Molecular Neurobiology

ISSN0893-7648/03/27(3): 277–324/$25.00

Neurotrophic Factors and Their Receptors in Axonal Regeneration and Functional Recovery After Peripheral Nerve Injury

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Abstract

Over a half a century of research has confirmed that neurotrophic factors promote the survival and process outgrowth of isolated neurons in vitro. The mechanisms by which neurotrophic factors mediate these survival-promoting effects have also been well characterized. In vivo, peripheral neurons are critically dependent on limited amounts of neurotrophic factors during development. After peripheral nerve injury, the adult mammalian peripheral nervous system responds by making neurotrophic factors once again available, either by autocrine or paracrine sources. Three families of neurotrophic factors were compared, the neurotrophins, the GDNF family of neurotrophic factors, and the neuropoetic cytokines. Following a general overview of the mechanisms by which these neurotrophic factors mediate their effects, we reviewed the temporal pattern of expression of the neurotrophic factors and their receptors by axotomized motoneurons as well as in the distal nerve stump after peripheral nerve injury. We discussed recent experiments from our lab and others which have examined the role of neurotrophic factors in peripheral nerve injury. Although our understanding of the mechanisms by which neurotrophic factors mediate their effects in vivo are poorly understood, evidence is beginning to emerge that similar phenomena observed in vitro also apply to nerve regeneration in vivo.

Index Entries: NGF; BDNF; GDNF; CNTF; axon regeneration; functional recovery; Schwann cells; motoneuron.

Received July 8, 2002; Accepted November 22, 2002
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General Introduction

Since the discovery of nerve growth factor (NGF) and its dramatic effects on the outgrowth of sympathetic fibres, many neurotrophic factors have been identified which promote neuronal survival and/or outgrowth of neurites in culture. The elucidation of the role of neurotrophic factors in vivo has progressively demonstrated their increasingly complex involvement in the nervous system during embryonic and postnatal development, adulthood, and following injury. In the past 10 yr, our knowledge of how neurotrophic factors transduce their signal from the cell surface to the nucleus in vitro has exploded exponentially. Interestingly, a paradox has arisen such that our understanding of the mechanisms of signal transduction now outweigh our understanding of their functional roles in vivo. Several recent reviews address specific areas of neurotrophic factor research, including their roles in development *(1–6),* plasticity in the central nervous system (CNS) *(7–21),* neuronal injury *(22–27),* and signal transduction mechanisms *(28–42).* The purpose of this review is to provide a comprehensive overview of neurotrophic factors and their receptors in the mammalian peripheral nervous system (PNS). The specific focus is to provide functional significance to what is known about the mechanisms by which neurotrophic factors exert their effects in the context of the motoneuronal response to injury, including survival, growth, and axonal regeneration.

Three separate and distinct families of neurotrophic factors will be compared: neurotrophins, GDNF family neurotrophic factors, and neuropoetic cytokines, based on molecular structure and receptor interactions, patterns of expression after peripheral nerve injury, and cellular effects, both in vitro and in vivo. In addition, a brief overview of the intracellular signal transduction pathways that are activated in response to these factors will provide insight into the considerable overlap, yet functional distinctiveness, in cellular responses to these neurotrophic factors.

Neurotrophic Factors and Their Receptors

Neurotrophin Family

Introduction

The neurotrophin family of neurotrophic factors is a family of structurally and functionally related peptides which mediate potent survival and differentiation effects on a wide variety of neuronal populations in the central and peripheral nervous systems. NGF, the prototypical member of the neurotrophin family, was isolated, purified, and identified as a diffusible factor which promoted the survival and neurite outgrowth of sympathetic and sensory neurons both in vitro and in vivo in the classic experiments by Viktor Hamburger and Rita Levi-Montalcini, almost half a century ago *(43).* Decades later, a second molecule belonging to this family, brain-derived neurotrophic factor (BDNF), was purified and cloned *(44).* With recent advances in cloning and molecular biology, the neurotrophin family now consists of 4 members in mammals, NGF, BDNF, neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Two additional members, NT-6 and NT-7 have been identified and cloned in fish, but mammalian homologs have not yet been identified *(45,46).*

Neurotrophin Structure

Neurotrophins exist and are biologically active as noncovalent homodimers due to highly conserved structural features, including a cysteine knot motif which confers considerable interaction between each molecule of the homodimer (reviewed in refs. *42,47*). Each molecule of the homodimer consists primarily of two pairs of antiparallel β strands which give the monomers their characteristic flat, elongated, and slightly asymmetrical shape *(48).* The β strands are connected by 3 short loops which are known to be highly flexible and are the regions in the neurotrophin structure where amino acid differences occur between different members of the neu-

rotrophin family *(42).* There is an approx 50% sequence homology between different members of the neurotrophin family *(42).*

Neurotrophin Receptor Structure

Neurotrophins are unique among neurotrophic factors in that they mediate their effects by binding to 2 classes of receptors, the tropomyosin receptor kinase (trk) family of receptor tyrosine kinases, and a member of the tumour necrosis factor (TNF)-α family of receptors, p75 (Fig. 1, reviewed in Ref. *42*). The p75 receptor binds all neurotrophins with similar affinity, but with different kinetics *(49).* In contrast, the trk family of receptors are more specific: NGF binds to trkA, BDNF and NT-4/5 bind to trkB, and NT-3 binds trkC, and to a lesser extent, trkA. In addition to the full length transmembrane trk receptors, in the case of trkB and trkC, multiple truncated forms exist, which either lack an intracellular tyrosine kinase domain or possess small inserts in the intracellular tyrosine kinase domain *(50).* The function of these truncated receptors remains largely unknown, but may exist to either sequester neurotrophins, thus limiting binding to full length receptors in a dominant-negative fashion, or serve to cluster neurotrophins and "present" them to their full length counterparts *(50–52).* As there is considerable homology in the extracellular domains of the 3 trk receptors (~50%), it is remarkable that such selectivity exists in distinguishing individual neurotrophins *(53).* Two separate binding sites for the neurotrophins have been identified on the extracellular domains of the trk receptors (Fig. 1): 1) A leucine-rich motif flanked by 2 cysteine clusters, and 2) the second of 2 immunoglobulin (Ig)-like domains which exist close to the cell membrane *(54).* The functional significance of the leucine-rich motif is poorly understood, but point mutation analysis has revealed that the latter binding site (i.e., the second Ig domain) is important in neurotrophin binding and receptor activation, as well as conferring specificity between neurotrophin ligands *(55,56).*

Interestingly, in addition to binding its ligands BDNF and NT-4/5, the trkB extracellular domain has been shown to participate in cellular adhesion. In vitro studies using NIH 3T3 cells stably transfected with the trkB receptor have demonstrated that trkB, together with cadherin, can mediate cellular adhesion *(57).* In addition, these studies showed that trkB was colocalized with the cell adhesion molecules, cadherin and cateinin, at cell–cell contact sites. The ability of the extracellular domain of trkB receptors to take part in cellular adhesion may suggest a novel role for the truncated trkB, and possibly trkC receptors in cellular mobility and motility *(57).*

The structural features of the p75 receptor which confer neurotrophin binding are substantially different from the trk family of receptors (reviewed in ref. *32*). Briefly, the extracellular domain of the p75 receptor consists of cysteine-rich domains tandemly arranged, the distinguishing feature of the tumor necrosis factor (TNF) receptor family (Fig. 1; *58,59*). Neurotrophins most likely interact with this ligand-binding domain on the p75 receptor via conserved positively charged residues. Targeted disruption of these positively-charged clusters abolishes neurotrophin binding to p75, but binding to their cognate trk receptors on trk-expressing fibroblasts, and survival promoting ability in isolated chick DRG neurons remains unaffected *(60).*

The original nomenclature of trk and p75 receptors conferring high and low affinity neurotrophin binding has recently been re-evaluated, based on experiments demonstrating that both p75 and trkA receptors cooperate to form high affinity binding sites for NGF (reviewed in ref. *42*). The differences in affinity can be attributed, in part, to substantial variations in binding kinetics. For example, NGF binds to trkA with relatively slow on- and off rates, but NGF associates and dissociates from p75 much faster *(61).* The net result of this accelerated on–off kinetic is an increase in the total amount of NGF that can bind trkA *(61,62).*

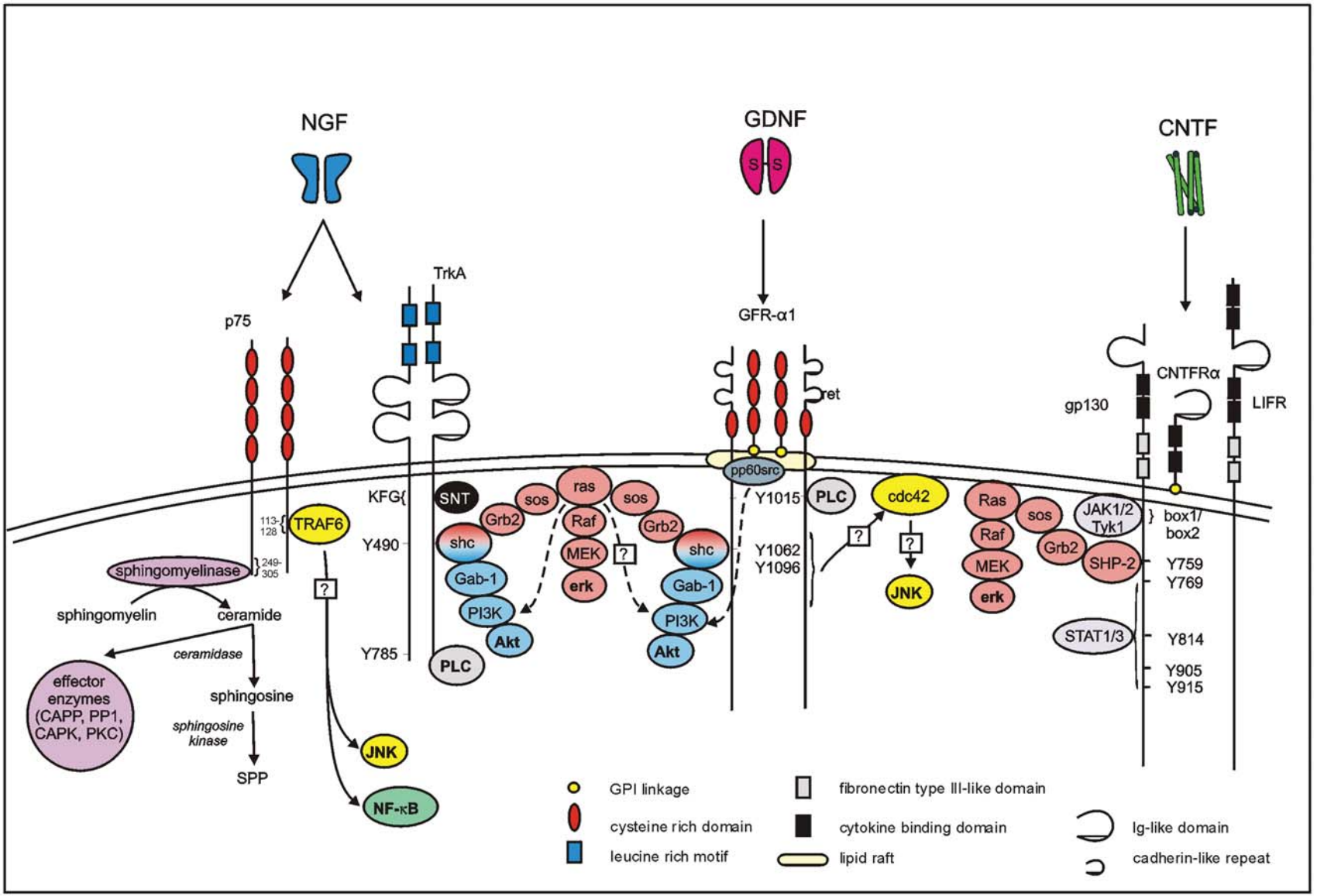


Fig. 1. Structural and functional overlap in neurotrophic factors and their receptors. Schematic representation of prototypical members of 3 families of neurotrophic factors: NGF-neurotrophin family, GDNF-GDNF family members, CNTF-neuropoetic cytokines. Although there are many adaptor proteins involved in linking neurotrophic factors and their receptors to intracellular signal transduction pathways, only key overlapping and distinct pathways are shown for simplicity (*see* text for details).

