NGF, BDNF, NT3, and NT4

M. Bothwell

Abstract

The discovery of nerve growth factor (NGF) was a seminal event in history of research in developmental neurobiology. The further discovery that NGF was just one of a family of structurally similar growth factors, neurotrophins, provided important insights into the way nerve cells communicate, during development of the nervous system, and in neuroplasticity, memory, and learning in the adult nervous system. Four neurotrophins, NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4), regulate a wide variety of neural functions, acting upon p75NTR, TrkA, TrkB, and TrkC receptors.

Keywords

Neurotrophin • NGF • BDNF • NT3 • NT4 • Evolution

1 Historical Background: Discovery of NGF and Other Neurotrophins

1.1 Discovery of NGF

The discovery and biochemical and functional characterization of nerve growth factor (NGF), by Rita Montalcini, Viktor Hamburger, and Stanley Cohen, was decades ahead of its time, in more ways than we can easily appreciate today. The discovery that the trophic effect of innervated tissues on sympathetic and sensory neuronal development was mediated by a diffusible factor (Levi-Montalcini and Hamburger 1953) was conceptually ground-breaking, and the successful isolation of this NGF from mouse salivary gland was remarkable given the primitive tools for

M. Bothwell (✉)
University of Washington, Seattle, WA, USA
e-mail: mab@uw.edu

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protein fractionation that were available at the time. It is also remarkable that the importance of NGF for neurons in vivo was established almost immediately by demonstrating that injection of NGF antibody caused death of sympathetic neurons (Cohen 1960). The precocious nature of these studies can only be understood by contrasting this to the history of experimentation with a variety of other growth factors that were discovered subsequently, since the loss-of-function experiments required to demonstrate the importance in vivo of other growth factors usually lagged the initial discovery of those growth factors by a decade or more.

It is hard to appreciate also, from a modern perspective, that the concept that a protein released by one cell could control the differentiation of neighboring cells was revolutionary at the time of discovery of NGF. Indeed, the possibility that NGF could be "instructive" for neuronal differentiation, rather than merely being "permissive" was still a hotly debated subject when the author of this chapter entered the field of NGF research in 1975.

The seed for the discovery of NGF was planted by the pioneering work of Viktor Hamburger in which it was shown that surgical removal of the wing buds of chick embryos reduced the ultimate number of motor neurons in the lateral motor column of the spinal cord and of sensory neurons in the dorsal root ganglia at segmental levels responsible for innervation of the missing target tissue, while transplantation of supernumerary limb buds had the opposite effect of allowing development of more motor neurons and sensory neurons (Hamburger 1934, 1939). Thus was born the so-called neurotrophic hypothesis, stating that "Each part of the peripheral field controls directly [development of] its own nervous center" along with the idea that some signal or substance must move from the axon terminus to the neuronal cell body to convey this signal.

The work of Rita Levi-Montalcini, initially independently (Levi-Montalcini and Levi 1943) and subsequently in collaboration with Viktor Hamburger (Hamburger and Levi-Montalcini 1949), demonstrated that these effects were not primarily an effect on neurogenesis, as initially supposed, but rather, largely reflected the ability of the innervated target to suppress developmental cell death of the innervating neurons. Attempts to model the limb bud effects with small pieces of sarcoma tumor, initially in vivo (Levi-Montalcini and Hamburger 1951), and subsequently in vitro (Levi-Montalcini and Hamburger 1953), using newly available tissue culture techniques demonstrated potent effects on development of sympathetic neurons as well as sensory neurons and importantly established that the effects were mediated by a diffusible factor. Subsequent studies revealed that a similar activity was present at much higher concentrations in cobra venom and mouse salivary gland, allowing the biochemical purification of the factors and importantly, demonstrating that an antiserum to the NGF protein caused degeneration of the nervous system of neonatal rodents (Cohen 1960; Cohen and Levi-Montalcini 1956). The later discovery that NGF was a member of a family of factors that control survival of sensory neurons was presaged by Levi-Montalcini's observation that NGF promoted the survival of small mediodorsally located sensory neurons in DRGs, while NGF had no effect on the larger ventrolaterally located sensory neurons.

