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Role of Neurotrophins and Trk Receptors in the Development and Maintenance of Sensory Neurons: An Overview

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Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons: an overview

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SUMMARY

The neurotrophins are a family of polypeptide neuronal growth factors related to the prototypical neurotrophic factor, nerve growth factor (NGF). In mammals this gene family encompasses NGF, brain-derived neurotrophic factor (BDNF) and neurotrophins-3 and -4/5, (NT-3, NT-4/5). The neurotrophins initiate signal transduction in responsive cells by ligand induced dimerization and activation of one of the *Trk* family of receptor tyrosine kinases; NGF being specific for TrkA, BDNF and NT-4/5 for TrkB, and TrkC the preferred receptor for NT-3. In accord with differential patterns of distribution of Trk receptors in peripheral ganglia, the neurotrophins show both distinct and overlapping specificity towards subpopulations of sensory neurons of both neural crest and neural placode origin. *In vitro* and *in vivo* studies, and transgenic mice baring targeted null mutations of the neurotrophin genes have established that BDNF, NT-3 and NT-4/5, like NGF, play critical roles as classical target-derived survival factors for subclasses of developing sensory neurons. However, much broader effects of neurotrophins on sensory neurons are now evident, including paracrine and autocrine actions on neuroblast proliferation, phenotypic differentiation, and survival and regeneration in the adult. This article provides an overview of the discovery and properties of the neurotrophin family, their receptors and their actions and specificity for both distinct and overlapping subpopulations of spinal and cranial sensory neurons.

1. NEUROTROPHIC FACTORS: AN INTRODUCTION

Neurotrophic factors constitute a class of protein molecules that are now considered critical for the development, maintenance and regeneration of the nervous system. Much of the conceptual framework surrounding the suspected function of neurotrophic factors has emerged from almost 50 years of study of the prototypical neurotrophic factor, nerve growth factor (NGF). The discovery of NGF itself arose from the search for a molecule that would satisfy the criterion of a target-derived effector molecule capable of promoting the survival of developing peripheral neurons at the stage when these neurons begin to innervate or terminate in their target peripheral tissues. The initial search for such 'neurotrophic factors' stemmed from the classical embryological observations of Viktor Hamburger. He showed through limb-bud ablation or transplantation experiments that the size of the peripheral target field had a direct influence on the number of peripheral or motor neurons that survived through the critical ontogenic phase of naturally occurring cell death, a period when there is elimination of neurons that are produced in excess of those ultimately required (Hamburger 1952). Identification and purification of NGF by Levi-Montalcini, Booker and Cohen provided the first molecular verification of such a target-derived neurotrophic factor hypothesis (reviewed in Levi-Montalcini & Angeletti 1968).

Within neurons of the peripheral nervous system (PNS), extensive tissue culture studies over several decades have established that NGF promotes the survival and differentiation of sympathetic neurons and subpopulations of neural crest-derived neurons, but has no demonstrable effect on cranial sensory neurons, enteric neurons, parasympathetic neurons or spinal motor neurons (reviewed in Thoenen & Barde 1980; Lindsay 1988*b*, 1992). This pattern of NGF specificity and the critical role of this molecule in ontogeny was confirmed *in vivo* first through neutralization of endogenous NGF with either autologous or heterologous antibodies. Sequestration of endogenous NGF in this manner leads to 'immunosympathectomy' – complete destruction of the superior cervical ganglion – and a loss of neurons in the dorsal root ganglion (Levi-Montalcini & Angeletti 1968). Elegant confirmation of this specificity of NGF among PNS neurons has recently been obtained from analysis of transgenic mice devoid of NGF through targeted disruption of the NGF gene (Crowley *et al.* 1994). Mice that are homozygous for a null mutation in the NGF gene are born with virtually no sympathetic neurons and a loss of $> 70\%$ of the normal complement of dorsal root ganglia (DRG) neurons.

Of the many different subclasses of growth factors that act upon neuronal or non-neuronal cells (or both), NGF remains as one of the factors with the highest degree of cellular specificity, with actions outside the PNS restricted to a very limited number of neurons in

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the brain (largely cholinergic neurons in the basal forebrain, and cholinergic interneurons in the striatum). Indeed this highly restricted specificity of NGF provided much of the impetus to search for related molecules with similar properties but distinct specificities. This search lead initially to the discovery and purification of brain-derived neurotrophic factor (BDNF; reviewed in Lindsay 1993) and subsequently to the identification of a larger family of NGF-related neurotrophic factors, the neurotrophins. Members of this family have both distinct and overlapping specificities for subclasses of sensory neurons (Barde 1989; Davies 1994; Lindsay *et al.* 1994).