Glial Cell-Lined Derived Neurotrophic Factor (GDNF) Family

Introduction

The GDNF family of neurotrophic factors consists of 4 members: GDNF, neurturin (NTN), persephin (PSP), and artemin (ART; reviewed in ref. *63*). Relative to the neurotrophins, the GDNF family of neurotrophic factors have only recently been discovered. GDNF was originally described as a potent survival factor for mesencephalic domaminergic cells, as well as motoneurons *(64–67).* Three years later, the second member of this family, NTN, was identified and cloned and found to promote the survival of rat sympathetic neurons *(68).* The remaining members, namely PSP and ART, were identified shortly thereafter and found to have similar survival promoting activities on sympathetic neurons in vitro *(69–70).*

Structure of GDNF Family Neurotrophic Factors

Like the neurotrophins, members of the GDNF family are typical secretory proteins, but differ from the neurotrophins in 2 main ways. First, members of the GDNF family contain 7 cysteine residues in the same relative spacing as members of the large transforming growth factor (TGF)-β superfamily *(63).* Secondly, GDNF is N-glycosylated at 2 amino acid residues, a chemical property which is rarely found on peptide growth factors. GDNF family members share structural similarities with the neurotrophins, such as possessing a cysteine knot which allows homodimerization, a conformation which confers biological activity. In contrast to neurotrophin homodimers which are held together by only hydrophobic interactions, GDNF family homodimerization is also supported an additional interchain disulphide bond (Fig. 1; *see* above; *42*). However, this interchain disulphide bond is not necessary to confer biological activity *(71).*

The second GDNF-family molecule to be discovered was NTN, which shares 42% homology with GDNF *(68).* The strong similarities between GDNF and NTN formed the basis for a new subfamily within the TGF-β superfamily of growth factors. PSP and ART show similar homology to GDNF, 40% and 36%, respectively *(69).*

Structure of GDNF Family Receptors

GDNF family members exert their effects via a receptor complex which consists of a high affinity ligand-binding subunit (GDNF family receptor [GFR]-α), and subsequent coupling to a common signal transduction subunit (ret; Fig. 1). Specificity is conferred by the high affinity ligand-binding subunits. GDNF binds GFR-α1, NTN binds to GFR-α2, PSP binds to GFR-α3, and ART binds to GFR-α4, although some overlap does occur (reviewed in ref. *63*). The GFR-α receptors are anchored to the plasma membrane via a glycosyl-phophotidylinositol (GPI) linkage (Fig. 1). Interestingly, although it was originally believed that GFRα proteins functioned solely to present ret with GDNF-family molecules, it has been recently suggested that these receptors possess an intrinsic signaling capacity (*63; see* "Ret-Independent Signaling"). Several research groups simultaneously identified ret as the signal-transducing component of GDNF family member receptors *(72–76).* The ret receptor shares similarities with the trk family of receptors for the neurotrophins. For example, ret is a transmembrane receptor tyrosine kinase and, like trk, was first identified as a proto-oncogene *(77).* Moreover, ret has two cadherin-like repeats in the extracellular domain, which may confer similar cell-adhesion properties as described above for trkB (*78;* Fig. 1). Thus, in light of the structural similarities between the neurotrophin and GDNF family of neurotrophic factors, and their respective receptors, functional overlap of these two families of neurotrophic factors may be predicted in the nervous system.

Neuropoeitic Cytokines

Introduction

Cytokines are a large family of pleiotropic glycoprotein molecules which mediate a wide variety of biological activities associated with

the induction of immune and inflammatory responses, such as initiation of both the cellular and humoral immune reactions, regulation of hematopoiesis, control of cellular proliferation and differentiation, and induction of wound healing (reviewed in Ref. *79*). The neuropoeitic cytokine family generally refers to molecules of the interleukin (IL)-6 family, which share a common signal-transducing receptor subunit termed glycoprotein (gp) 130, and include IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin (CT)-1, and the newest member, novel neurotrophin-1/B-cell stimulating factor-3 *(80,81).* Since the first identification of the effect of LIF in changing the normal cardiac sympathetic noradrenergic innervation to cholinergic *(82,83),* a wide variety of neuronal responses have been ascribed for members of this family. These include neuronal survival, differentiation, and neurite outgrowth, which will be described in detail below.

Structure of Neuropoeitic Cytokines

The neuropoeitic cytokines belong to a larger family of long-chain α-helix bundle cytokines, which also includes erythropoeitin, granulocyte colony-stimulating factor, IL-12, growth hormone, prolactin, IL-10, interferon-α/β, and leptin *(84).* Neuropoeitic cytokines are secretory proteins with amino terminal signal peptides, with the exception of CNTF which is only released upon cellular damage *(85).* Unlike the neurotrophins and the GDNF-family of neurotrophic factors, the neuropoeitic cytokines are not biologically active as homodimers, but as single, four-α-helix bundle proteins (Fig. 1; *80*). Like GDNF, certain members of the neuropoeitic cytokine family, namely IL-6, CT-1, and OSM, have N-glycosylation sites, the function of which is unknown. Neuropoetic cytokines bind to their receptors via 3 distinct binding sites (reviewed in Ref. *86*).

Structure of Neuropoeitic Cytokine Receptors

All members of the neuropoetic cytokine family signal via recruiting the common signal transduction receptor subunit, gp130 *(80,86,87).* Like the GDNF family members which do not bind ret directly (Fig. 1), many neuropoetic cytokines do not activate gp130 directly, but first bind to specific ligand-binding subunits. IL-6 binds the IL-6 receptor (IL-6R), LIF binds the LIF receptor (LIFR), CNTF binds the CNTF receptor (CNTFR), OSM binds both LIFR and the OSM receptor (OSMR) *(80,86,87).* The ligand-binding receptor for CT-1 remains to be identified. Together with gp130, these receptors belong to the cytokine receptor class 1 family, defined by at least 1 cytokine-binding module and at least one extracellular immunoglobulin-like domain *(88).* Members of this family have one transmembrane domain, except CNTFR, which like GFR-α receptors, is a GPI-linked receptor (Fig. 1).

In Vitro Response of PNS Neurons to Neurotrophic Factors: Mechanisms of Differentiation, Survival, and Neurite Outgrowth

Neurotrophins

Summary of trk Signaling Events Following Neurotrophin Binding

The events following neurotrophins binding to their respective receptors have been extensively characterized in vitro, and have been the subject of many recent and extensive reviews *(28–42)* and will be summarized briefly here and illustrated in Fig. 1.

Much of what is known about the intracellular events resulting from neurotrophins binding to trk receptors stems from research on the response of NGF binding to trkA on PC 12 cells. PC 12 cells are derived from a rat pheochromocytoma cell line, express trkA and p75 receptors, and respond to NGF by differentiating into cells that resemble adult sympathetic neurons *(89,90).* Neurotrophin binding to trk receptors induces receptor dimerization and autophosphorylation of intracellular tyrosine residues of the "activation loop" *(91).* It has been suggested that phosphorylation of

the tyrosine residues in this region can induce conformational changes within the catalytic domain, thus allowing greater interaction with downstream substrates *(92).* The phosphorylation of specific tyrosine residues serves as specific docking sites for important adaptor proteins which initiate intracellular signaling events (Fig. 1; activation loop not illustrated for simplicity; *93–96;* reviewed in refs. *42* and *44*). Many of these adaptor proteins such as shc, Grb2, GAB-1 (Fig.1) contain src-homology-2 (SH2) or phosphotyrosine binding (PTB) domains *(97,98).*

As diagrammed in Fig. 1, one of these tyrosine residues that is phosphorylated upon trkA receptor dimerization is Y490, located in the juxtamembrane region of the intracellular domain of the receptor, and is responsible for the NGF-induced trkA association with both the Shc and fibroblast growth factor receptor substrate (FRS)-2 adaptor proteins (*99–102,* reviewed in Refs. *28,39).* Y484 and Y485 are the homologous tyrosine residues on the trkB and trkC molecules, respectively *(92,103).* In addition, Y785, at the extreme C-terminal region of trkA and trkB receptors, and Y789 at the extreme C-terminus of trkC, when phosphorylated are the likely binding sites of the SH2-containing enzyme phospholipase-C (PLC)-γ (Fig. 1; *99,103,104*), as well as the Csk homologous kinase (CHK; *105*). In addition to the Shc and PLC-γ-binding sites, another site on the trkA molecule that is important in mediating downstream events is a lysine-phenylalanine-glycine (KFG) sequence located in the juxtamembrane region which stimulates the phosphorylation of suc1-associated neurotrophic factor-induced tyrosine phosphorylated target (SNT; reviewed in Refs. *30,39*).

The downstream events following trk autophosphorylation and recruitment of adaptor molecules have been extensively characterized in vitro, not only in PC 12 cells, but primary sympathetic and sensory dorsal root ganglion neurons as well *(28,30,31,39,40).* In general, the functional outcomes of neurotrophin binding to trk receptors on PC12 cells, or isolated primary sympathetic and sensory neurons in vitro can be divided into 3 major categories-differentiation, survival, and neuritogenesis, and are very roughly associated with activation of the ras-erk, PI3K-Akt, and SNT pathways, respectively; however, considerable overlap does occur (Fig. 1).

Ras-erk Pathway

Phosphorylation of Shc at Y490 leads to its interaction with Grb2-SOS complexes, thus activating the small G-protein p21ras (Fig. 1). Ras can also be activated by other upstream pathways including FRS-2/SHP2/Grb/SOS, Crk/SOS, CHK, rAPS/Grb/SOS and SH2-B/Grb/SOS *(31,39).* Activated ras initiates the extracellular related kinase (erk) pathway, also known as the mitogen associated protein kinase (MAPK) pathway. This intracellular cascade is characterized by a series of phosphorylation events which include the serine-threonine kinase raf, the tyrosine-threonine kinase map/erk kinase (MEK), and the serine-threonine kinase erk (Fig. 1). These elements in the ras-erk pathway have been demonstrated to be necessary and sufficient to mediate the differentiation, but not survival, of PC12 cells in response to NGF stimulation *(106).* In contrast, blocking ras, but not raf, MEK, or erk prevents the survival-promoting effects of NGF primary sensory and sympathetic neurons *(40).* In another study, pharmacological inhibition of MEK with PD98059 decreased sympathetic neuron survival in the presence of constitutively activated ras by only 20% *(107).* This suggests that, although elements of the erk pathway downstream of ras are essential in mediating the differentiation response to NGF in PC12 cells, ras can mediate survival by multiple pathways in postmitotic differentiated primary sensory and sympathetic neurons.

How does activation of the ras pathway promote neuronal survival? In vitro, ras activity in sympathetic neurons suppresses the apoptosis that normally occurs 24–48 h after NGF-withdrawal *(107).* The mechanism has been demonstrated to be the suppression of the p53 pro-apoptotic pathway, which includes c-jun amino-terminal kinase (JNK), the tumour sup-

pressor protein p53, and Bax (not illustrated in Fig. 1; *109;* reviewed in ref. *31*). Specifically, sympathetic neurons that have been transfected with constitutively activated ras inhibited the increases in c-jun, p53, and Bax that typically follow NGF withdrawal *(107).* Moreover, reducing endogenous ras activity augmented the increase in expression of c-jun, p53, and Bax following NGF withdrawal *(107).* The link between ras activation and JNK expression has not been elucidated and is not illustrated in Fig. 1. Although sustained JNK activation leads to apoptosis, transient JNK induction is actually associated with cellular proliferation *(109).* Thus it is the kinetics of JNK expression that are important in determining cellular fate.

PI3K Pathway

PI3K is central to the survival-promoting effects of NGF on PC12 cells, as well as primary sympathetic and sensory neurons (reviewed in ref. *31*). It has been demonstrated that activation of PI3K is dependent on the Shc binding site of trkA in PC12 cells *(110),* and that activation of the PI3K pathway may require ras activation (*40,107;* Fig. 1). It has been suggested that the association and activation of PI3K are mediated by additional adaptor proteins, such as the insulin-receptor substrate (IRS)-1 and 2, and/or the Grb-associated binder (Gab)-1 proteins (not shown in Fig. 1; reviewed in ref. *39*). The downstream target of PI3K critical for mediating the survival effects is the serine-threonine kinase Akt (Fig. 1; *28,30,31,39,40*).

SNT and PLC-γ

Two additional downstream signaling events which occur following NGF activation of trkA are the activation of SNT and PLC-γ (Fig. 1). As mentioned above, SNT activation is dependent on the presence of the conserved KFG sequence in the juxtamembrane region of the trkA receptor. SNT appears to be involved in neurite outgrowth, as targeted deletion of this KFG region results in a loss of SNT phosphorylation, and a failure to demonstrate neurite outgrowth *(111).* Phosphorylated PLC-γ cleaves phophotidylinositol 4,5 bisphosphate into 2 intracellular messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). The generation of IP_3 leads to a rise in intracellular calcium via binding to its receptor on the endoplasmic reticulum, whereas DAG stimulates protein kinase C (PKC) activation *(112,113).* It is possible that the increase in intracellular calcium may serve to induce neurotrophin stimulation in an autocrine manner, as it has been demonstrated that calcium influx can trigger BDNF transcription via phosphorylation of a calcium response element binding protein (CREB) dependent mechanism *(114,115).* The link between BDNF transcription and axonal growth has been demonstrated in vivo (*116; see* "Axonal Sprouting, Regeneration, and Functional Recovery"). Although it is known that the activation of PLC-γ can be an alternative pathway for the activation of ras, the mechanism by which this occurs is unknown *(28).*

Other trk Receptors

Although most experiments have focused on the interactions between NGF and trkA on PC12 cells, sympathetic, and sensory neurons, due to the high homology between the neurotrophins and their receptors it is anticipated that these events can be generalized to other members of the neurotrophin family. In fact, it was recently demonstrated that in sympathetic neurons stably expressing trkB, phosphorylated trkB stimulated signaling proteins and induced survival and growth in a manner similar to trkA *(117).* Analogous to trkA: 1) mutations at the Shc binding site reduced the ability of BDNF to promote survival and growth, whereas mutations at the PLC-γ were with little effect; 2) survival was dependent primarily on PI3K, and to a lesser extent MEK; and 3) growth depended on both MEK and PI3K activity *(117).* The importance of PI3K in mediating the survival-promoting effects of BDNF may be extended to motoneurons as well. BDNF activation of PI3K, but not erk kinase promoted the survival of isolated embryonic chick motoneurons *(118).* However, elements of the erk pathway are important in mediating the survival-promoting

effects of neurotrophic factors in mammalian motoneurons, as embryonic motoneurons isolated from mice carrying a homozygous deletion of B-raf no longer survive in response to BDNF, GDNF, or CNTF *(119).*

p75 Signaling in Response to Neurotrophin Stimulation

In contrast to the well-delineated pathways and their functional significance that have been identified for trk signal transduction, the physiological role of the p75 receptor has been more difficult to define. Barker *(32)* delineated the effects of the p75 receptor into 2 broad categories: 1) p75 signals independently to induce apoptosis, in a manner similar to other members of the TNF-α family of receptors; and 2) p75 functionally cooperates with trk receptors to either a) enhance, or b) reduce neurotrophin-mediated trk activation. This section will focus on p75 independent signaling, and p75-trk interactions will be discussed in the following section.