1.2 Discovery of the Neurotrophin Gene Family

The sensory ganglia that are segmentally distributed along the trunk of vertebrates derive from the neural crest, but many cranial sensory ganglia derive instead from epithelial placodes. Target-derived trophic factors are required for developmental survival of both neural crest-derived and placode-derived peripheral sensory neurons, yet NGF only promotes survival of neural crest-derived sensory neurons. Such observations motivated experiments leading to the discovery and molecular cloning of brain-derived neurotrophic factor (BDNF) as a trophic factor for placode-derived sensory neurons (Barde et al. 1982). Nucleotide sequence analysis revealed that NGF and BDNF were structurally related (Leibrock et al. 1989). Several teams of investigators recognized independently that short highly conserved regions of NGF and BDNF transcripts permitted the design of primers for polymerase chain reaction that would jointly amplify both NGF and BDNF sequences and employing these primers discovered additional members of the NGF gene family—neurotrophin-3 (NT3) (Hohn et al. 1990; Jones and Reichardt 1990; Maisonpierre et al. 1990; Rosenthal et al. 1990) and neurotrophin-4 (NT4) (Berkemeier et al. 1991; Hallbook et al. 1991; Ip et al. 1992). The fourth mammalian neurotrophin identified was variously named NT4 or NT5, according to whether the discoverers included a previously described fish neurotrophin in their numbering scheme. As a compromise between the alternative nomenclatures, the fourth mammalian neurotrophin is frequently referred to as NT4/5. It will be called NT4 in this chapter. Some fish possess an additional neurotrophin family member, while birds lack NT4. The term neurotrophin was originally coined to describe members of the NGF gene family. A few present day investigators use the term "neurotrophin" as a synonym for "neurotrophic factor." Most investigators prefer to reserve the term "neurotrophin" for its original purpose, as a means to refer to NGF gene family members collectively.

2 Neurotrophin Structure

Mature neurotrophins exist as noncovalently associated dimers of ~13,500 Da protomers (Bothwell and Shooter 1977; Radziejewski et al. 1992). The affinity of the dimeric NGF association is sufficient to prevent dissociation even at the pM concentrations at which NGF acts physiologically (Bothwell and Shooter 1977) and this is probably also true for the other neurotrophins. High resolution structures have been determined for each of the neurotrophins (Butte et al. 1998; McDonald et al. 1991; Robinson et al. 1999). Each neurotrophin subunit has a backbone consisting of two pairs of antiparallel β-strands generating an elongated shape and stabilized by three disulfide bonds. The structure has been referred to as a cystine knot, and a similar folding organization has been observed in several other growth factors, including TGF-beta and PDGF family members (McDonald and Chao 1995). The highly conserved interaction interface of the neurotrophin dimers permits the formation of heterodimers between different neurotrophins in vitro but

evidence is lacking for the existence of such heterodimers in vivo (Jungbluth et al. 1994; Philo et al. 1994; Robinson et al. 1995).

Early studies of the biochemistry of NGF placed particular attention on the high molecular weight complex in which NGF could be isolated from mouse submaxillary salivary gland (Greene et al. 1969; Nichols and Shooter 1985). The sedimentation velocity of this complex was 7 svedbergs—accordingly the complex was known as 7S NGF. Curiously, this complex was ultimately found to represent NGF (known at the time as low molecular weight NGF, 2.5S NGF or beta-NGF) in association with alpha and gamma subunits which represent two different members of the glandular kallikrein family of proteases (Bothwell et al. 1979; McDonald and Blundell 1991). The physiological relevance of the high molecular weight complex of NGF is unclear, as this form of NGF appears to exist only in mouse, and in mouse, only in salivary glands. The manner in which this peculiar biological adaptation of NGF may have evolved in mice is discussed below.

3 Neurotrophin Receptors

The four mammalian neurotrophins interact with four receptors; $p75^{NTR}$, TrkA, TrkB, and TrkC. The function of these multiple receptors is complex, as $p75^{NTR}$ and Trk receptors can function independently, but in neurons that express both $p75^{NTR}$ and Trk receptors, the receptors interact physically and functionally in ways that may alter the signaling properties of each. The structure and signaling functions of these receptors are discussed in detail elsewhere in this book. Briefly, all four neurotrophins, both as proneurotrophins and as mature fully processed neurotrophins, can bind and activate signaling by $p75^{NTR}$, whereas the Trk receptors prefer to bind mature neurotrophins and are selective for particular neurotrophins. NGF preferentially binds and activates TrkA, NT3 preferentially binds and activates TrkC, and BDNF and NT4 preferentially bind and activate TrkB. For this reason, BDNF and NT4 are typically functionally redundant in mammals, and reflecting this redundancy, the NT4 gene has apparently been lost during evolution of birds. NT3 is the most promiscuous of the neurotrophins as alternative splicing of the TrkA transcript can generate forms of TrkA that are effectively activated by NT3 (Clary and Reichardt 1994). Importantly, however, NGF and NT3 are not functionally equivalent with respect to TrkA activation, as they influence TrkA signaling differently (Harrington et al. 2011).