2. PRIMARY SENSORY NEURONS

Primary sensory neurons of the spinal DRG and cranial ganglia represent the main conduit for conveying information from the periphery to the brain and spinal cord. Sensory neurons of the DRG function primarily as detectors of noxious stimuli (nociceptors), pressure (mechanoceptors) and positional information (proprioceptors). Neurons of the cranial sensory ganglia, although less numerous than DRG neurons have more varied functions, acting for example as baroreceptors and chemoreceptors (nodose ganglion neurons) and transducers of the specialized senses such as sound (spiral ganglion neurons). Although these peripheral neurons may be considered less complex in either structure or function than many classes of central nervous system (CNS) neurons, the diversity of primary sensory afferents seems to increase in direct relation to any attempts to classify them by biochemical, molecular or physiological phenotype. This seems to hold true when attempting to correlate the chemical or functional phenotype of these neurons with their responsiveness to particular neurotrophic factors (see below). For simplicity in this article, I will use some generalization with regard to categorizing subpopulations of DRG neurons (for a more detailed review of the biology of sensory neurons see Scott 1992). Furthermore, I make the general assumption that the origins of sensory neurons that have been well established for DRG and cranial ganglia of the chicken embryo may hold true in rodents.

Before the discovery of the neurotrophin family, it was clear from *in vitro* studies that most, if not all, sensory neurons that respond to NGF are of neural crest origin (Davies & Lindsay 1985). Response or lack of response to NGF was initially quantitated by whether or not NGF elicited neurite outgrowth from explanted ganglia. Later, and most contemporary, studies tend to additionally define responsiveness of developing neurons to a neurotrophic factor as the ability to promote neuronal survival (or a more mature phenotype) in neuron-enriched cultures. Using the latter measure, it became clear that in embryonic chickens or rats 50–70 % of DRG neurons appeared to be responsive to NGF; conversely one-third to one-half of DRG neurons appeared unresponsive to NGF. Taken together, many *in vivo* studies have lead to a general consensus that the majority of NGF responsive DRG neurons are primarily nociceptive C-fibres: neurons with a relatively small soma and small, unmyelinated axons. Conversely, DRG neurons with larger axon caliber's (and larger cell soma) that subserve mechanoceptive or proprioceptive modalities have generally been considered to be unresponsive to NGF.

In marked contrast to sensory neurons of neural crest origin, neurons derived from the neural placodes that give rise to the majority of the sensory ganglia of the specialized cranial nerves appear to be unresponsive to NGF (Davies & Lindsay 1985; Lindsay & Rohrer 1985; Davies 1989). However, as with DRG neurons, neurons of cranial ganglia such as the nodose ganglion do not survive in culture in the absence of appropriate neurotrophic support. Before the discovery of the neurotrophins, various tissue extracts were found to contain non-NGF neurotrophic activities that were capable of supporting the survival of various classes of cranial sensory neurons such as those of the nodose ganglion (Lindsay & Rohrer 1985). Taken together, the very restricted specificity of NGF for a subpopulation of DRG neurons and the lack of response of most cranial sensory neurons to NGF initiated the search and subsequent isolation of other neurotrophic factors.

3. THE NEUROTROPHINS: DISCOVERY OF BDNF, NT-3 AND NT-4/5

As was the case with the identification and purification of NGF, sensitive *in vitro* assays using explants of PNS ganglia or enriched cultures of different classes of PNS neurons were instrumental in the discovery of BDNF. Barde and colleagues first described an activity in the conditioned medium of glioma cells that: (i) supported the survival of cultured chicken embryo dorsal root ganglion cells in culture; but (ii) was not NGF by virtue of the fact that this activity was could not be neutralized by antibodies to NGF (Barde *et al.* 1978). Similar or related non-NGF activities were subsequently identified in cultured astrocytes and in extracts of both rodent and porcine brain (reviewed in Lindsay 1993). The first molecular characterization of one of these activities was achieved with the painstaking purification of very small quantities of BDNF from pig brain (Barde *et al.* 1982). Partial sequencing of the purified BDNF protein subsequently lead to molecular cloning of the BDNF gene (Liebrock *et al.* 1989). Whereas the physical properties of purified BDNF had already suggested that it might be closely related to NGF, the primary structure of the mature BDNF protein revealed a high degree of amino acid identity to NGF (55 %) and, in particular, conservation in BDNF of all six cysteine residues (and a very high degree of homology in sequences flanking these residues) found in NGF. Although these data confirmed that BDNF and NGF were closely related, differences in their biological activity and neuronal specificity had already been identified. Whereas BDNF and NGF appeared to have overlapping actions on chicken DRG neurons that might have indicated similar neuronal specificity, BDNF, unlike NGF, was found to promote the survival of nodose ganglion

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neurons (Lindsay *et al.* 1985). Conversely BDNF was found to have no effect upon sympathetic neurons, whereas in tissue culture NGF promotes the survival of the majority of the latter class of neurons.