There are several salient features to p75 receptor signaling which have been the subject of many recent reviews and will only be highlighted here *(29–37,39,41).* Generally speaking, a cohesive picture of p75 signaling has been difficult because the effects of neurotrophin binding to p75 depend greatly on cell type, developmental stage, and ligand.

A well-documented downstream event following neurotrophin binding to the p75 receptor is the activation of sphingomyelinases and induction of sphingomyelin hydrolysis, which leads to the production of ceramide (Fig. 1; reviewed in Ref. *37*). Ceramide is a lipid second messenger that is also produced as a result of TNF-α family members binding to their cognate receptors, an event intimately linked with the pro-apoptotic functions of these proteins (reviewed in Ref. *120*). It is not known how ligand binding to p75 leads to sphingomyelinase activation, but appears to require a sequence within amino acids 249–305 of the cytoplasmic tail (Fig. 1), as targeted deletion of this sequence abolishes sphingomyelinase activation, but not NGF binding or expression in the membrane *(121).*

In the nervous system, a wide variety of responses have been attributed to the generation of ceramide. These responses include the induction of JNK in oligodendrocytes leading to cell death *(122),* induction of NF-κB in Schwann cells *(123),* promoting survival of isolated sensory neurons *(124),* promoting neurite outgrowth of cultured hippocampal neurons *(125),* inhibiting neurite outgrowth of sympathetic neurons *(126),* and modulating trkA signaling in PC12 cells *(127,128).*

To explain the diverse cellular responses to ceramide, Perry and Hannun *(120)* identified several factors important in determining a cell's response to ceramide, such as the number of downstream effector enzymes which are activated by ceramide, and the activities of various enzymes involved in ceramide metabolism. The downstream effector enzymes activated by ceramide include ceramide activated protein phosphatase (CAPP; a member of the 2A class of protein phosphatases), protein phosphatase-1 (PP1), ceramide-activated protein kinase (CAPK), and protein kinase C-ζ (Fig. 1; reviewed in Ref. *120*). The role of these effector enzymes in mediating the effects of ceramide in neurons remains to be examined. However, experiments in other nonmammalian, nonneuronal cell types suggest there are many levels at which ceramide effector enzymes may interact with known neuronal signaling pathways, contributing to our difficulty in isolating a physiological role for ceramide in neurons. For example, in transformed human epithelial cells, both CAPP and PP1 have been demonstrated to activate c-jun *(128).* Also, in *Drosophila,* CAPK has been demonstrated to be the kinase suppressor of ras *(129)* and directly involved in the activation of raf, and thus the erk pathway *(130).*

The second factor that may determine a cell's response to ceramide is the presence of enzymes involved in ceramide metabolism into biologically active metabolites, namely ceramidase and sphingosine kinase. Ceramidase cleaves ceramide to form sphingosine and a

free fatty acid, the sphingosine can be phosphorylated by sphingosine kinase to form the biologically active sphingosine-1-phosphate (Fig. 1; SPP; *131*). SPP has been shown to reverse the pro-apoptotic functions of ceramide, thus suggesting that cellular fate may depend on a balance between ceramide and SPP *(132),* and further, that regulation of the enzymes involved in ceramide metabolism may serve as important sites for regulation of cellular survival. Indeed, trkA activation via NGF induces a biphasic increase in sphingosine kinase activity in PC12 cells, and treatment with SPP protected these cells from apoptosis induced by serum withdrawal *(133).* Furthermore, not only does SPP act as an intracellular messenger, but it also mediates ligand-specific effects via binding to the EDG-1 family of G-protein coupled receptors *(134).*

Recent experiments suggest that neurotrophin binding to p75 may activate pathways distinct from the induction of sphingomyelinase activity. Other members of the TNF family of receptors interact with TNF receptor associated factors (TRAFs) via a conserved intracellular element termed the "death domain," to modulate apoptosis, as well as JNK and NF-κB activity *(135,136).* The cytoplasmic juxtamembrane region tail of p75 has been shown to associate with TRAF6 in a ligand-dependent manner, and requires sequences between residues 113 and 128 (Fig. 1; *137*). In the same study, a dominant negative form of TRAF6 was shown to inhibit the p75-dependent NF-κB nuclear translocation in response to NGF treatment. Furthermore, several other adaptor proteins have been shown to interact with the p75 receptor in glutathione s-transferase pull-down assays, such as neurotrophin receptor-interacting factor (NRIF), SC-1, and neurotrophin receptor-interacting melanoma antigen gene homolog (NRAGE) (reviewed in Ref. *31*). These adaptor proteins are associated with apoptosis (NRIF) and cell-cycle arrest (SC-1 and NRAGE), but their role in axonal growth or regeneration remains undetermined.

A possible link between p75 receptors and the regulation of axonal growth comes from recent experiments which investigated the role of RhoA in p75 signaling in response to neurotrophin stimulation *(138).* The Rho family of small GTPases, includes Rho, Rac, and Cdc42, and are involved in regulating growth-cone morphology (reviewed in Ref. *139*). In vitro, p75 receptors constitutively activate the small GTP-binding protein RhoA, and neurotrophin binding to p75 reduces this activation in vitro *(138).* There was an increase in axonal growth associated with the reduced RhoA activity in response to neurotrophin binding to p75. Thus the authors suggested that neurotrophin binding to p75 receptors may facilitate axonal growth, which was consistent with delayed axonal outgrowth of embryonic spinal sensory and motor neurons in p75 knockout mice *(138).* Alternatively, it may also be possible that the 30–50% reduction in the number of motor and sensory neurons present in p75 homozygous knockout mice *(140–144),* make it more difficult to detect axon growth during these early developmental periods.

p75-trk Interaction

As described above, trkA receptors require coexpression of p75 for formation of high-affinity binding states, and evidence from p75 homozygous knockout mice demonstrate that coexpression of these receptors are required for maximal survival of neurons during periods of development when neurons are competing for limiting amounts of neurotrophins. For example, in p75 knockout mice, there is significantly more developmental cell death in sensory *(140–142)* and motoneuronal *(143,144)* cell populations. The enhanced cell death may be explained by a rightward shift in the dose-response curves for the amount of neurotrophin required to elicit maximal survival responses. Weise et al. *(145)* found that embryonic motoneurons isolated from p75 deficient mice required 5 times more BDNF to elicit maximal survival responses than wild-type control motoneurons, consistent with higher concentrations of NGF to promote survival of sympathetic neurons in p75 knockout mice *(141).*

Two models have been proposed to explain the collaborative interaction between p75 and trk receptors (reviewed in refs. *34,35*). Briefly, the first model suggests that the high-affinity state is a result of the p75 receptor "presenting" trk receptors with bound neurotrophin *(62),* whereas the second model proposes that p75 and trk receptors are able to demonstrate neurtrophin-independent association, in other words, p75 induces a conformational change in trk receptors which confers high affinity binding *(146).* The latter is supported by experiments which demonstrate that high molecular weight receptor complexes that bind NGF can be recognized by both p75 and trkA antibodies *(147).* The specific nature of p75-trk collaborative interaction remains to be elucidated.

There are several recent lines of evidence from both PC12 cells and sympathetic neurons that suggest that activation of the p75 receptor antagonizes trk-signal activation *(107,126,148).* In PC12 cells expressing p75 and trkA, BDNF-mediated activation of p75 reduced the ability of NGF to induce tyrosine phosphorylation of trkA receptors in a dose-dependent manner *(126).* The reduction in trkA phosphotyrosine content was associated with: 1) a reduction in c-fos activation, a transcription factor normally activated in response to trkA; and 2) an increase in phosphoserine content. This effect may be mediated by the p75-induced generation of ceramide, as transient stimulation with a short-chain ceramide analog mimicked this effect *(126).* It is not currently understood how this increase in phosphoserine content confers reduced trkA activation, however, it provides an enticing mechanism by which p75 activation can modulate trkA activity. Interestingly, although transient stimulation with short chain ceramide analogs decreased trkA responsiveness to NGF, long-term ceramide application actually enhanced trkA responsiveness to NGF *(127).*

Further in vitro studies have characterized p75 antagonism of trkA activity. If sympathetic neurons are maintained at suboptimal concentrations of NGF, BDNF activation of p75 can antagonize trkA mediated survival *(107,149)* and axonal growth *(148,150)* in a dose-dependent fashion. In addition, preventing autocrine activation of p75 with a function-blocking anti-p75 antibody directed towards the extracellular domain of p75 *(151),* or with an anti-BDNF antibody also increased neurite outgrowth in these cells *(148).* Furthermore, sympathetic neurons isolated from p75 knockout mice show increased neurite outgrowth, and do not show an inhibitory effect on axonal growth in response to BDNF compared to wild-type control neurons *(148).* It is possible that p75 antagonism of axonal growth, like trkA autophosphorylation, involves ceramide as elevation of intracellular ceramide and application of short-chain ceramide analogs to the distal axons of sympathetic neurons inhibits axonal elongation in vitro *(152).*

In vivo experiments have also suggested a role for p75 in negatively regulating trk-mediated neurite growth. Sympathetic target organs such as the pineal gland which normally contain high levels of BDNF are hyperinnervated by tyrosine hydroxylase positive, trkA/p75 expressing, sympathetic axons in BDNF knockout mice *(148).* There is also extensive CNS sprouting of sympathetic and sensory fibres in p75 knockout mice which overexpress NGF under a glial-specific promoter *(153).* This negative regulation of trk by p75 is not necessarily restricted to trkA, and may also extend to trkB, or trkC, as evidenced by increased motoneuronal survival and axonal sprouting following facial nerve crush in p75 knockout mice *(143),* and increased number of motoneurons which regenerated their axons in p75 knockout mice compared to wild-type controls (*144; see* "Neurotrophins"). In addition, administration of BDNF exerts a biphasic dose-dependent effect on the survival of isolated embryonic motoneurons from wild-type mice, where lower doses promote survival, but at progressively higher doses BDNF becomes progressively less effective in mediating this effect *(145).* However, this decline in effectiveness is not apparent in embryonic motoneurons isolated from p75 homozygous knockout

mice. It may be possible that increasing doses of BDNF shift the balance between trkB and p75 activation, thus at high doses of BDNF, p75 activation predominates and antagonizes the survival signal mediated by trkB.

The antagonism between trk and p75 can be bidirectional (reviewed in ref. *30*). Not only does p75 negatively regulate trk activity, but high levels of trk were shown to inhibit p75 activation. In PC12 cells which do not express trkC, NT-3 induced significant sphingomyelin hydrolysis and the production of ceramide; presumably via p75 receptors *(121).* However, NGF did not induce sphingomyelin hydrolysis in these cells, perhaps because NGF signaling via trkA inhibited NGF signaling via p75, an idea supported by the fact that NGF was able to induce sphingomyelin hydrolysis in the presence of K252a, an inhibitor of trk tyrosine kinase activity *(121).* The downstream target of activated trkA which is responsible for mediating the negative regulation of p75 is PI3K, as pharmacolocial inhibition of PI3K restored the ability of NGF to induce sphingomyelin hydrolysis *(154).*

Neurotrophins as Axon Guidance Molecules

Neurotrophins are unique among neurotrophic factors in their ability to act as guidance molecules for growth cones *(155–158).* Studies of growth-cone turning responses of *Xenopus* spinal neurons have identified several molecules which can attract or repulse growth cones, and have divided these molecules into two groups. BDNF is considered a group 1 guidance molecule. Group 1 guidance molecules are defined according to the following criteria: 1) their ability to induce turning responses in growth cones of *Xenopus* spinal neurons; 2) their dependence on extracellular calcium; and 3) their regulation by cAMP and PKC. In contrast, NT-3 is considered a group 2 guidance molecule because it is not dependent on extracellular calcium, and is regulated by cGMP or protein kinase G (PKG; reviewed in ref. *159*). Until recently, the role of NGF in growth-cone turning was unknown because *Xenopus* spinal neurons do not express endogenous trkA. In vitro experiments evaluating the turning responses of growth cones of *Xenopus* spinal neurons which have been transfected with either wild-type trkA, or mutant trkA unable to activate Shc, PI3K, or PLC-γ, have demonstrated that NGF behaves as a group 1 guidance molecule, and coactivation of PLC-γ, and PI3K is essential for turning responses to a gradient of NGF *(156).*

The role of the neurotrophins in the chemotaxis of the mammalian growth cone has been newly elucidated. By expressing enhanced green fluorescent protein under a neuron-specific promoter, Tucker et al. *(158)* were able to observe spinal neuron growth in response to gradients of neurotrophins in real time. Chromatographic beads soaked in NGF, NT-3, BDNF, and NT-4/5 were all able to induce chemotaxis in sensory, but not motor neurons. Functional-blocking antibodies to the neurotrophins abolished the turning response of both motor and sensory neurons. The authors suggest that adhesive forces between "neurotrophin dependent" sensory and "neurotrophin independent" motor axons could result in inhibition of the growth of both populations when the outgrowth of one is potently inhibited *(158).* Thus specific chemotactic molecules for developing motor axons remain to be identified.