One or more of the four neurotrophin receptors are expressed in a wide variety of types of neurons and glia in both the central and peripheral nervous system and also in a variety of non-neural cell types. Thus, neurotrophins have an extraordinary range of biological functions, with the neurotrophin preference of various cell populations being determined by the particular neurotrophin receptor or receptors they express.

4 Neurotrophin Processing and Secretion

Like most other secreted biologically active polypeptides, protein synthesis of neurotrophins occurs in the rough endoplasmic reticulum, where the proneurotrophins are packaged into secretory vesicles. Proneurotrophins, which range from about 210 to 270 amino acid residues in length, are processed within these vesicles by proteases of the proprotein convertase family (Seidah et al. 1996), producing the mature neurotrophins which are about 120 residues in length. In the case of BDNF, the cleaved prodomain is stored with and cosecreted with mature BDNF (Dieni et al. 2012). Whether this pro-peptide has any biological function, and whether the pro-domains of other neurotrophins are secreted, is unknown. In some cases, vesicular processing of neurotrophins is incomplete, leading to secretion of unprocessed pro-neurotrophins from which the mature neurotrophin may be released by plasmin and matrix metalloproteinases following secretion (Lee et al. 2001).

The seminal experiments that lead to the proposal of the "neurotrophic hypothesis" and the discovery of neurotrophins examined neuronal populations that innervated non-neural peripheral target tissues. However, in many cases, and particularly in the central nervous system, the neurotrophin-producing innervated target cell may also be a neuron. Neurotrophin secretion by neuronal and non-neuronal cells differs in several important ways that were not immediately appreciated by investigators. Firstly, neurons (and neuroendocrine cells) have distinct regulated and constitutive secretory pathways, whereas non-neuronal cell types typically have only the constitutive secretory pathway (Kelly 1985). Thus, while the manner of secretion of the four neurotrophin is similar in non-neural cell types, in neurons, this is not the case, as NGF, NT3, and NT4 traffic mainly through the constitutive secretory pathway in neurons and neuroendocrine cells, whereas BDNF selectively traffics through the regulated secretory pathway (Farhadi et al. 2000; Griesbeck et al. 1999; Hibbert et al. 2003; Mowla et al. 1999). This distinction is particularly important in the context of BDNF functions in learning and memory, where control of BDNF secretion by neural activity is likely to be essential, as discussed elsewhere in this book. The second important distinction between neurons and many non-neural cell types is that neurons are highly polarized cells. Initially, no doubt with a mindset influenced by early studies of neurotrophic functions with non-neural neurotrophin-producing cells, the expectation was that neurons would secrete neurotrophins principally at the somatodendritic membrane domains, as these are normally the site of axonal synaptic contacts. However, in neurons, much of BDNF secretion follows the same secretory pathway as neuropeptides, being packaged in dense core vesicles, which are transported anterogradely down axons and secreted at the axon terminus (Conner et al. 1997; Dieni et al. 2012; von Bartheld et al. 1996; Zhou and Rush 1996).

Two proBDNF-binding proteins have been implicated in directing proBDNF to the regulated secretory pathway, carboxypeptidase E (Lou et al. 2005), and sortilin (Chen et al. 2005). Importantly, a common allelic variant of the human *BDNF* gene

encodes a Val/Met substitution within a region of the BDNF pro-domain that binds sortilin. Consequently, the Met-containing proBDNF variant is poorly sorted into the activity-regulated secretory pathway, resulting in poor performance in some memory tasks (Chen et al. 2004, 2005; Egan et al. 2003).

5 Differential Activity of Neurotrophins and Proneurotrophins

It is beyond the scope of this chapter to provide a detailed discussion of the various functions of neurotrophins or of the structure and function of neurotrophin receptors. For these topics, the reader may consult other chapters in this volume. For the purposes of this chapter, it is sufficient to say that all four neurotrophins interact effectively with the $p75^{NTR}$ neurotrophin receptor, whereas TrkA functions primarily as an NGF receptor, TrkB as a receptor for BDNF and NT4, and TrkC as a receptor for NT3 (Bothwell 1991). Additional complexity is provided by the ability of NT3 to interact weakly with TrkA and TrkB receptors and by the ability of $p75^{NTR}$ to influence neurotrophin/Trk receptor interactions (Huang and Reichardt 2003).