It appears that when expressed as target-derived neurotrophic factors, growth factors such as NGF and BDNF are present in relevant tissues in strictly limiting amounts. This was evident in the purification of BDNF, where a purification factor of several million-fold was required to obtain homogenous protein. These findings support one of the original premises of the target-derived neurotrophic hypothesis that the selection of the appropriate numbers and types of neurons that make up the final complement of neurons that innervate a particular structure results from competition for limiting amounts of survival factor(s) produced by that tissue. For many years this low abundance was a major barrier to the molecular characterization of novel neurotrophic factors. The cloning of BDNF was thus not only a major feat in itself (Thoenen 1991) but the homology of BDNF to NGF immediately offered the prospect of identifying further 'relatives' of a gene family by homology cloning strategies.

Using the regions of NGF and BDNF that are most conserved, several groups used a homology cloning approach to identify firstly a third family member, neurotrophin-3 (NT-3; Hohn *et al.* 1990; Maisonpierre *et al.* 1990*b*; Rosenthal *et al.* 1990) from whence derives the family name 'neurotrophin', and subsequently two other family members (NT-4; also known as NT-5 or NT-4/5: see Berkemeier *et al.* 1991; Hallböök *et al.* 1991; Ip *et al.* 1992) and NT-6 (Götz *et al.* 1994). At present NT-6 has been identified only in lower species, with no evidence, as yet, of a mammalian gene. Cloning of the neurotrophin genes has allowed production of each factor as a recombinant protein in relatively unlimited amounts. This has played a crucial role in the rapid advances that have taken place in the last five years in understanding the role of each neurotrophin in the development and maintenance of multiple neuronal types of both the CNS and PNS (reviewed in Lindsay *et al.* 1994), especially primary sensory neurons.

4. PROPERTIES OF THE NEUROTROPHINS

The neurotrophins are expressed as prepro-precursors that are processed to yield highly basic (pI 9–10.5) mature proteins of around 13 kDa monomers (120 amino acids). Between any two family members there is around 50% identity in primary amino acid sequence, with complete conservation of 6 critical cysteine residues (3-disulfide bridges; for review see Glass & Yancopoulos 1993). At physiological concentrations the neurotrophins exists as homodimers (heterodimer formation may possibly exist in cells that produce more than one neurotrophin). Resolution of the crystal structure of NGF has revealed that the highly conserved regions form a hydrophobic core of four anti-parallel beta strands, whereas obvious structural diversity among family members occurs in several exposed variable loops, as well as at both the N and C termini (McDonald *et al.* 1991; McDonald & Chao 1995). Although there is some evidence from mutagenesis studies, and the creation of many chimeric neurotrophin proteins (Ibáñez *et al.* 1991), that these variable regions may account directly for differences in the distinct receptor (and thus functional) specificities of each of the neurotrophins, it is not yet clear which portions of the neurotrophins are involved in binding to either the low affinity neurotrophin receptor ($p75^{LNTFR}$) or to individual members of the Trk family of high-affinity neurotrophin receptors. Ongoing analyses of co-crystals of each of the neurotrophins with either their common low affinity receptor ($p75^{LNTFR}$) or cognate Trk receptor should hopefully shed light on this intriguing issue.

5. NEUROTROPHIN RECEPTORS: P75 AND THE TRK FAMILY

Radiolabelled ligand binding studies with ^{125}-NGF indicated the existence of different classes of NGF receptors that could be distinguished by different binding affinities and different kinetics of binding and displacement (for review see Bothwell 1995). The first receptor to be molecularly characterized and cloned was the so-called low-affinity NGF receptor or LNGFR. Although the precise function of this protein, particularly when expressed in neurons, remains controversial, it is clear that it acts as a 'low' affinity (nM) receptor for all the neurotrophins and is generally referred to as $p75^{LNTR}$ or simply p75 (Meakin & Shooter 1992).

A serendipitous histological observation lead to the recent discovery that a previously identified proto-oncogene, a transmembrane tyrosine kinase termed *Trk* (tropomyosin related kinase) is a high affinity receptor for NGF (reviewed in Barbacid 1994). As shown by *in situ* hybridization and ligand binding studies, Trk is expressed in all NGF responsive cell in both the PNS and CNS. Studies with both NGF responsive neurons and cell lines (PC12 cells, 3T3 or MG87 fibroblasts) transfected to express Trk have confirmed that like other receptor tyrosine kinases, binding of NGF to Trk induces receptor dimerization, autophosphorylation and activation of an intracellular amplification cascade that includes several known pathways, including activation of MAP kinases etc. (reviewed in Glass & Yancopoulos 1993).