GDNF Family Members

GDNF Family Member Signaling via Ret

Elements involved in Ret-signal transduction following dimerization with GFRα receptors has been recently reviewed *(160)* and will be summarized here to compare and contrast with elements involved in signal-transduction pathways activated by other neurotrophic factor families. Upon dimerization, several key tyrosine residues on Ret become phosphorylated and serve as docking sites for intracellular adaptor proteins. As shown in Fig. 1, PLC-γ associates with Y1015, Shc with Y1062, and Grb2 with Y1096 *(161–169).* PLC-γ, Shc, and Grb2 also associate with phosphorylated trk

receptors (*see* above), and may at least partially explain the functional redundancy of these 2 distinct families of neurotrophic factors on cellular populations which express both types of receptors. Subsequently, similar downstream-signaling pathways are activated, such as the ras-erk pathway *(170–173),* PI3K *(172–174),* and PLC-γ *(162,173).* The GDNF-induced activation of PI3K and survival-promoting effects have also been shown to involve the association of the src kinase, pp60src, with activated ret *(175).*

In addition, GDNF can activate Rho/Rac-related small GTPases, such as Cdc42, thus leading to the activation of JNK (Fig. 1; *176*). As mentioned above (*see* "Summary of trk-Signaling Events Following Neuroprophin Binding"), although the expression of JNK is often associated with apoptosis *(122,149,177),* it is the kinetics of JNK expression that are important in determining cellular fate, as transient JNK induction is associated with cellular proliferation, whereas sustained activation leads to apoptosis *(109).*

In PC12 cells, activation of the ras-erk kinase pathway appears to be necessary for the survival and neurite outgrowth stimulating actions of GDNF and NTN *(169,174,178).* Also, PI3K signaling is required for GDNF-induced formation of lamellipodia, which are important for neuritogenesis *(174,179).* In contrast, as with exogenous BDNF *(118),* activation of PI3K, but not the erk-MAPK pathway was responsible for mediating the survival-promoting effects of GDNF family members on isolated embryonic motoneurons *(172).*

Ret-Independent Signaling

Saarma and Sariola *(63)* identify 3 lines of evidence that GFRα receptors can signal independently of ret. First, in some tissues GFRα receptors are expressed without ligand or ret. Secondly, GDNF supports the survival of postnatal cochlear ganglion neurons, which express GFRα1, but not ret. Third, GDNF increases intracellular calcium levels in sensory neurons isolated from mice carrying a homozygous mutation for ret. GFRα receptors, like other GPI-linked receptors, may be associated with lipid rafts, which are detergent insoluble "microdomains" within the plasma membrane which are rich in cholesterol and sphingomyelin (Fig. 1). Lipid rafts are considered specialized signaling organelles within the plasma membrane (reviewed in ref. *180*). Lipid rafts are generally enriched in signaling proteins which localize to the intracellular leaflet of the plasma membrane, thus GFRα receptors may mediate intracellular signaling events such as activation of src family kinases and elevation of intracellular calcium *(160).* This is supported by recent studies which demonstrate that in cell lines which express high levels of GFRα1, but not ret, GDNF stimulation promotes cell survival, and is associated with activation of src-like kinases in detergent insoluble membrane fractions, suggesting the involvement of lipid rafts *(173,181).*

Interestingly, in cells that express both GFRα1 and ret, GDNF binding to GFRα1 leads to ret translocation to lipid rafts as well as ret association with pp60 src, and disrupting ret's ability to associate with lipid rafts leads to attenuated cellular responses to GDNF, such as survival, as well as phosphorylation of erk and Akt *(175,182).* Thus, it is becoming clear that both ret-dependent, and ret-independent signaling depends on association with lipid rafts (Fig. 1; *175,182*).

Neuropoeitic Cytokines

Neuropoeitic Cytokine Signaling via gp130

The intracellular events involved in neuropoetic cytokine signaling have recently been reviewed and will only be briefly summarized here *(80,86,183).* The best described intracellular pathway that is activated in response to neuropoetic cytokine stimulation is the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway which mediates the transcription of target genes. This pathway is common to other cytokines, such as interferons as well as other growth factors. The first event in the induction of the JAK-STAT pathway is ligand-dependent homo- or

heterodimerization of signal transduction subunits such as gp130, LIFR, and OSMR. Upon ligand binding, all neuropoeitic cytokines recruit the signal-transducing gp130 to the receptor complex, which can either signal alone, or in combination with either LIFR or OSMR. The signal-transducing receptors gp130, LIFR, and OSMR are all able to induce the phosphorlyation of JAKS, and thus recruit STAT proteins to the intracellular domain of the receptor complex. The exact composition and conformation of the ligand-receptor complex is ligand specific. For example, IL-6 binding to IL-6R induces gp130 homodimerization, but CNTF, LIF, and CT-1 binding to their cognate ligand-binding receptors induces heterodimerization of gp130 and LIFR (Fig. 1; *80*). As with the GDNF family of neurotrophic factors, IL-6 and CNTF first bind to specific α-receptor units which are not directly involved in the signal transduction cascade (Fig. 1; shown as CNTFRα). A ligand-binding alpha subunit has been proposed for CT-1, but so far has not been identified. Another similarity between GDNF family receptors and IL-6/CNTF receptors is that the membrane bound α-receptor subunit can be functionally replaced by soluble forms which lack the transmembrane domain and cytoplasmic domain. In contrast, LIF and OSM bind directly to their signal-transduction receptors LIFR and OSMR, respectively. Upon ligand binding and receptor homo- or heterodimerization, gp130 associated JAKs, Jak1, Jak2, and Tyk2 become activated via their association with Box1/Box2 domains of gp130, and the cytoplasmic tail of gp130 becomes phosphorylated at several phosphotyrosine residues which serve as docking sites for STAT proteins which contain SH2-binding sequences (Fig. 1; STAT1 and STAT3). The association of STATs with gp130 can induce STAT phosphorylation and dimerization, at which time the STATs can translocate to the nucleus where they regulate transcription of target genes.

Phosphorylated gp130 also serves as a docking site for SHP2 at Y759, in addition to STAT activation (Fig. 1). SHP2 is a tyrosine phosphotase that forms a link to the ras-erk pathway, which like the neurotrophins and GDNF family neurotrophic factors, is also activated in response to neuropoeitic cytokine stimulation (Fig. 1; *80,184,185*). As described above, SHP2 also serves as a adaptor protein for activation of ras via its association with Grb2 in response to neurotrophin stimulation *(40).* Recently, Shc, the adaptor protein involved in linking trk (neurotrophin family signaling) and ret (GDNF family signaling) activation to the ras-erk, as well as the PI3K pathways (Fig. 1), has been shown to be recruited to activated OSMR *(186),* suggesting further points of overlap between neurotrophin-, GDNF-, and neuropoeitic families of neurotrophic factors.

In contrast to the functional significance that has been attributed to the different aspects of both neurotrophin and GDNF family neurotrophic factor signal transduction, little is known about the specific role of the different elements involved in neuropoeitic cytokine-signal transduction. It is known, however, that these factors do promote the survival and differentiation of many different peripheral neuronal populations in vitro. For example, IL-6, CNTF, and LIF all promote the survival of embryonic sensory and motoneurons *(187–195).* Recently, exogenous CT-1 has also been shown to promote the survival of embryonic motoneurons *(196–198).* The ability of CNTF, LIF, and CT-1 to promote the survival of embryonic motoneurons depends on coexpression of LIFR *(197).* In addition, IL-6 can promote the differentiation of PC12 cells in a manner similar to NGF *(199)*

Thus, although it is well established that members of the neuropoeitic cytokine family are important in mediating the survival and differentiation of different populations of peripheral neurons in vitro, the specific intracellular signaling mechanism(s) by which this occurs remain(s) to be determined.

In Vitro Interactions Between Neurotrophic Factors

Due to the significant overlap in adaptor proteins recruited to activated receptors and signal transduction mechanisms utilized by

the 3 distinct families of neurotrophic factors outlined in the above sections (Fig. 1), one may predict that these factors also demonstrate significant interactions in biological responses that are elicited upon application to responsive cell types. This is supported by several lines of experimental evidence which demonstrate that combinations of members of the neurotrophin family, GDNF family, and neuropoetic cytokines interact to elicit either additive or synergistic effects.

In studying the effects of BDNF, GDNF, and CNTF on embryonic motoneuron differentiation, all 3 neurotrophic factors promoted the expression of ChAT, as well as neurite outgrowth, albeit to varying degrees *(200).* BDNF had the largest effect on ChAT activity, whereas GDNF had the largest effect on neurite outgrowth. Combinations of GDNF and BDNF had additive effects on ChAT activity, whereas GDNF and CNTF, or BDNF and CNTF had synergistic effects *(200).* This difference may be explained in part, by the fact that the intracellular signaling pathways activated by GDNF and BDNF are largely overlapping, whereas CNTF may activate both the ras-erk and JAK-STAT pathways (Fig. 1). This is consistent with synergistic effects of GDNF and CT-1 on promoting the survival of cultured embryonic motoneurons *(201).* Substantial convergence of the intracellular pathways activated by the 3 families of neurotrophic factors is also supported by the fact that although BDNF, GDNF, and CNTF all promote the survival of embryonic motoneurons in vitro, the ability of all 3 of these factors to promote survival is abolished in mice lacking B-raf *(119),* an essential component in the ras-erk kinase pathway (Fig. 1). This suggests a common "survival" signal transduction pathway that is activated by distinct families of neurotrophic factors.

There is also evidence for neurotrophic factor interaction in embryonic sensory neuron survival. The ability of IL-6 to promote the survival of embryonic DRG neurons depends on a basal level of endogenous BDNF activity, as anti-BDNF antibodies and antitrkB antibodies reduce the ability of IL-6 to support the survival of a subpopulation of sensory neurons *(202).*

Summary

Neurotrophins, GDNF family members, and neuropoetic cytokines display many key similarities and differences in their structure, receptor systems, intracellular signal transduction pathways, and in vitro biological activity. Specifically, neurotrophins and GDNF family members exist and are biologically active as homodimeric molecules, whereas the neuropoetic cytokines are long chain α-helix bundle proteins. Although the neurotrophins bind to 2 distinct classes of receptors, trk and p75, the trk-p75 interaction in the formation of high-affinity binding sites for neurotrophins may be considered analogous to the receptor systems used by GDNF family members and neuropoetic cytokines, which include the ligand-binding α-subunits and the transmembrane signal transduction subunits. Furthermore, we have highlighted both the redundant and distinct signal transduction pathways that are activated by these 3 families of neurotrophic factors which is consistent with the ability of these neurotrophic factors to exert similar biological effects on many neuronal populations such as survival, differentiation, and neurite outgrowth.

In the following sections, we provide a detailed description of the regulation of neurotrophic factors and their receptors, as well as several key signal transduction molecules, following peripheral nerve injury. We focus on the expression of the neurotrophins, GDNF family members, and the neuropoetic cytokines, in axotomized motoneurons and in the regenerative environment of the distal nerve stump. By examining the temporal expression of these neurotrophic factors after injury, we aim to provide insight into the mechanisms by which neurotrophic factors exert their effects on axotomized motoneurons in vivo. The effects include promoting survival, regenerative sprouting, and functional recovery.

Spatio-Temporal Expression of Neurotrophic Factors Relative to Peripheral Nerve Injury

Introduction

Following a discussion of the signal transduction mechanisms and pathways activated by neurotrophic factors binding to their receptors, it is evident that not only is it important to know what neurotrophic factors are present following peripheral nerve injury, but also their; 1) kinetics of expression; and 2) the relative expression of neurotrophic factors and their receptors. Thus, prior to examining the roles of neurotrophic factors in peripheral nerve injury, we first describe the spatial and temporal regulation of neurotrophic factors and their receptors in motoneurons (Fig. 2), and the distal nerve stump (Fig. 3) following peripheral nerve injury.

Motoneurons

Neurotrophins and Their Receptors

Motoneuronal expression of neurotrophins after injury has been well described. NGF is not expressed by motoneurons, nor is it upregulated after injury *(203,204).* In contrast, BDNF is expressed at relatively low levels in intact motoneurons, and is rapidly induced after injury. As early as 8 h after axotomy, BDNF mRNA is increased in facial motoneurons *(205),* as detected by *in situ* hybridization (Fig. 2A). Two days after injury, most facial motoneurons express high levels of BDNF mRNA *(205).* However, this increase in BDNF gene expression is transient, as BDNF mRNA expression begins to decline at 4 d, and returns to baseline by 7 d after injury. Quantification of the serial dilution reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of BDNF mRNA isolated from the facial motoneuron pool demonstrates a twofold increase at 8 h, four fold increase at 12 h, returning to twofold increase at 1 d, declining to 1.5-fold increase at 4 d, and returning to baseline at 7 and 14 d after injury (Fig. 2A; *205*). The upregulation of BDNF mRNA in axotomized facial motoneurons, as detected by *in situ* hybridization, RT-PCR, and RNAse protection assay corresponds with BDNF protein expression. BDNF protein is detected as early as 1 d following facial nerve transection, reaches a peak at 7 d, and remains elevated compared to contralateral intact control motoneurons as late as 14 d post injury *(205).* Using *in situ* hybridization, our laboratory has recently demonstrated that the kinetics of BDNF mRNA expression is substantially slower in regenerating femoral motoneurons than in axotomized facial motoneurons, with a twofold increase not being detectable until 7 d after nerve repair *(116).* Interestingly, the same short periods of continuous electrical stimulation that accelerated femoral motor axonal regeneration across the injury site *(206)* significantly accelerated the kinetics of BDNF mRNA expression *(116).* Specifically, electrical stimulation shifted the peak BDNF mRNA expression from 7 to 2 d after nerve repair.

