (Yu et al. 2007). NPB is more likely to be involved in this role in vivo, due to the expression of NPB mRNA in the PVN. However, *NPBWR1*$^{-/-}$ mice have normal blood pressure and heart rate in basal states (our unpublished observation).

8 Emotion and Behavior

Amygdala is a well-defined subcortical nuclear group that is the center of emotion including fear (Phelps and LeDoux 2005). A sensory stimulus that predicts an aversive outcome will change neural transmission in the amygdala to produce the somatic, autonomic, and endocrine signs of fear, as well as increased attention to that stimulus. Fear learning involves the lateral and basolateral amygdala (BLA), where the association between incoming sensory stimuli leads to potentiation of synaptic transmission. The BLA receives sensory information from the thalamus, hippocampus, and cortex and then activates or modulates synaptic transmission in target areas appropriate for the reinforcement signal with which the sensory information has been associated. The BLA projects to the CeA and BSNT, whose efferents to the hypothalamus and brainstem trigger the expression of fear. NPW-ir and NPBWR1 are strongly expressed in the CeA and BSNT. However, despite the discrete and strong expression pattern of NPBWR1 in the CeA, BST, and hippocampus, the role of NPBWR1 regarding these functions has not been elucidated. These anatomical evidences suggest that NPB/W systems might have important roles in the modulation of output from the extended amygdala (Kitamura et al. 2006) (Fig. 4). Studying the behavioral phenotypes of

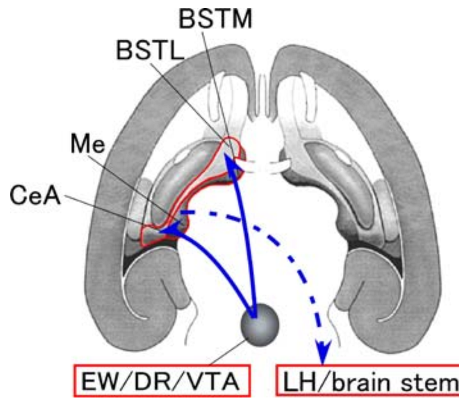

Fig. 4 Schematic representation of the NPW neuropeptide system. NPW neurons are localized in discrete areas in the brain stem, including the VTA, PAG, and EW, which send out projections to the lateral extended amygdala (CeA and BNST). NPBWR1 is localized in the CeA and BNST. These observations suggest that NPW neurons in the brain stem send feedback information to the output regions of the extended amygdala

$NPBWR1^{-/-}$, $NPB^{-/-}$, and $NPW^{-/-}$ mice would help to clarify the potential role for NPB/W in regulating these behaviors.

8.1 Effects on Circadian Rhythm

NPBWR1 are abundantly expressed in the suprachiasmatic nucleus. This strongly suggests that this neuropeptide/receptor system has a role in regulating circadian rhythm (Lee et al. 1999; Singh et al. 2004; Tanaka et al. 2003). However, we did not observe any effects of NPB/W on circadian activity in rats or mice when administered by i.c.v. injection (our unpublished observations). Furthermore, $NPBWR1^{-/-}$ mice displayed a normal circadian pattern of behavior in both light-dark and constant dark conditions. Both light-entrainable and food entrainable oscillation were also normal in these mice (our unpublished observations).

9 Peripheral Actions

Expressions of NPB, NPW, and NPBWR1 mRNAs in both adrenal cortex and adrenal medulla have been reported (Andreis et al. 2005; Hochol et al. 2007; Mazzocchi et al. 2005). NPB and NPW were shown to stimulate adrenal glucocorticoid secretion by an ACTH-independent mechanism when administered intravenously. It was also reported that NPW stimulates in vitro aldosterone

Table 1 In vivo pharmacological effects of NPB/NPW

Substance	Effects	Animal	Refs.
NPW (i.c.v.)	Food intake ↑ Body weight ↑	Rats (male)	Shimomura et al. Tanaka et al.
NPW (i.c.v.)	Body temperature ↑ Heat production ↑	Rats	Mondal et al.
NPW	ACTH ↑ Estradiol ↑	Rats	Hochol et al.
NPW30 (i.c.v.)	Arterial blood pressure (ABP) ↑ Heart rate (HR) ↑ Plasma catecholamine concentration ↑	Rats	Yu et al.
NPB (i.c.v.)	Food intake (light period) — Food intake (dark period) ↑	Mice	Tanaka et al.
NPB (i.c.v.)	Prolactin ↑ Growth hormone ↓	Rats (male)	Samson et al.
NPW23/NPB (i.t.)	Inflammatory pain ↓	Rats	Yamamoto et al.
NPW/NPB (i.c.v.)	Corticosterone in circulation ↑	Rats (male)	Samson et al., Taylor et al.
NPW/NPB (i.p.)	(plasma level) Parathyroid hormone ↑ (plasma level) Corticosterone ↑ (plasma level) Testosterone ↑	Rats	Hochol et al.
NPW/NPB (i.c.v.)	Circadian rhythm —	Rats/mice	Our unpublished observations

secretion by enhancing the release of medullary catecholamines, which activate beta-adrenoceptors located on zona glomerulosa cells (Hochol et al. 2007).