Almost in parallel with the discovery of the neurotrophin family, relatives of Trk have been identified, such that three Trk genes are now known to constitute the signal transducing element of cellular responses to NGF, BDNF, NT-3 and NT-4/5 (for review see Barbacid 1994; Bothwell 1995). As the obligate high affinity receptor for NGF signalling, Trk is now widely referred to as TrkA. TrkB serves as the unique signal transducing receptor for both BDNF and NT-4/5. Whereas TrkC appears to be the preferred receptor for NT-3, NT-3 has gained notoriety as being promiscuous, due to the capability of this neurotrophin to activate all three Trk receptors, albeit showing different potencies and possibly eliciting different types

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of responses when bound to TrkA or TrkB (as compared to activation of the latter with their cognate ligands, NGF and BDNF/NT-4/5, respectively).

A possible further level of complexity to neurotrophin signalling in sensory neurons lies in the fact that their appears to be multiple transcripts of each of the Trk family members. The extent to which these transcripts are translated or how such variants may function as receptor proteins is not entirely clear. With regard to TrkA, two variants that differ in their extracellular domains have been identified (Barker *et al.* 1993). With both TrkB and TrkC, truncated receptors that lack any kinase domain have been found to be widely expressed in the brain and in peripheral ganglia; truncated TrkB protein is particularly abundant in the mature nervous system with estimates that this form of TrkB is found at tenfold higher levels than the catalytic full length TrkB protein. Finally, several transcripts of TrkC have been found to encode proteins with small inserts (up to 40 amino acids) in their kinase domain (Valenzuela *et al.* 1993). Taken together these findings, and as yet uncharacterized splice variants of the three Trk genes, suggest that the specificity of neurotrophin signalling in sensory neurons will prove to be more complex than that which may be revealed from a simple analysis of the distribution of the expression of mRNA for each of the Trk genes in DRG. Furthermore, it is now clear that in sensory neurons, as in the CNS, individual neurons often express more than one Trk gene. For example studies by McMahon *et al.* (1994) indicate that in the adult rat DRG the expression of TrkB largely overlaps with either TrkA or TrkC, such that few DRG neurons express TrkB alone. In addition it has recently been suggested that a significant percentage of neurons that express TrkA also express TrkC (V. M. K. Verge, personal communication). This coexpression of Trk receptors is not only important in understanding the actions of neurotrophins in mature neurons, but also raises the issue of temporal changes in the response of individual neurons to different neurotrophins during development.

6. NEUROTROPHIN AND TRK RECEPTOR EXPRESSION

Northern analyses and *in situ* hybridization studies have shown widespread expression of each of the neurotrophins and their Trk receptors throughout the CNS and periphery (Maisonpierre *et al.* 1990*a*; Glass & Yancopoulos 1993; Altar *et al.* 1994; Lindsay *et al.* 1994). In the CNS almost all neurons appear to express TrkB or TrkC (or both), whereas TrkA expression is highly restricted to cholinergic neurons of the basal forebrain or striatum. In parallel with their receptors, BDNF and NT-3 are expressed in many parts of the developing and adult CNS; clues from developmental analysis of several CNS structures suggest that NT-3 levels are highest during ontogeny, whereas BDNF levels only peak upon maturity (Maisonpierre *et al.* 1990*a*). This has suggested that in addition to any actions as a target derived factor, NT-3 may act early in development as a mitogenic or early post-mitotic signal for certain neurons in the spinal cord and granule cells of both the hippocampus and cerebellum. In contrast the later appearance of peak levels of BDNF in the CNS as a whole has predicted a role for BDNF in maintenance and regulation of the morphological and neurochemical phenotype of mature neurons (Lindsay 1993).

Trk receptors have been detected on all classes of PNS neurons with the notable exception of parasympathetic neurons of the ciliary ganglion. With regard to sensory neurons, TrkA is expressed only in DRG and other neural crest-derived ganglia, whereas TrkB and TrkC are expressed to some degree in all sensory ganglia. However, the expression pattern of Trk receptors is not as simple as first envisaged. Firstly, the pattern of expression appears to change during development. For example, during embryogenesis up to 70% of DRG neurons express TrkA but this number declines to around 40% in the adult rat. Secondly coexpression in a single neuron of two members of the Trk family is common, e.g. in adult rat DRG few cells express TrkB alone rather TrkA+TrkB or TrkB+TrkC (McMahon *et al.* 1994). In addition overlap of TrkC with TrkA has recently been reported (V. M. K. Verge, personal communication). More detailed accounts of Trk and p75 ^{LNTR} expression in developing and mature DRG will be found elsewhere in this volume.