Less is known about the kinetics of NT-3 and NT-4/5 expression after injury. Evaluation of spinal cord homogenates after sciatic nerve injury has suggested that the expression of both NT-3 and NT-4/5 in motoneurons decreases after injury (Fig. 2A; *203*). NT-3 mRNA shows a biphasic response: decreasing twofold 12 h after axotomy, recovering to baseline by 3 d, but again decreasing twofold 2 wk later *(203).* NT-4/5 decreases slightly 6 h after sciatic nerve injury, but returned to control levels at 12 h *(203).* However, it is not clear whether the mRNA detected in these studies truly reflects motoneuronal expression, or expression in other cells present in the spinal cord such as interneurons, astrocytes, oligodendrocytes, or microglia.

Motoneuronal expression of neurotrophin receptor mRNA following peripheral nerve injury is summarized in Figure 2B. Motoneurons do not express nor upregulate trkA after injury *(204). In situ* hybridization analysis of full length trkB mRNA shows that it does not

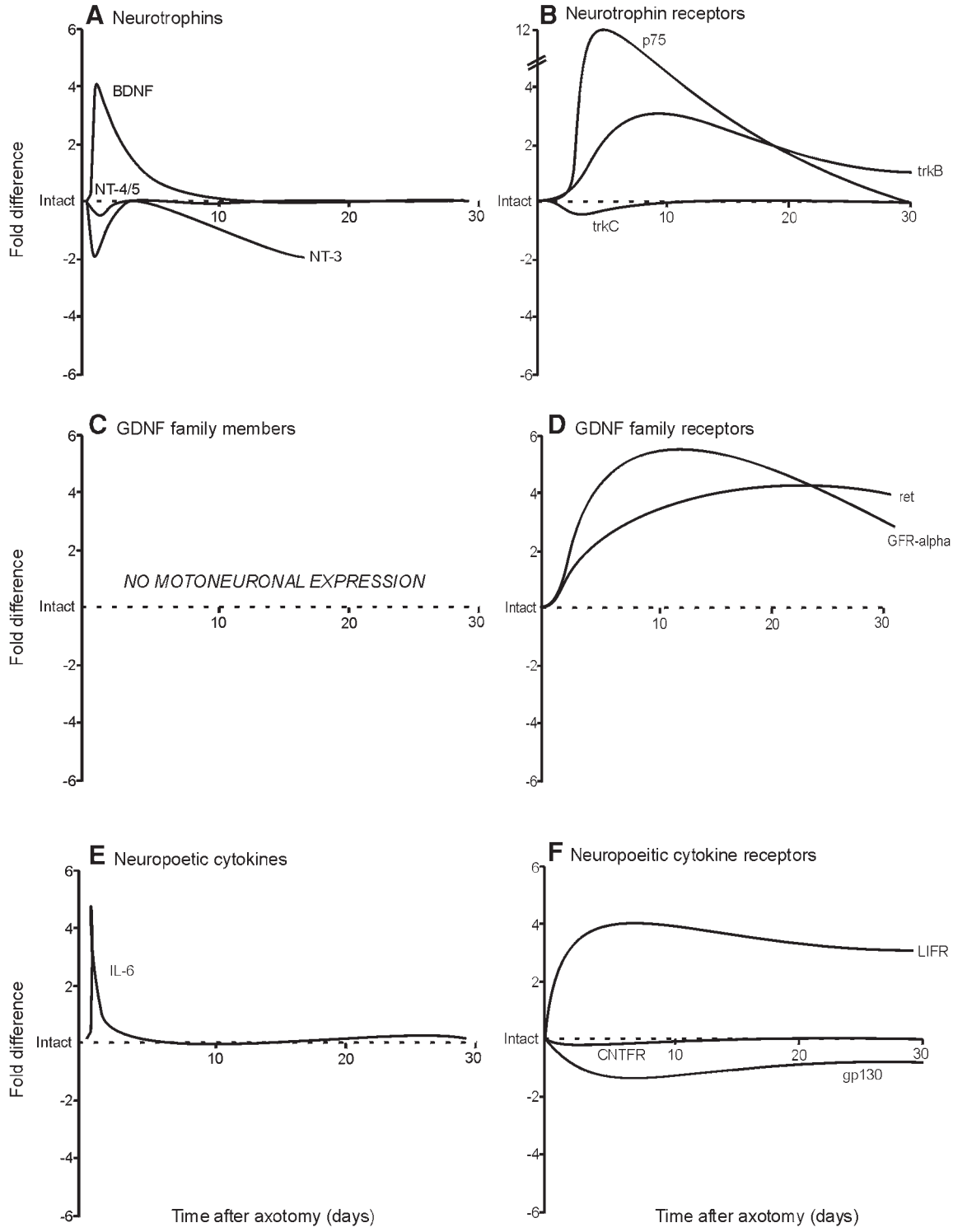


Fig. 2. Temporal expression of neurotrophic factors and their receptors in axotomized motoneurons. All values are normalized to contralateral intact control motoneurons (*see* text for details).

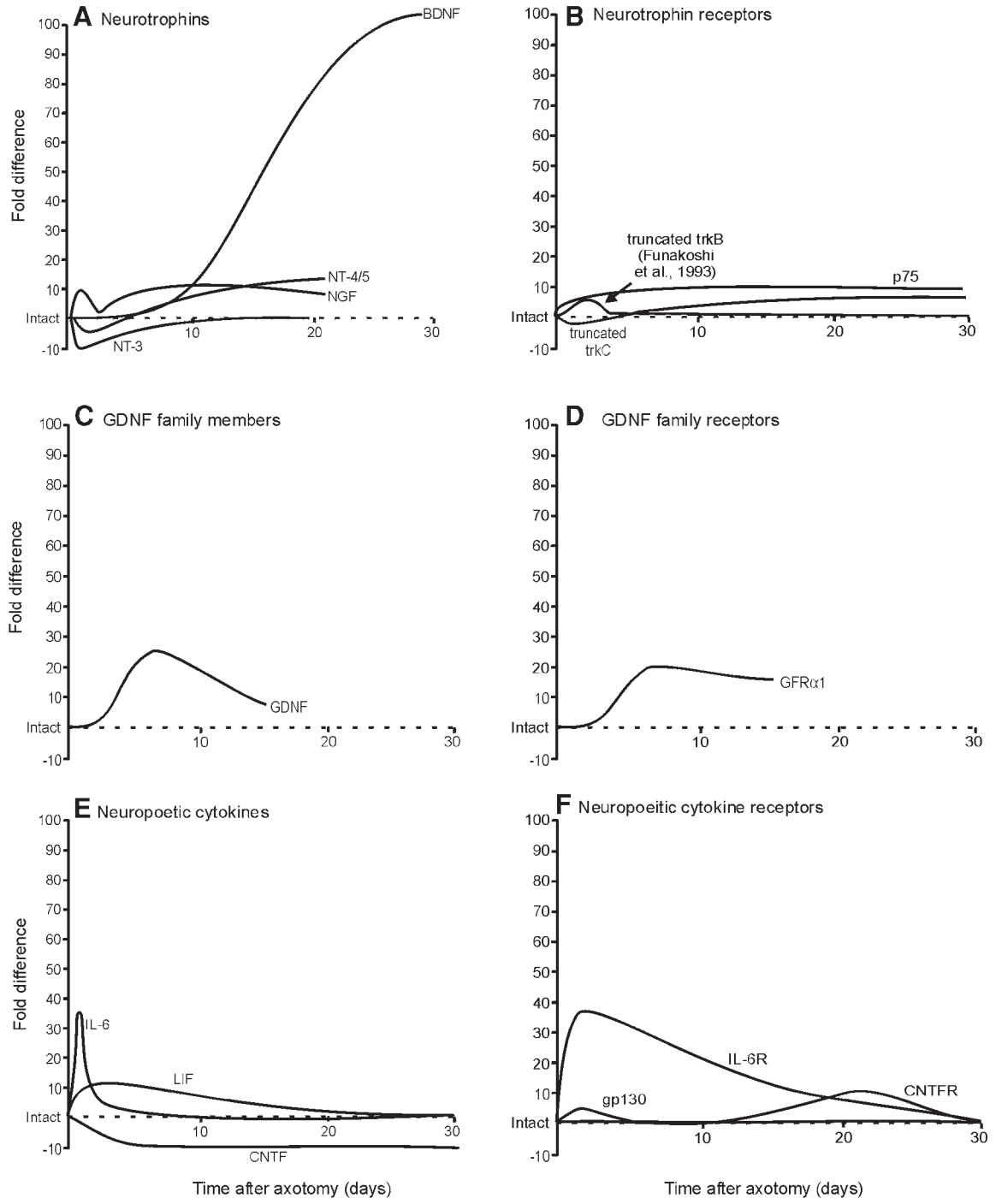


Fig. 3. Temporal expression of neurotrophic factors and their receptors in the nonneuronal cells of the distal nerve stump. All values are normalized to contralateral intact control motoneurons (*see* text for details).

begin to increase until 2 d after facial nerve axotomy, peaks at a three fold increase by 7 d, and remains elevated between 1.5- and threefold 14 and 21 d after injury *(205).* Expression of trkB mRNA following sciatic nerve axotomy follows a similar time course *(207).* Similar to what we have observed with BDNF mRNA, the kinetics of trkB mRNA expression can be significantly accelerated in femoral motoneurons by applying short-term periods of continuous electrical stimulation *(116).* Although trkC mRNA remains relatively unchanged following sciatic nerve axotomy, it is massively downregulated after sciatic nerve avulsion, and remains well below intact levels at least 42 d after injury *(207).* Thus, it is possible that only more severe injuries cause downregulation of trkC. This is consistent with RT-PCR analysis of inserted and noninserted isoforms of trkC which suggest that 7 d after facial nerve axotomy (which is considered more severe than a sciatic axotomy due to its proximity to the cell body), all isoforms are downregulated below contralateral intact control levels *(208).*

Expression of the p75 receptor is barely detectable in adult motoneurons, but is rapidly upregulated following injury, with mRNA levels reaching 12-fold greater levels 7 d after axotomy (Fig. 2B; *209–214*). This is supported by an increase in p75 immunoreactivity in adult sciatic motoneurons at 2 and 7 d after axotomy *(214–216).* The increased expression of p75 is maintained for several weeks after axotomy, returning to baseline by 30 d *(214).* Interestingly, a crush injury induced a larger and more sustained increase in p75 expression than transection injury *(214).*

In summary, based on the expression of neurotrophins and their receptors (Fig. 2A,B), a strong role is suggested for BDNF in mediating motoneuronal response to injury. As BDNF, trkB, and p75 are rapidly upregulated in axotomized motoneurons, BDNF may play an autocrine or paracrine role in motoneuronal response to axotomy, but the exact function of BDNF and the receptors which mediate these effects remain to be determined.

GDNF and Its Receptors

Although the regulation of GDNF and its receptors in response to motoneuronal injury has been well documented *(207, 217–219),* the role of other GDNF family members such as NTN, ART, and PSP remains to be determined. In contrast to members of the neurotrophin family such as BDNF, GDNF mRNA has not been detected in intact or injured motoneurons (Fig. 2C; *217,218,220*). Thus, it is not likely that GDNF mediates its effects on motoneurons in an autocrine fashion.

Motoneuronal responsiveness to GDNF is conferred by GFR-α1 and Ret mRNA upregulation as early as 18 h after hypoglossal nerve transection, expression which peaks at 5 d and remains elevated for at least 5 wk postinjury *(219).* The kinetics are slightly longer in duration following facial nerve axotomy *(219),* and longer still after sciatic nerve axotomy (Fig. 2D; *207*), with: 1) ret mRNA increasing approx twofold, 3 d following sciatic nerve transection, peaking at approx 4.5-fold at 21 d, and returning near baseline 42 d later; and 2) GFR-α1 mRNA increasing twofold by 1 d, peaking at approx sixfold between 7 and 14 d, and returning to baseline 42 d later *(207).* It is not clear whether this discrepancy can be attributed to differences in various populations of motoneurons or species differences.