Bolus intraperitoneal (i.p.) injection of NPB or NPW increased the plasma levels of parathyroid hormone, corticosterone and testosterone. NPB was also reported to increase the blood concentration of thyroxine, and NPW was shown to increase ACTH and estradiol levels. These findings suggest that NPB and NPW play a role in the regulation of the endocrine system (Hochol et al. 2006).

Existence of NPW in rat stomach antral cells was reported. It was also reported that levels of NPW in stomach is decreased in fasted animals, while it was increased by re-feeding (Mondal et al. 2006), which is consistent with the notion that NPW may act as a suppressant to feeding. However, we did not observe any effects on feeding in mice when NPB or NPW were intravenously administered suggesting that peripheral NPB/W has limited, if any, significant role in modulating feeding behaviors (our unpublished observations).

10 Phenotypes of NPBWR1- and NPB-Deficient Mice

Genetically engineered mice are powerful tools for elucidating the physiological roles of particular genes. In this next section, we discuss the phenotypes of *NPBWR1*$^{-/-}$ mice and *NPB*$^{-/-}$ mice.

10.1 NPBWR1-Deficient Mice

Male *NPBWR1*$^{-/-}$ mice develop an adult-onset obesity that progressively worsens with age and was greatly exacerbated when animals are fed a high-fat diet (Ishii et al. 2003). These mice were hyperphagic and had decreased energy expenditure and locomotor activity resulting in obesity. *NPBWR1*$^{-/-}$ male mice showed decreased hypothalamic neuropeptide Y mRNA levels and increased proopiomelanocortin mRNA levels, a set of effects opposite to those evident in *ob/ob* mice. Furthermore, *ob/ob NPBWR1*$^{-/-}$ and *Ay/a NPBWR1*$^{-/-}$ double mutant male mice had an increased body weight compared with normal *ob/ob* or *Ay/a* male mice, suggesting that the obesity of *NPBWR1*$^{-/-}$ mice is independent of leptin and melanocortin signaling. Female mice did not show any significant weight increase or associated metabolic defects. These data suggest a potential role for NPBWR1 and NPB/W in regulating energy homeostasis independent of leptin and melanocortin signaling in a sexually dimorphic manner (Ishii et al. 2003).

10.2 NPB-Deficient Mice

Consistent with the phenotype of the *NPBWR1*$^{-/-}$ mice, *NPB*$^{-/-}$ mice also manifest mild adult-onset obesity. NPB-deficient mice also exhibit hyperalgesia in response to inflammatory pain. Hyperalgesia was not observed in response to chemical pain, thermal pain, or electrical stimulation. NPB-deficient mice demonstrated intact behavioral responses to pain, and learning from the negative reinforcement of electrical stimulation was unaltered. Baseline anxiety was also unchanged as measured in both the elevated plus maze and time spent immobile in a novel environment (Kelly et al. 2005). These data support the idea that NPB can modulate the responses to inflammatory pain and body weight homeostasis.

11
Discussion

From the hypothalamic distribution of NPBWR1, NPB and NPW were initially hypothesized to modulate feeding behavior (Shimomura et al. 2002; Tanaka et al. 2003), and many studies have focused on the roles of NPB/NPW in the regulation of feeding and energy homeostasis. The biphasic (early orexigenic followed by anorexic) effect of NPB/NPW suggest a complex role for NPB and NPW in the regulation of food intake. Both *NPB*$^{-/-}$ and *NPBWR1*$^{-/-}$ mice both show late onset obesity and hyperphagia, suggesting that the endogenous NPB-NPBWR1 pathway negatively regulates feeding behavior and positively regulates energy expenditure (Ishii et al. 2003; Kelly et al. 2005). This notion is further supported by several pharmacological studies that show that i.c.v. NPB/W increase heat production and sympathetic outflow (Yu et al. 2007).

Many studies have also shown that the NPB/W system is involved in the modulation of inflammatory pain. I.c.v. or i.t. administered NPB/W decreased sensitivity to inflammatory pain, while having no significant effect on chemical pain, heat sensation, or nociception. Consistent with these results, *NPB*$^{-/-}$ mice are hypersensitive to inflammatory pain but display no significant differences in chemical or thermal pains. These data in aggregate strongly support a physiological role for central NPB in pain regulation, and agonists for NPBWR1 or NPBWR2 might be good candidates for analgesic drugs for chronic inflammatory pain.

Finally, strong and discrete expression of NPBWR1 in the CeA and BNST, and abundant projection of NPW fibers in these regions suggest that this neuropeptide system has a role in the regulation of fear and anxiety. The CeA and BNST are the output nuclei of the extended amygdala, which has been implicated in a variety of emotional functions including expression of fear, modulation of memory, and mediation of social communication (Davis and Shi 1999) (Figs. 3, 4). Therefore, the expression of GPR7 in the CeA and BST suggests modulatory roles of GPR7 in these functions. Studies of these functions using genetically engineered mice in NPB/W and their receptors would help to clarify these roles.

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