The low abundance of the neurotrophins in peripheral tissues has hampered the generation of a clear picture of their expression patterns in developing and mature animals. However, Northern analyses and *in situ* hybridization has confirmed expression of relevant neurotrophin mRNA in the target tissues of particular classes of sensory neurons (see below). In addition to target tissues, neurotrophin mRNA and protein have been detected in peripheral nerve support cells (Schwann cells and fibroblasts) and indeed in sensory neurons themselves. These sources of neurotrophins have suggested important roles of these factors beyond their classical role as developmentally critical target-derived survival promoting factors. The presence of neurotrophins in PNS support cells implicates a possible role in nerve regeneration and remodelling, whereas the finding of neurotrophin expression within DRG neurons themselves has suggested paracrine and autocrine actions in the maintenance of mature neurons (see below).

7. EFFECTS OF NEUROTROPHINS ON SENSORY NEURONS *IN VITRO*

(*a*) *Target derived survival factors for early post-mitotic sensory neurons*

Largely due to the 'template' derived from extensive studies of NGF, initial characterization of the actions of BDNF, NT-3 and NT-4/5 towards sensory neurons focused on either neurite promoting or survival promoting effects of these factors towards different

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subclasses of either spinal (DRG) or cranial sensory neurons. These studies have largely been carried out in tissue culture using explants or dissociated neuron-enriched cultures of sensory ganglia derived from chicken embryos. Detailed accounts of these studies appear elsewhere in this volume, but broadly speaking such studies have shown that DRG and other neural crest-derived primary sensory neurons show some degree of responsive to NGF, BDNF or NT-3, whereas neural placode-derived cranial sensory ganglia show little or no response to NGF, but varying degrees of response to BDNF or NT-3.

In the chicken embryo during the period between E6-E12, NGF supports survival of approximately 50% of DRG neurons, whereas BDNF supports survival of around 30–40% (Lindsay *et al.* 1985) of these cells. The discovery that the effects of each neurotrophin were additive, suggested for the first time that NGF and BDNF might have distinct specificities for different subpopulations of sensory neurons. The magnitude of the effects of NGF or BDNF were found to be essentially the same on DRG taken from different segmental levels over the period of E6-E12 i.e. there was no obvious spatial or temporal variance in the percentage of neurons supported by each factor. NT-3, like NGF and BDNF, was found to promote neuronal survival and outgrowth of neurites from explants of chick embryo dorsal root ganglia (Maisonpierre *et al.* 1990*b*). In this respect, NT-3 was clearly less effective that NGF, but qualitatively similar to BDNF. The observation that NT-3 produced much greater neurite outgrowth from explants of chick embryo DRG excised from the cervical or lumbar enlargement than from thoracic or sacral segments (Hory-Lee *et al.* 1993) suggested that NT-3 might act on a specific subclass of DRG neurons. More detailed analysis using dissociated cultures of E9 chick DRG neurons, showed that NT-3 promoted the survival of 25–30% of neurons from lumbar ganglia as compared to less than 10% of neurons in thoracic DRG. The greater percentage of proprioceptive neurons in the DRG of the cervical and lumbar enlargement suggested that NT-3 might be relatively specific for those neurons. This was confirmed in experiments in which the neurons of the DRG that innervate skin or muscle in the developing chick were selectively labeled by retrograde tracers *in ovo* before excision and use in tissue culture assays. When cultured as dissociated neurons, NT-3 was found to promote the survival of muscle afferents to a greater degree than NGF, conversely NGF selectively promoted the survival of small DRG neurons back labeled from skin (Hory-Lee *et al.* 1993).

It should be noted that substantial differences may arise when comparing the response of avian and rodent neurons to neurotrophins. For example, despite clear effects on DRG from chicken embryos, BDNF has little or no effect upon the survival of cultured DRG neurons derived from perinatal rats. The latter may also be reflected in the recent finding that mutant mice lacking in expression of BDNF do not apparently have any deficit in their complement of DRG neurons at early post-natal stages (Conover *et al.* 1995) although loss may occur later in maturation.