Neuropoeitic Cytokines and Their Receptors

In comparison to the well-characterized temporal kinetics of motoneuronal expression of neurotrophin family members, less is known about the regulation of neuropoeitic cytokines after injury. It is known that IL-6 mRNA is rapidly upregulated after facial nerve axotomy with a peak at 24 h, and a return to baseline shortly thereafter (Fig. 2E; *221*). Motoneuronal regulation of neuropoeitic cytokine receptors after axotomy appears to depend greatly on both the nature as well as the location of the injury. After axotomy of the facial nerve in neonatal animals, motoneuronal CNTFR mRNA was not

Table 1
Summary of Axotomy-Induced Changes in Motoneurons and the Distal Nerve Stump

Location	Peptide phenotype	Neurotrophins	GDNF family	Neuropoetic cytokines
Motoneurons	ChAT ↓ AChE ↓ VANP-1 ↓ neurofilament ↓	BDNF ↑ trkB ↑ p75 ↑	ret ↑ GFR-α1 ↑	IL-6 ↑ LIFR ↑
	CGRP ↑ VAMP-2 ↑ GAP-43 ↑ tubulin ↑ actin ↑	NT-3 ↓ NT-4/5 ↓ trkC ↓		CNTFR ↓ gp130 ↓
Distal nerve stump		NGF ↑ BDNF ↑ p75 ↑	GDNF ↑ GFR-α1 ↑	IL-6 ↑ LIF ↑ gp 130 ↑ CNTFR ↑ IL-6R ↑
		NT-3 ↓ NT-4/5 ↓ trunc. trkB ↓ trunc. trkC ↓		CNTF ↓

detected *(222)*, consistent with very little change in sciatic motoneuronal expression of CNTFR following axotomy *(207)*. In contrast, CNTFR is downregulated after adult facial nerve axotomy *(223)*. The common signal transduction subunit for the neuropoetic cytokines ngp130, is unchanged after adult facial and hypoglossal nerve transection *(223,224)*, but is drastically downregulated 3 d after sciatic nerve transection and virtually abolished after sciatic ventral root avulsion *(207)*. In contrast, LIFR is upregulated 1 and 3 d after adult facial and sciatic axotomy, respectively, and remains elevated for at least 6 wk *(207,223)*.

Intracellular Signal Transduction Molecules

As described above in "Neurotrophic Factors and Their Receptors", there are several key intracellular protein molecules which serve as common signal transduction molecules for the neurotrophin family, GDNF family members, and neuropoetic cytokines. These molecules include elements of the ras-erk pathway, the PI3K pathway, as well as the JAK-STAT pathway activated by the neuropoetic cytokines (Fig. 1). In addition to the regulation of neurotrophic factors and their receptors, axotomized motoneurons also show a characteristic regulation of downstream signal transduction molecules. Specifically, Shc *(225)*, ras *(226)*, PI3K *(227)*, and Akt *(228)* all demonstrate transient mRNA upregulation following hypoglossal nerve injury. This provides strong evidence for the involvement of the PI3K-Akt pathway, and to a lesser extent the ras-erk pathway, in mediating the motoneuronal response to injury. The role of

the ras-erk pathway is weakened by distinct downregulation of Grb2 *(226),* the adaptor protein involved in linking activated trkB to the ras-erk pathway *(117).* In addition, facial and hypoglossal axotomy also induce upregulation of JAK2 and JAK3 mRNA upregulation, as well as phosphorylation and nuclear translocation of STAT3 *(223,224),* indicative of neuropoetic cytokine signal transduction via gp130 or LIFR *(229).*

Distal Nerve Stump

Introduction

The cellular and molecular changes which occur in the nerve stump distal to the site of injury are initially degenerative, with phagocytosis of myelin and axonal debris initially by Schwann cells, then by invading macrophages; a process that is collectively known as Wallerian degeneration (reviewed in ref. *24*). After injury, Schwann cells of the distal nerve stump rapidly convert from a mitotically quiescent myelinating phenotype to a rapidly proliferating non-myelinating phenotype and upregulate many growth associated proteins, including neurotrophic factors, cell adhesion molecules, and many basement membrane components *(24).* Proliferating Schwann cells form linear bands within the endoneurial sheath, known as Bands of Bungner, which are important in guiding regenerating axons across the injury site and into the distal nerve stump (reviewed in ref. *24,230*). The temporal expression of 1) neurotrophins, 2) GDNF-family neurotrophic factors, and 3) neuropoetic cytokines, as well as their receptors in the distal nerve stump following injury is summarized in Figure 3 and will be described in detail in the following sections. It is important to note the change in the scale for the change in expression is substantially larger for Figure 3, compared to Figure 2, suggesting that the changes in neurotrophic factor and receptor expression are much more dynamic in the distal nerve stump than in the axotomized motoneurons. A summary of the axotomy-induced changes in motoneuron, and the distal nerve stump can be found in Table 1.

Neurotrophins and Their Receptors

The temporal expression of NGF and BDNF in the rat sciatic nerve following injury has been well characterized *(231–233).* Using Northern blot analysis, NGF mRNA is barely detectable in intact nerve, but shows a biphasic upregulation after sciatic nerve transection; being rapidly upregulated 10-fold in the distal nerve stump compared to intact nerve as early as 12 h after injury, falls at 2 d, and returns to at least fivefold elevated levels at 3 d and persists for at least 3 wk thereafter (Fig. 3A; *233*). The second phase of NGF mRNA elevation is likely induced by IL-1β secreted by macrophages, as 1) this second phase is temporally correlated with macrophage invasion *(232),* 2) can be mimicked in vitro by addition of activated macrophages or recombinant IL-1β *(232),* and 3) can be blocked by IL-1β antibodies *(232,234).*

The kinetics of BDNF mRNA upregulation in the distal nerve stump following injury are substantially slower than what has been observed for NGF *(233).* Like NGF mRNA, BDNF mRNA is expressed at very low levels in intact nerve *(203).* An upregulation of BDNF mRNA is detectable at 7 d after sciatic nerve transection, and continues to increase up to 28 d later (Fig. 3A; *203,233*). Furthermore, the maximal levels of BDNF mRNA are at least 10 times higher than what was observed for NGF mRNA *(233).*

In comparison to what is known about temporal expression of NGF and BDNF mRNA in the distal nerve stump after injury, less is known about other members of the neurotrophin family, namely NT-3 and NT-4/5. Using an RNAse protection assay, NT-3 mRNA is clearly detectable in intact nerve, but is rapidly downregulated ninefold 6 and 12 h after sciatic nerve transection, and progressively returns to baseline by 2 wk later (Fig. 3A; *203*). In the same study, NT-4/5 mRNA showed a similar, albeit smaller, decline in expression 6 and 12 h after injury, but increased progressively thereafter with eightfold higher levels than in control nerves by 2 wk postinjury.

Although the nonneuronal cells of the distal nerve stump do not express full length trkA, trkB, or trkC receptors, they do express truncated trkB and trkC isoforms, as well as the p75 receptor after injury (Fig. 3B; *24,50*). Truncated trkB receptor mRNA increases slightly in the distal nerve stump 1 d after sciatic nerve transection and returns to baseline by 3 d (Fig. 3B; *203*). Another study demonstrated that truncated trkB expression decreases after peripheral nerve injury *(235).* The reasons for this discrepancy are unclear, but the changes in truncated trkB receptor mRNA were relatively small in both cases. Truncated trkC receptor mRNA decreases slightly in the distal nerve stump 6 and 12 h after sciatic nerve transection, recovers to baseline, and continues to increase to twofold elevated levels by 2 wk after injury *(203).*

The p75 receptor is not expressed by mature, myelinating Schwann cells, but is rapidly upregulated in the distal nerve stump as early as 36 h after peripheral nerve transection *(231,232,236–238).* At 1 wk after injury, p75 mRNA expression is increased approximately ninefold, and continues to increase at one month to a peak of 12-fold compared to background levels (Fig. 3B; 239). The p75 mRNA levels are maintained by denervated Schwann cells for at least 4 mo, at which time they return to basal levels *(239).*

It was initially thought that nonneuronal expression of neurotrophin receptors, like p75 and the truncated trkB and trkC receptors may function to "present" regenerating axons with neurotrophins *(50,236,240).* However, these neurotrophin receptors on the non-neuronal cells of the distal nerve stump may play a much more complex role in peripheral nerve regeneration. For example, expression of p75 on the Schwann cells has been linked to promoting cellular migration or the Schwann cells *(241)* and apoptosis *(242).* The apoptotic signal via p75 in Schwann cells is independent of the anti-apoptotic protein Bcl-2 *(243).* In terms of the truncated trk receptors, their role may not be presenting regenerating axons with neurotrophins; in fact, the opposite may be true. A substrate of fibroblasts stably expressing truncated trkB receptors actually inhibits neurite outgrowth by endocytosing bound neurotrophin and essentially removing trkB ligands from the environment of a growing neurite *(52).* We have recently provided evidence that nonneuronal expression of truncated trkB receptors may limit the initial stages of motor axonal regeneration in vivo *(144).* Specifically, 2 and 3 wk after nerve repair, motor-axonal regeneration is significantly increased in mice expressing a heterozygous mutation in both full-length and truncated trkB receptors. It is possible that the reduced expression of truncated trkB receptors on the nonneuronal cells of the distal nerve stump increases the local availability of BDNF and/or NT-4/5 to regenerating motor axons. Thus expression of truncated trk receptors on the nonneuronal cells of the distal nerve stump may possibly serve to restrict axonal growth and regeneration.

GDNF Family Members and Their Receptors

Similar to motoneuronal response to injury, the expression of GDNF and its receptors has been well characterized in the distal nerve stump following injury, but the pattern of expression for other GDNF family members remains to be determined (Fig. 3C,D). Using an RNAse protection assay, GDNF mRNA was detected in intact nerve, and its levels were found to increase in the distal nerve stump, peak at 7 d, and remain elevated for at least 2 wk following sciatic nerve crush in adult mice *(220).* It is not known, however, whether or not GDNF mRNA expression would follow a similar time course following sciatic nerve transection.

Sciatic nerve crush *(220),* and transection *(217,244)* induces a marked increase in GFRα1 mRNA in the distal nerve stump 3 d postinjury, peaking at 7 d (21-fold increase above intact), and remained elevated by 12-fold at least 14 d after injury, whereas ret mRNA was not detectable in either intact or injured nerves (Fig. 3D; *217,220*). Two models have been proposed to explain this discordant expression of

GFRα1 and ret in the distal nerve stump following injury. First, it has been suggested that GFRα1 expression on Schwann cells of the distal nerve stump serves to "present" regenerating axons with GDNF molecules in a manner analogous to one of the proposed functions of the p75 receptor on Schwann cells *(217,220).* It has also been proposed that soluble GFRα1 is secreted by Schwann cells to bind with GDNF and form a ligand-coreceptor complex that can activate ret receptors on regenerating axons in *trans (220).* However, *trans*-activation of ret is much less effective in eliciting biological responses, such as survival, than *cis*-activation *(182).*

Neuropoeitic Cytokines

The temporal expression of CNTF, LIF, and IL-6, as well as their receptors, following either nerve crush or nerve transection has recently been described by Ito et al. *(245)* and is summarized in Figures 1–3D,E. Briefly, using RT-PCR relatively high levels of CNTF mRNA were detected in intact nerve, but LIF and IL-6 mRNA were undetectable. After injury, CNTF mRNA declines as early as 1 d *(246–248),* and continues to fall to a fivefold decrease compared to intact levels by one week *(245).* In the crushed nerve CNTF mRNA levels recovered by 4 wk, but remained low after transection *(245).* In contrast, LIF and IL-6 mRNA are upregulated in the distal nerve stump *(193,245,248,249).* LIF mRNA rapidly increased 10-fold 1 d after both crush and transection injuries, and returned to baseline by 1 mo later *(245).* IL-6 expression was more transient, increasing 35-fold compared to intact levels 1 d after injury, and returned to baseline 24 h later *(245).*

The rapid decline in CNTF mRNA may be compensated for by the upregulation of CNTFRα mRNA, its ligand-binding receptor, which increases gradually reaching a 10-fold increase by d 21, then returning to baseline levels by d 28 *(245).* IL-6α mRNA also demonstrates a massive upregulation in the distal nerve stump after injury. Peak levels were observed at 2 d, reaching 8-and 35-fold elevations compared to intact levels after nerve crush and nerve transection, respectively, and again returning to baseline by 28 d later *(245).* Little change was seen in the ligand-binding receptor for LIF, LIFRβ *(245).* The common signal transduction component gp130 for CNTF, IL-6, and LIF, showed only a transient increase in mRNA expression.

The distinct temporal expression of these neuropoeitic cytokines and their receptors in the distal nerve stump suggests the possibility that CNTF, IL-6, and LIF may have discrete roles in mediating peripheral neuronal as well as non-neuronal responses to injury. For example, as CNTF lacks a secretory sequence (*see* "Structure of Neuropoeitic Cytokines"), it is thought that nerve injury damages Schwann cells and causes the release of CNTF into the environment of regenerating axons, thus acting as a "lesion factor" *(247,250).* On the other hand, the massive but transient increase in IL-6 expression may serve to initiate a local inflammatory response, such as recruitment of macrophages to the site of injury (reviewed in ref. *79*). In addition to being a potent survival factor for many populations of injured neurons *(191,193),* LIF also plays an important role in promoting local axonal sprouting after injury, because cultured DRG neurons failed to show normal axonal sprouting in response to predegenerated nerves isolated from LIF homozygous knockout mice *(251).*

In Vivo Effects of Neurotrophic Factors After Peripheral Nerve Injury: Survival, Reversing the Effects of Injury, Axonal Sprouting, Regeneration, and Functional Recovery

Survival

Introduction

Neuronal survival after injury depends on several factors, such as neuron type, developmental stage, and the degree and proximity of

the injury *(24).* Generally, sensory neurons are more susceptible than motoneurons and neonatal neurons are more susceptible than adult neurons. Further, more traumatic injuries induce more cell death. Injuries close to the cell body are more damaging than distal injuries. For example, in the case of adult spinal motoneurons, following distal injury, they often remain viable for long periods of time, whereas an avulsion injury may induce substantial cell death in the same cell type *(207,252–254).* As described above, a mosaic of neurotrophic factors are available to injured neurons from both autocrine and paracrine sources. In vivo exogenous application of many of these neurotrophic factors has been demonstrated to support the survival of both sensory and motoneuronal cell populations.