The structural basis governing the selectivity of neurotrophin/receptor interactions has been extensively characterized by X-ray crystallographic analysis of neurotrophin/receptor complexes and by mutagenic structure/function studies. The extracellular domain of Trk receptors contains leucine-rich repeat domains and two C2-immunoglobulin-like domains, and the second immunoglobulin-like domain represents the main neurotrophin-binding region (Holden et al. 1997; Urfer et al. 1998), although leucine-rich repeat domains may also contribute to binding (Windisch et al. 1995). The interaction of neurotrophins with Trk receptors is mediated largely by two neurotrophin interaction surfaces. One important interaction involves amino acid residues that are highly conserved among neurotrophins, whereas a second neurotrophin/Trk interaction surface employs neurotrophin amino acid residues that are not conserved among neurotrophins. The former surface (known as the conserved patch) contributes importantly to the affinity of binding, whereas the latter surface (known as the specificity patch) clearly is largely responsible for determining the selectivity of binding of particular neurotrophins to particular Trk isoforms (Hirata-Fukae et al. 2008). A similarly detailed comparison of the binding interactions of all the neurotrophins with $p75^{NTR}$ has not been published, although high resolution structures of both NGF and NT3 with $p75^{NTR}$ have been reported (Aurikko et al. 2005; Gong et al. 2008; He and Garcia 2004).

Initially it appeared that it might be possible to generalize neurotrophin function by stating that the different neurotrophins have a similar range of functions, but directed against different cell populations, depending on the receptors expressed. It has become clear, however, that the truth is much more complicated than this, as the four different neurotrophin receptors can mediate very different and in some cases even opposite cellular responses.

Although the function of neurotrophins in the peripheral nervous system contributes to the neurotrophic support of various neuronal populations, in complete accord with the "neurotrophic hypothesis" originally developed by Victor Hamburger and Rita Levi-Montalcini, the neurotrophins mediate an extraordinary range of other functions, which are not amenable to simplified generalizations. For example, neurotrophins can either promote neuronal survival or promote neuronal cell death, depending on the circumstance, the receptor employed, and whether mature or proneurotrophins forms are present. In some cases neurotrophin/receptor association promotes pro-survival signaling, in other cases, neurotrophin/receptor association promotes pro-death signaling (Rajagopal et al. 2004), and in still other cases, neurotrophin/receptor association terminates activity of a receptor that constitutively signaling pro-death (Huang and McNamara 2010; Lee et al. 2002). Further, while neurotrophin action sometimes conforms to the original neurotrophic hypothesis, which predicts that neurotrophins should convey trophic signals from the axon terminus retrogradely back to the neuronal soma, in some cases, and particularly for BDNF, neurotrophins may be transported anterogradely down axons to convey signals to postsynaptic cells (Altar et al. 1997; Conner et al. 1997; Smith et al. 1997; von Bartheld et al. 1996; Zhou and Rush 1996). Other neurotrophin functions, such as control of neuronal dendritic branching (McAllister et al. 1997) and control of synaptic function (Kang and Schuman 1995; Lohof et al. 1993; Patterson et al. 1992), differ radically from the original concept of the neurotrophic hypothesis. Other chapters in this volume provide a more comprehensive discussion of the range of neurotrophin functions.

6 Evolution of Neurotrophins

6.1 Invertebrate Origins of Neurotrophins

The neurotrophin-signaling system, including neurotrophin-like ligands and $p75^{NTR}$ and Trk-like receptors, evolved before the evolution of vertebrates. The genome of the arthropod Daphnia pulex encodes clearly recognizable neurotrophin, $p75^{NTR}$, and Trk orthologs (Wilson 2009). Bilaterian organisms fall within two major branches, protostomes, including arthropods such as Daphnia, and deuterostomes, from which vertebrates evolved. The genomes of prechordate deuterostomes *Strongylocentrotus purpuratus* (sea urchin) and *Saccoglossus kowalevskii* (acorn worm) also encode neurotrophin, $p75^{NTR}$, and Trk orthologs (Bothwell 2006). Thus, the shared ancestor of protostomes and deuterostomes must have possessed neurotrophin, $p75^{NTR}$, and Trk orthologs.

It remains to be determined whether the invertebrate neurotrophin orthologs bind and activate the invertebrate neurotrophin receptor orthologs. Interestingly, however, the sea urchin neurotrophin has a pro-domain, a mature neurotrophin domain, and a predicted site for proteolytic processing that are closely similar to these domains in vertebrate neurotrophins, suggesting that proteolytic processing might control selective association of proneurotrophin and mature neurotrophin

with $p75^{NTR}$ and Trk receptors, in invertebrates as in vertebrates. Indeed, the sea urchin proneurotrophin is processed by the proprotein convertase furin, releasing mature 13 kDa neurotrophin, when expressed in human HEK293 cells (Mark Bothwell and Mark Hudson, unpublished results).