(*b*) *Autocrine role as survival factors for mature DRG neurons*

Unlike the absolute requirement of developing sensory neurons for neurotrophic factors to support their survival and differentiation, DRG neurons isolated from the adult rat survive *in vitro* in the absence of any exogenous neurotrophic factor(s) (Lindsay 1988*a*). This is true not only at low density, but also when these cells are cultured as single neurons in microwells. This transition of DRG neurons from exogenous growth factor dependence to autonomous survival seems to occur between 10–12 days after birth in the rat. At first no mechanism for achieving autonomy was apparent, but the finding that a considerable proportion of DRG neurons express BDNF (Ernfors *et al.* 1990; Schecterson & Bothwell 1992) suggested the possibility of an autocrine loop in which BDNF made by a mature neuron is sufficient to promote survival of that neuron. As documented in detail elsewhere in this volume, evidence of such an autocrine loop has been reported in studies which have shown that disruption of BDNF synthesis by antisense oligonucleotide treatment of mature DRG neurons cultured as single cells does indeed produce selective neuronal death (see paper by A. Acheson & R. M. Lindsay this volume and Acheson *et al.* 1995). The fact that this can be demonstrated at the single cell level and that antisense induced cell death can be rescued by the addition of exogenous BDNF provides strong evidence for an autocrine BDNF loop (as opposed to a solely paracrine action of BDNF on neighbouring cells).

Although the hypothesis that target-derived neurotrophic factors play a critical role in sculpting the developing PNS has a certain elegance, the notion that mature neurons may become autonomous from their targets by deriving survival-sustaining neurotrophic support from other sources, autocrine or paracrine, has particular attractions with regard to PNS neurons. In essence the role of target-derived neurotrophic factors during development is to match the appropriate numbers and types of peripheral neurons to the needs of specific tissues. Having established a precise match, it would seem prudent that the match be maintained without retaining an absolute dependence of each neuron on a constant supply of a survival factor from a potentially distant target. Retaining such a requirement would only appear to increase the vulnerability of PNS neurons to trauma of even a minor nature. Obvious, but rarely stated, sensory neurons are essentially in a constant state of remodelling in response to microtrauma resulting from simple growth or turnover of cells, as well as normal wear and tear in peripheral tissues such as skin and muscle. Thus maintaining the integrity of a mature neuron by a mechanism independent of its target would appear to reduce the risk of neuronal death resulting from target disconnection, or target damage or atrophy. Most conservatively, however, the signalling capabilities of a target-derived factor may be retained upon maturation, but deployed to a different function. This may well be the case in the interactions of NGF with sensory

neurons, target-derived NGF playing an essential, survival promoting role during development, but switching to a role of regulating transmitter phenotype and possibly regenerative sprouting in the mature animal. Given our suggestion that autocrine BDNF in the mature sensory neuron may take over from the role of target-derived NGF in the developing neuron, it will be interesting to determine if indeed BDNF is synthesized (and thus the autocrine loop) in adult DRG neurons that express TrkA and/or also express genes such as substance P or CGRP that appear to be regulated by NGF (see below).

(*c*) *Phenotypic maintenance of mature neurons*

Axotomy of developing sensory neurons *in vivo* leads to marked cell death. This is presumed to result from the loss of target-derived neurotrophic support that reaches the cell soma by receptor mediated retrograde axonal transport. In contrast, axotomy of adult sensory neurons does not lead to significant cell loss. This may result from the burden of providing survival promoting trophic support switching from a target-derived mode to an autocrine mode during maturation, as suggested above. Nonetheless crush or cut injury *in vivo* or culturing of adult neurons without exogenous neurotrophic factors leads to a loss of specific phenotypic markers such as the neuropeptides substance P or CGRP. This decrease in neuropeptide expression can be reversed *in vitro* and *in vivo* by application of NGF, and *in vivo*, neuropeptide levels do return in a crush injury after regeneration of axons to their appropriate targets (Lindsay 1992). As yet there is no clear evidence that BDNF or NT-3 regulate the expression of important phenotypic traits in mature DRG or other neurons, but generalizing from the actions of NGF, it seems that the role of target-derived neurotrophins may switch from one of survival promotion during development to one of regulating phenotype and local sprouting or regeneration in the adult.

8. TARGET DERIVED ROLE OF NEUROTROPHINS CONFIRMED IN NEUROTROPHIN NULL MUTANTS

Some of the first experiments to firmly establish a crucial role of NGF *in vivo*, relied on the sequestration of endogenous NGF by administration of neutralizing antibodies (Levi-Montalcini & Angeletti 1966). Such experiments demonstrated that NGF deprivation *in vivo* or *in ovo* during development leads to loss of almost all sympathetic neurons and to loss of small neural crest-derived sensory neurons. The recent creation of mutant mice which are null in expression of NGF has elegantly confirmed this critical role of NGF during ontogeny (Crowley *et al.* 1994). For a recent review of deficits reported in neurotrophin and trk null mutant mice see (Snider 1994).