Although adult lumbar motoneurons rarely die after peripheral nerve injury and remain viable up to a year after peripheral nerve injury *(255–257),* substantial motoneuronal cell death follows: 1) avulsion of adult lumbar motoneurons; 2) axotomy of neonatal lumbar motoneurons; and 3) axotomy of both adult and neonatal cranial motoneurons, death being more severe in the latter (*see* ref. *24,* 1997 and references cited therein). Neurotrophic factors have been demonstrated to promote the survival of axotomized or avulsed motoneurons in many different experimental paradigms. The methods of administering neurotrophic factors range from exogenous application, adenoviral transfer, lentiviral transfer, and several other strategies to ensure sustained continuous release to produce long-lasting survival.

Neurotrophins

Application of NGF to axotomized neonatal rat sciatic *(258),* and facial *(259)* nerves may actually potentiate motoneuronal cell death, an effect attributed to NGF binding to p75 receptors, as motoneurons do not express trkA *(260).* This effect however, may be species specific, as NGF did not potentiate motoneuronal cell death following facial nerve lesion in several strains of neonatal or adult mice; only in mice which had a conditioning crush lesion to the sciatic nerve did application of NGF induce motoneuronal cell death *(261).*

It has been well documented that BDNF acts as a survival factor for injured mammalian motoneurons *(262).* Exogenous BDNF rescues: 1) axotomized neonatal facial *(259,263,264);* 2) axotomized neonatal sciatic *(264–267);* and 3) adult lumbar motoneurons following avulsion injury *(252–254,268).* The ability of BDNF to promote survival of axotomized neonatal motoneurons likely depends on functional trkB receptors, as axotomy-induced motoneuronal cell death is significantly exacerbated in neonatal mice carrying a homozygous deletion in full length trkB receptors *(269).* The survival-promoting effects of exogenous BDNF on axotomized neonatal sciatic motoneurons has been demonstrated to be transient. For example, 4 to 5 wk after lesion, up to 95% of the motoneurons which were originally rescued by exogenous BDNF had died, despite continuous treatment *(266).* In contrast, continuous exogenous BDNF exerted long-term survival promoting effects on adult motoneurons after sciatic nerve avulsion *(252).* As with isolated embryonic motoneurons *(145),* the effects of BDNF on motoneuronal survival after sciatic nerve axotomy are dose-dependent, with rescue effects of high doses being significantly lower compared to optimal doses *(265,266).*

There is considerable controversy concerning the relative potency of NT-3 and NT-4/5 in promoting the survival of injured motoneurons in vivo *(262).* In one study, NT-3 AND NT-4/5 were as effective as BDNF in promoting the survival of axotomized neonatal sciatic motoneurons *(265).* In other studies, NT-3 (264,270) and NT-4/5 (270) have been demonstrated to promote the survival of axotomized neonatal cranial and lumbar motoneurons, but to a lesser extent than BDNF. In contrast, Koliatsos et al. *(263)* reported that NT-3 did not promote the survival of axotomized neonatal facial motoneurons compared to controls. These discrepancies are difficult to reconcile, but may be due to slight variations in experimental procedures, or methods of evaluating survival of axotomized motoneurons.

GDNF Family Members

There is considerable evidence that GDNF promotes the survival of several motoneuronal populations after injury. In neonatal animals, exogenous GDNF promotes the survival of axotomized facial *(67),* and sciatic *(198,266,267)* motoneurons, in addition to sciatic motoneurons following ventral root avulsion *(267).* In adult animals, exogenous GDNF rescues axotomized rat facial motoneurons *(67),* in addition to avulsed sciatic motoneurons *(271).* However, like BDNF, a single local dose of exogenous GDNF only produced a transient survival promoting effect *(266).* Thus to promote long-term survival, methods to obtain a more sustained release of GDNF have been examined, such as polymer-encapsulated cells which secrete GDNF *(266),* adenoviral- *(272–275)* and lentiviral-mediated gene transfection *(276)* of motoneurons with GDNF. Adenoviral transfection of adult facial *(274)* and cervical *(275)* motoneurons protected these motoneurons from axotomy- and avulsion-induced cell death. Lentiviral mediated transfection of adult facial motoneurons conferred nearly complete (~95%) survival effects which lasted as long as 3 mo *(276).*

Although there is sufficient evidence that NTN and PSP support motoneuronal survival in vitro *(70,277,278)* it remains to be determined whether or not other members of the GDNF family also prevent axotomy-induced cell death.

Neuropoeitic Cytokines

Although exogenous application of IL-6 is ineffective in promoting the survival of axotomized neonatal motoneurons *(279),* there is extensive evidence that CNTF and LIF are potent survival factors for axotomized neonatal motoneurons in vivo *(192,247,250,270,279–281).* Similar to members of neurotrophic factor families, such as BDNF and GDNF, the effects of exogenous CNTF on motoneuronal survival are transient *(265).* Thus strategies other than exogenous administration have been employed to provide long-term continuous administration of CNTF. Adenoviral transfer of CNTF elicited long-term protection of axotomized facial motoneurons, and when combined with adenoviral transfer of BDNF, showed significantly better long-term protection *(282).*

Reversing the Effects of Injury

In response to nerve injury, the many changes which occur in motoneurons has been denoted as a transition from a mature "transmitting" to a "regenerating" phenotype *(24).* In particular, motoneurons downregulate enzymes associated with neurotransmission, for example: ChAT *(67,214,215,252,283–285),* and acetylcholinesterase (AChE; *208,252*), as well as proteins associated with transmitter release, such as the synaptobrevin isoforms vesicle-associated membrane protein (VAMP)-1 *(285).* In close temporal correlation with the decline in ChAT and AChE is an upregulation of regeneration associated genes that include actin, GAP-43, and Tα1-tubulin (*286,287;* reviewed in ref. *288*), calcitonin gene-related peptide (CGRP; *214,284,285*), as well as neurotrophic factors and their receptors (*see* Fig. 2, and "Motoneurons").

There is substantial evidence that BDNF and NT-4/5, but not NGF or NT-3, play important roles in maintaining the cholinergic phenotype after injury. For instance, intrathecal administration of BDNF prevents the loss of ChAT and AChE in motoneurons after sciatic nerve avulsion in a dose-dependent fashion *(252,289).* Intracerebral ventricular infusion of BDNF and NT-4/5, but not NT-3 or NGF maintained the expression of ChAT in axotomized hypoglossal motoneurons *(284).* Also, peripheral application of BDNF and NT-4/5 maintained the expression of ChAT in axotomized sciatic motoneurons *(215).* The ability of BDNF and NT-4/5, but not NGF or NT-3, to promote the cholinergic phenotype after injury is consistent with motoneuronal regulation of neurotrophin receptors after injury, namely rapid upregulation of trkB, but not trkC or trkA (*see* "Neurotrophins and Their Receptors"). It is not clear however, if and how this regulation of ChAT/AChE expression is linked to the other biological effects of BDNF and NT-4/5 in vivo,

that include sprouting and axonal regeneration (*see* below).

In addition to the phenotypic changes in motoneurons after injury, there are a number of electrophysiological changes which occur, such as decline in axonal conduction velocity, rheobase, EPSP amplitude and duration of afterhyperpolarization, and increase in input resistance *(290,291).* In contrast to the regulation of the cholinergic phenotype which seems to depend on only trkB ligands, maintaining axonal conduction velocity in axotomized motoneurons depends on both trkB and trkC ligands. This is despite the downregulation of NT-3 and trkC by axotomized motoneurons (*see* above) as peripheral application of NT-4/5 or NT-3 improved motor axonal conduction velocity 5 wk after tibial nerve axotomy *(291).* Furthermore, reducing levels of endogenous trkB and trkC ligands using IgG sequestering molecules, reduced motor axonal conduction velocity to a level similar to what is observed after axotomy *(291).*

In contrast to the neurotrophins, little is known about the roles of GDNF family members and neuropoetic cytokines in reversing the effects of motoneuronal injury, such as the downregulation of cholinergic enzymes and the reduced conduction velocity. However, in light of the considerable overlap in the signal transduction mechanisms used by these 3 families of neurotrophic factors, in addition to their ability to promote the survival of injured motoneurons, one may predict a similar overlap in their ability to reverse the effects of axotomy. Surprisingly however, exogenous CNTF did not attenuate the increase in CGRP, nor strongly reverse the ultrastructural changes in motoneurons (fragmentation of rough endoplasmic reticulum, decrease in Golgi apparatus size) following facial nerve axotomy *(292,293).*

Axonal Sprouting, Regeneration, and Functional Recovery

Introduction

Despite the compelling experiments that demonstrate that neurotrophic factors effectively promote motoneuronal survival after injury, the evidence showing a link between exogenous neurotrophic factors and axonal regeneration after injury has been largely indirect *(294–297).* Evaluations of peripheral nerve regeneration have relied heavily on outcomes such as axon counts distal to the site of injury, as well as functional evaluations (e.g., the "pinch test" to evaluate sensory regeneration and walking track analysis to evaluate motor regeneration; *220,295,296*). Although these approaches have the potential to be clinically relevant, they are confounded by the fact that they do not take into account the outgrowth of several axonal sprouts from the proximal stump (reviewed in ref. *24*). Hence they do not provide a direct measure of the absolute number of axotomised peripheral neurons which regenerate their axons in response to exogenous neurotrophic factors. Techniques of counting the number of retrograde-labelled neurons which have regenerated axons through the injury site and into distal nerve stumps have provided important insight into the mechanisms by which short periods of continuous electrical stimulation can promote the speed and accuracy of motor axonal regeneration *(206).* Thus, only combined with quantitative measures of axonal regeneration, such as fluorescent retrograde labelling, can the more qualitative measures of axonal regeneration (pinch test, walking track analysis, axon counts) provide a comprehensive picture as to the functional roles of neurotrophic factors in peripheral nerve regeneration.

Neurotrophins

In line with the ability of BDNF to promote neurite outgrowth in vitro *(117,118),* it has been shown that BDNF can induce motor axonal outgrowth after ventral root avulsion *(252–254),* although this regeneration was primarily restricted to the avulsion site. After less traumatic injuries, such as sciatic nerve transection and repair, BDNF did not enhance sciatic functional recovery as evaluated by sciatic function index *(297),* gait analysis *(294),* or force recovery *(294).* However, force measurements often con-

ceal the failure of many motoneurons to regenerate their axons. Motoneurons may reinnervate 5–8 times their normal number of fibres to compensate for considerable reduction in the numbers of motoneurons that do regenerate *(298–300).* Hence full recovery of muscle mass and twitch/tetanic forces may conceal the failure of up to 80% of the motoneurons to regenerate and remake functional neuromuscular connections. These data highlight the extensive axonal branching ability of axotomized motoneurons which functionally compensate for the reduction in motor axons which reinnervate denervated muscle. In addition, these data also illustrate the difficulty in interpreting functional data, such as walking track analysis or force measurements alone.

The inability of exogenous BDNF to promote functional recovery after immediate sciatic nerve injury and repair is consistent with recent studies from our laboratory. We have demonstrated that local continuous long term administration of low doses of BDNF has no effect on the number of tibial motoneurons which regenerate their axons after immediate cross suture to a freshly cut distal common peroneal nerve stump *(301).* However, this inability of exogenous BDNF to increase motor axonal regeneration and functional recovery is quite surprising in light of recent evidence that the accelerated femoral motor axonal regeneration induced in response to short-term electrical stimulation *(206)* is correlated with accelerated temporal kinetics of BDNF and trkB mRNA expression *(116).* There are at least two possible explanations for this apparent contradiction regarding the role of BDNF in motor axonal regeneration after immediate nerve repair. First, it is plausible that the effects of short-term continuous electrical stimulation in accelerating endogenous motoneuronal expression of BDNF and trkB mRNA, and application of exogenous BDNF to the site of injury and repair exert distinct biological effects. A second possibility is that the short periods of continuous electrical stimulation described by Al-Majed et al. *(116)* increase the expression of other factors involved in accelerating motor axonal regeneration which have not yet been determined.