Curiously, the neurotrophin-signaling system has apparently been lost during evolution of several bilaterian classes. Clear orthologs of neurotrophin, $p75^{NTR}$, and Trk genes cannot be identified in protostome species such as *Drosophila melanogaster* or *Caenorhabditis elegans* or in deuterostome species such as *Ciona intestinalis* (Bothwell 2006). Lack of a neurotrophin-signaling system in *Ciona* is especially curious, as tunicates such as *Ciona* are commonly believed to the closest living prechordate relatives of vertebrates.

The lack of a neurotrophin-signaling system in *Drosophila* is a subject of mild controversy, as a family of *Drosophila* neurotrophic factors have been named as neurotrophins (Zhu et al. 2008). These secreted proteins, like neurotrophins, do contain a cystine knot structural fold, but they are only marginally more similar in sequence to neurotrophins than they are to a variety of non-neurotrophin proteins that contain cystine knot structures, and rather than signaling via $p75^{NTR}$-like or Trk-like receptors, they apparently employ receptors of the toll-like receptor family.

The chordate ancestor of modern vertebrates probably had only one neurotrophin and one Trk gene. The multiple neurotrophin and Trk paralogs in modern vertebrates (four neurotrophins in mammals, three neurotrophins in birds, three Trks in both mammals and birds) apparently arose as a result of the two genome duplications that occurred as an early event in vertebrate evolution (Hallbook et al. 2006).

6.2 Evolutionary Adaptations of NGF

6.2.1 Mouse Saliva NGF

The abundance of NGF in mouse salivary glands and in the venoms of various snakes (Cohen and Levimontalcini 1956), which greatly aided the original isolation of NGF, somewhat distorted understanding of the biochemistry of NGF in the early years after its discovery, since these sites of NGF synthesis represent peculiar species-specific adaptations with associated specializations of NGF synthesis and storage. Mouse salivary gland NGF is stored in secretory vesicles as a zinc ion-stabilized heterotrimeric complex, 7S NGF, consisting of alpha, beta, and gamma subunits. The beta subunits contain all of the neurotrophic activity (hence the name beta-NGF, which is still in common use), whereas the alpha and gamma subunits are members of the tissue kallikrein family of proteases (Bothwell et al. 1979). The tissue kallikrein gene family has undergone substantial expansion in rodents—orthologs of alpha and gamma NGF subunits do not exist in most other mammals, including humans. Thus, the 7S complex of NGF is only of interest as a peculiar mouse-specific specialization, and even in mice, there is little evidence for this heterotrimeric complex in tissues other than salivary glands.

6.2.2 NGF as a Noxious Component of Venoms

The question inevitably arises—why is NGF so highly enriched in the submaxillary salivary glands of mice and in the venom glands of snakes? The question is particularly poignant since NGF is abundant in the venoms of rattle snakes, vipers, and Australian elapid snakes, and the venom glands of these snake families are believed to have evolved independently (Jackson 2007), indicating that the highly enriched production of NGF in salivary or venom glands has evolved on at least four separate occasions, and implying that there is a strong selective pressure for this adaptation. It is reasonable to speculate that the selective pressure derives from the ability of NGF elicit pain. Ironically, the ability of NGF to elicit profound pain was not fully appreciated until it emerged as a serious side effect of human clinical trials of NGF for treatment of peripheral neuropathy (Petty et al. 1994). Although venomous snakes use their venom to immobilize prey, this effect is too slow to be a reliable defense mechanism against predators of snakes. The ability of snake venoms to cause immediate pain, mediated by NGF, may be a specialization to discourage predators. NGF is secreted at much higher concentrations in the saliva of male mice than in female mice and is released specifically in a specialized nondigestive saliva produced in response to epinephrine (Wallace and Partlow 1976), which is presumably elevated during the aggressive encounters that typify the behavior of male mice. Thus, salivary NGF in mice may also function as a pain-producing weapon.

6.2.3 Fowlpox NGF

One other evolutionary adaptation of NGF deserves comment. The genome of the fowlpox virus contains genes encoding two NGF orthologs (Afonso et al. 2000). Although the functional properties of the encoded NGF proteins have not been characterized, viruses commonly capture host genes that enhance the ability of the virus to evade host defenses. It would be interesting to learn what feature of avian skin biology is modified by the virally encoded NGFs. As conventional forms of NGF act through both $p75^{NTR}$ and TrkA receptors, it would also be interesting to learn whether the viral NGF has become specialized for activation of one or the other of these two receptors.

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