Confirming tissue culture studies that first suggested that NT-3 might be relatively specific for large fibre proprioceptive sensory neurons (Hory-Lee *et al.* 1993), analysis of mice homozygous for an NT-3 null mutation (*NT-3* $-/-$) has shown that such animals completely lack type 1a sensory afferents and have no muscle spindles (Ernfors *et al.* 1994). Interestingly, heterozygotes (*NT-3* $+/-$) have half the normal number of muscle spindles, indicating a rather tight gene dosage relationship and providing further strong evidence of an important tenet of the target-derived hypothesis; neurotrophic factor levels in peripheral targets are strictly limited.

Recent analysis of BDNF and NT-4/5 null mutants (Conover *et al.* 1995; Bianchi *et al.* 1996) has also confirmed the important role of these TrkB ligands in the development of neural placode-derived sensory neurons that was first suggested from *in vitro* studies (reviewed in Lindsay 1993). In *BDNF* $-/-$ mice, for example, there are virtually no vestibular sensory neurons by two weeks of age. Again a clear gene dosage relationship was found in that two week old BDNF $+/-$ heterozygote mice had half the number of vestibular neurons of wild-type mice. Analysis of the number of neurons in the nodose ganglion showed a loss of neurons in both BDNF and NT-4/5 null mutants. Interestingly, BDNF/NT-4/5 double mutants had virtually no nodose ganglion neurons, reflecting additive losses contributed from the absence of both factors (Conover *et al.* 1995).

9. EFFICACY OF NEUROTROPHINS IN *IN VIVO* STUDIES

The potent effects of neurotrophins *in vitro* and the devastating effects of disrupting neurotrophin levels *in vivo* either by gene knockout or by antibody sequestration has prompted much interest in the possibility that pharmacological doses of neurotrophins may attenuate nerve damage to sensory neurons resulting from trauma, metabolic insults or neurodegenerative disease (for review see Lindsay *et al.* 1994). With regard to sensory neurons there are a wide range of clinical disorders that pose severe health problems. Among the most common ailments are neuropathies that result from widely used chemotherapy agents, complications of diabetes or genetic disorders. Secondary loss of auditory neurons following acoustic hair cell damage is a major side effect of aggressive treatment with aminogylcoside antibiotics and chemotherapy agents such as cisplatin.

Although much attention has been given to the potential use of NGF as a therapeutic agent in Alzheimer's disease, there has if anything been a stronger rationale to explore therapeutic properties of NGF on peripheral neurons, given very extensive studies that have documented the ability of systemically administered NGF to attenuate a variety of insults to primary sensory neurons (reviewed in Snider & Johnson 1989). However, a major issue that may now hinder any therapeutic use of NGF, are the consistent findings that systemically delivered NGF produces hyperalgesia in both rodents and man (Lewin & Mendell 1993). Given what we now know about the specificity of BDNF and NT-3, there is an extensive effort to assess the efficacy of these two neurotrophins in animal models of either motor or sensory neuron

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dysfunction. To this end, as outlined below, we have been exploring the efficacy of NT-3 in an animal model of large sensory fibre damage.

(*a*) *NT-3 and mature large sensory neurons*

As indicated above both *in vitro* studies and analysis of NT-3 null mutants indicate that NT-3 is a critical target derived neurotrophic factor for proprioceptive sensory neurons. The first indication that mature DRG neurons are likely to remain responsive to NT-3 came from analysis of the labelling pattern of DRG neurons by receptor mediated retrograde axonal transport, following injection of radiolabelled neurotrophins into the sciatic nerve of adult rats. In these experiments ^{125}I-NT-3 was preferentially found to label the largest neurons of lumbar DRG L4/L5 (DiStefano *et al.* 1992). Consistent with this pattern of labelling are the findings that TrkC is expressed predominantly on the largest diameter DRG neurons, plus a small percentage of very small diameter DRG neurons (McMahon *et al.* 1994).

(*b*) *Neuroprotective action of NT-3 In large fibre sensory neuropathies*

The majority of the largest cell diameter/largest fibre diameter DRG neurons are muscle sensory afferents that subserve the modality of proprioception. Damage to these cells or their axons leads to large fibre sensory neuropathy that manifests as a loss of position sense of the limbs. Such large fibre sensory neuropathies are a major clinical problem that arise in diabetics and in cancer patients being treated with cytotoxic drugs. In the latter category, the dose-limiting side-effect of chemotherapy drugs, such as cisplatin, is a profound loss of proprioception. Ingestion of large doses of vitamin B6 (pyridoxine) produces a similarly selective large fibre neuropathy in man. Given the specificity of NT-3 for proprioceptive neurons, we have explored the possibility that systemic NT-3 administration may attenuate proprioceptive loss in animal models of large fibre neuropathy, as a prelude to assessing such potential in human clinical trials.

Unlike in man, severe nephrotoxicity and general morbidity seem to precede any signs of sensory neuropathy in rats chronically treated with cisplatin. In view of this we developed a rat model of large fibre neuropathy which results from repeated high-dose intoxication with pyridoxine (Helgren *et al.* 1994). Chronic treatment of adult rats for 2–3 weeks with high doses of pyridoxine (vitamin B6) produces a profound proprioceptive loss similar to that found in humans overdosed with this vitamin or treated with cisplatin. Pyridoxine toxicity was manifest as deficits in simple and precise locomotion, sensory nerve function and degeneration of large diameter/large fibre spinal sensory neurons.

As assessed quantitatively in a beam walking task, in analysis of gait in simple overground locomotion and analysis of locomotion across an open grid, chronic pyridoxine treatment greatly impaired simple and precise locomotion such that animals were virtually unable to cross a 6-foot long one-inch diameter beam nor able to navigate across an open wire mesh. These deficits were associated with increased base of support and changes in stride length and intrastep distance. In all cases co-administration of NT-3 during pyridoxine intoxication greatly attenuated these deficits in simple and precise locomotion (Helgren *et al.* 1994). Motor function was not impaired in these animals, confirming that pyridoxine toxicity is selective for proprioceptive sensory neurons.

Consistent with selective toxicity towards large fibre muscle sensory afferents, EMG recordings of H and M wave latencies and amplitudes showed that the H reflex was severely impaired in rats receiving pyridoxine. Cotreatment with NT-3 largely and significantly restored H wave properties to normal (Cliffer *et al.* 1994). Sensory conduction velocity was similarly reduced in animals treated with pyridoxine alone as compared to animals co-treated with pyridoxine and NT-3. Finally, histological analysis of the spinal cord of control, pyridoxine, and pyridoxine + NT-3 treated groups revealed that NT-3 prevented marked pyridoxine-induced degeneration of sensory fibres in the dorsal column of the spinal cord (M. Helgren, unpublished results).

(*c*) *Clinical trials*

Taken together, the above data are consistent with other evidence indicating that NT-3 is a target-derived neurotrophic factor for muscle sensory afferents and suggest that pharmacological doses of NT-3 may be beneficial in the treatment of large fibre sensory neuropathies. Based on these observations a clinical trial has been initiated to determine any efficacy of NT-3 in attenuating the large fibre sensory neuropathy produced in cancer patients receiving cisplatin chemotherapy and in patients with a similar large fibre neuropathy resulting from long term diabetes.

10. CONCLUSIONS

The discovery of NGF was a major milestone in neurobiology and despite intensive study in the last two decades, new and exciting aspects of the effect of this molecule on sensory neurons are still emerging. The more recent discovery of NGF's interesting relatives has had an equally important impact on our understanding of the epigenetic factors that help to sculpt and maintain the integrity of the nervous system as a whole, and sensory neurons in particular.

There is no doubt that the modern techniques of molecular biology have had (and will continue to have) a major impact in defining the players that participate in the reciprocal interactions that initiate and sustain functional connections between neurons and their neural or non-neural targets. The formidable task of characterizing BDNF by purification and protein sequencing was undoubtedly the critical step that led to the discovery of the neurotrophin family and subsequently to the identification of the Trk family as their high affinity receptors. There is no question that from that point on the rapid pace of elucidation of

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the biology of neurotrophins and Trk receptors in the last few years has been propelled by the power of molecular biology: success in homology cloning of NT-3 and NT-4/5, cloning of the Trk family, production of all of these proteins by recombinant techniques and the generation and analyses of null mutants of each of the neurotrophins, their common low affinity receptor p75, and their respective high affinity receptor. Although this is an exciting and technically challenging era, none of the recent advances would have been possible without a basic premise, a robust assay system and *in vitro* and *in vivo* systems to test out ideas. What is perhaps rewarding for some, is that the refined scrutiny of molecular analysis has largely confirmed the validity of the neurotrophic hypothesis as it pertains to the role of neurotrophic factors in the development and maintenance of sensory neurons in particular. Also of note is that fact that a detailed understanding of factors that are essential for development and maintenance of sensory neurons may lead to novel therapeutic strategies to treat disorders of sensory neurons. Several critical clinical experiments to validate this notion are in progress.

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