Despite the incapability of exogenous BDNF to promote functional motor recovery after immediate nerve repair *(294,297),* it is clear that endogenous BDNF is important for peripheral nerve regeneration, as application of an anti-BDNF antibody significantly reduces: 1) the length of regenerating axons; 2) the number and density of myelinated axons; and 3) the amount of sensory axon regeneration, as evaluated by the "pinch test" *(302).*

In contrast to the limited effectiveness of exogenous BDNF in promoting axonal regeneration after sciatic nerve repair, NT-3 delivered to the site of axonal regeneration via fibronectin mats that bridged a 10 mm gap between proximal and distal nerve stumps significantly increased the number of axons which crossed the gap and penetrated the distal nerve stump compared to fibronectin mats alone *(303).* In addition, there were more myelinated axons in the NT-3/fibronectin groups 8 mo after nerve repair compared with fibronectin mats alone *(303).* Using the same surgical model, it was found that the NT-3 enhanced peripheral nerve regeneration was associated with increased muscle recovery from denervation atrophy, and NT-3 acted to selectively improve the cross-sectional area of fast-type muscle fibres which express the type 2b myosin heavy chain *(304).* The ability of NT-3 to promote selective reinnervation of fast muscle fibres is supported by the fact that NT-3 increased the number and size of reinnervated neuromuscular junctions in the fast extensor digitorum longus muscle, but had no effect on reinnervation of the slow soleus muscle after sciatic nerve repair *(305).*

Compelling evidence supporting a role for neurotrophins in peripheral nerve regeneration comes from experiments using a multifunctional chimeric neurotrophin, pan-neurotrophin-1 (PNT-1). It was engineered by combining the active domains of NGF, BDNF, and NT-3 into an NT-3 backbone *(306);* its expression after peripheral nerve injury was driven by coupling the PNT-1 gene to the fourth promoter of the

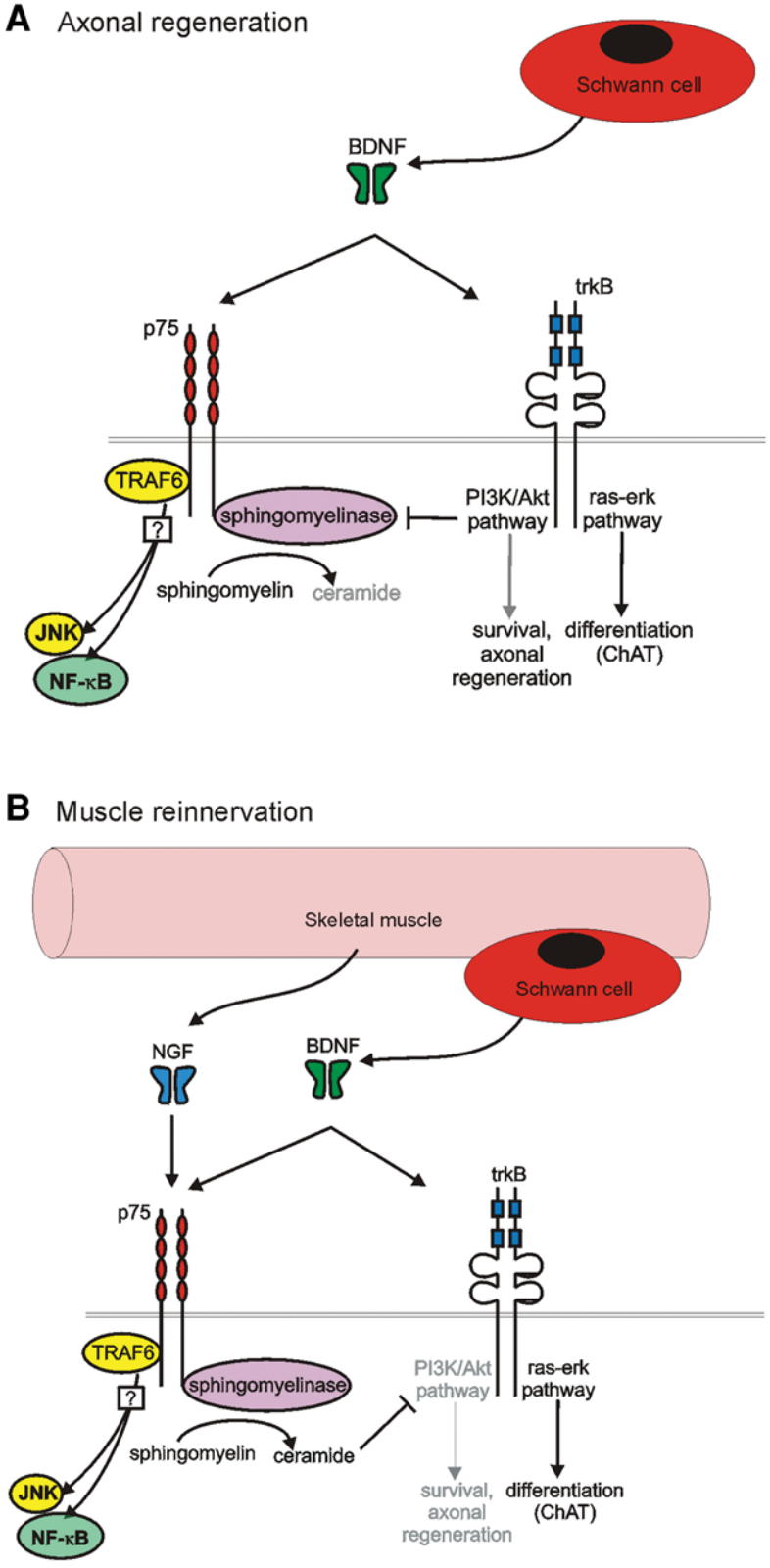
BDNF gene *(307).* Therefore, the expression of PNT-1 after injury followed a similar time course after injury as BDNF (Fig. 2A,3A; *203,205,233*). Expression of PNT-1 after a combined sciatic crush/freeze injury initially caused increased axonal sprouting, as well as accelerated the elimination of axon sprouts at later time points, suggesting accelerated maturation of regenerated axons *(307).* In the same study, compared to wild-type control mice, in PNT-1 transgenic mice, there was: 1) increased motor and sensory innervation of the plantaris muscle and skin, respectively; 2) increased compound action potential recovery in both dorsal and ventral spinal roots; and 3) attenuated reduction in muscle mass, suggestive of increased functional motor recovery.

BDNF and Axonal Regeneration of Chronically Axotomized Motoneurons

One of the major contributing factors to the poor recovery of muscle function after peripheral nerve injury is the progressive decline in a motoneuron's inherent ability to regenerate its axon. Compared to immediate nerve repair, there is a time-dependent decline in the number of chronically axotomized motoneurons which regenerate their axons *(301),* and reinnervate denervated muscle fibres *(308).* We have used a quantitative method of counting the number of retrogradely labelled motoneurons to extensively characterize the role BDNF in motor axonal regeneration. Continuous administration of low doses of exogenous BDNF (2 μg/d for 28 d) are extremely effective in reversing the negative effects of chronic axotomy, on both motor axonal regeneration and recovery of motoneuron cell body size *(301).* In addition, the same low doses of BDNF significantly increase the number of motor units in the reinnervated tibialis anterior muscle compared to saline-treated controls (J. G. Boyd, N. Tyreman, and T. Gordon, unpublished observations). The ineffectiveness of exogenous BDNF to promote axonal regeneration and functional recovery after immediate nerve repair *(294,297),* contrasts with this strong ability of low doses of BDNF to completely reverse the negative effects of chronic axotomy. Thus, these experiments provide a much more clearly defined role for neurotrophic factors in motor axonal regeneration. Specifically, in line with the derivation of the word neurotrophic, which literally means "food for neurons," these experiments demonstrate that neurotrophic factors act to provide sustenance for regenerating motor axons over their long and difficult journeys to reinnervate denervated muscle fibres. Furthermore, the early upregulation of BDNF and trkB by axotomized motoneurons (*see* "Neurotrophic Factors" section 4) may be sufficient to sustain initial axon growth after immediate nerve repair, but the transient nature of this expression (Fig. 2) fails to support axonal regeneration for extended periods of time.

In addition to the beneficial effects of low dose BDNF in promoting motor axonal regeneration of chronically axotomized motoneurons, progressively higher doses (>4 μg/d for 28 d) dramatically reduce the number of both acutely and chronically axotomized motoneurons which regenerate their axons *(301).* Specifically, a dose of 20 μg/d exogenous BDNF exhibits a 92% inhibition of the axonal regener-

Fig. 4. **(A)** Proposed mechanism of neurotrophin's bimodal regulation of motor axonal regeneration **(B)** and muscle reinnervation. **(A)** During early periods of motor axonal regeneration, BDNF (secreted in low levels by denervated Schwann cells) that bind to trkB receptors activates the PI3K-Akt and ras-erk pathways. Activation of the PI3K pathway promotes motoneuronal survival and axonal regeneration and/or inhibits sphingomyelinase and thus the production of ceramide (shown in gray). **(B)** During the later periods of axonal regeneration (approaching denervated skeletal muscle) activation of p75 (possibly by large amounts of NGF secreted by denervated muscle) may lead to generation of ceramide. In turn, ceramide may inhibit the growth-promoting activites of the PI3K-Akt pathway. The ras-erk pathway remains unaffected, and thus motoneurons are able to upregulate ChAT and initiate neuromuscular cholinergic transmission.


A Axonal regeneration
Schwann cell
BDNF
trkB
p75
TRAF6
?
sphingomyelinase
PI3K/Akt pathway
ras-erk pathway
sphingomyelin
ceramide
JNK
NF-κB
survival, axonal regeneration
differentiation (ChAT)
B Muscle reinnervation
Skeletal muscle
Schwann cell
NGF
BDNF
trkB
p75
TRAF6
?
sphingomyelinase
PI3K/Akt pathway
ras-erk pathway
sphingomyelin
ceramide
JNK
NF-κB
survival, axonal regeneration
differentiation (ChAT)

ation of chronically axotomized motoneurons compared to vehicle controls. We propose that there is a dose-dependent interaction between exogenous BDNF and its receptors, such that the bimodal facilitatory and inhibitory effects of low and high dose exogenous BDNF are attributed to binding to trkB and p75 receptors, respectively. This hypothesis is supported by several lines of evidence. For instance, the inhibitory effects of high dose exogenous BDNF can be blocked by preventing BDNF binding to p75 receptors with a function-blocking antibody directed towards the extracellular domain of the p75 receptor *(144)*. Furthermore, using the same quantitative method of evaluating motor axonal regeneration, we have shown that there is increased motor axonal regeneration in homozygous p75 knockout mice, but reduced axonal regeneration in heterozygous trkB knockout mice, especially at long periods after nerve repair *(144).* Recent work is also consistent with this hypothesis. GDNF which does not bind p75 receptors, only has facilitatory effects on motor axonal regeneration, and these effects are not dose-dependent (*see* "GDNF Family Members" below *309*). Also, analogs of ceramide, a lipid second messenger downstream of p75 activation (*see* "p75 Signaling in Response to Neurotrophin Stimulation" above), mimics the inhibitory effects of high dose exogenous BDNF on motor axonal regeneration after immediate nerve repair *(310).* Thus motor axonal regeneration appears to depend on a delicate balance between trkB and p75 activation.

The concept of a trk-p75 rheostat is not a novel idea. It has been known for some time that sensory and sympathetic neuronal survival and neurite outgrowth depend on a balance between trkA and p75 activation in vitro and in vivo (*see* "p75 trk Interaction" above). Recent evidence suggests that motoneuronal survival after injury is also regulated by an analogous trkB-p75 equilibrium *(143,269),* and our data suggest that motor axonal regeneration is regulated in the same manner.

Mechanisms of BDNF's Biphasic Regulation of Motor Axonal Regeneration

What are the downstream mechanisms involved in mediating these facilitatory and inhibitory effects on motor axonal regeneration? A proposed mechanism for BDNF's biphasic regulation of motor axonal regeneration is shown in Figure 4. During the early phases of motor axonal regeneration, motor axon growth cones are exposed to a large number of neurotrophic molecules (*see* Figs. 2 and 3), including BDNF, while regenerating through the permissive environment of the distal nerve stump (Fig. 4A). It is possible that BDNF binding to trkB receptors on motor axonal growth cones promotes motor axonal regeneration through the PI3K-Akt pathway. This is consistent with our results demonstrating the critical dependence of motor axonal regeneration on trkB receptors *(144).* These trkB receptors have been shown to activate both the PI3K pathway and the ras-erk pathway in vitro (*see* above *117,118*). Furthermore, activation of the PI3K-Akt pathway has been demonstrated to be important in neonatal motoneuronal survival, as well as motor axonal regeneration in the adult *(311).* The ras-erk pathway is also activated in response to BDNF binding to trkB *(117,118).* In contrast to the PI3K-Akt pathway, less is known about the role the ras-erk pathway mediates in response to BDNF, as it does not appear to be necessary nor sufficient for motoneuronal survival *(117,118,311).* However, as described above, the ras-erk pathway is important in neuronal differentiation in vitro. Thus the possibility exists that activation of the ras-erk pathway is responsible for the regulation of ChAT activity in motoneurons (i.e., cholinergic differentiation), since the upregulation of ChAT activity is one of the earliest signs of differentiation of neurons into the motoneuronal phenotype during early development. Furthermore, ChAT activity can be induced in embryonic motoneurons in vitro by neurotrophic factors such as BDNF, GDNF, and ciliary neurotrophic factor (CNTF), which all activate the ras-erk pathway (*200; see* Fig. 1). This model suggests that motor

axonal regeneration and ChAT activity are regulated by distinct mechanisms. Differential regulation of ChAT and axonal regeneration is consistent with reports of a BDNF dose-dependent increase in ChAT immunoreactivity in motoneurons following ventral root avulsion *(289)* across the same range of doses that we have demonstrated a biphasic dose-dependent facilitation and inhibition of motor axonal regeneration *(301).*

During periods of muscle reinnervation the environment surrounding regenerating growth cones changes dramatically (Fig. 4B). Regenerating motor axional growth cones come in contact with denervated muscle fibres which express and secrete distinct molecules from the Schwann cells of the distal nerve stump. Specifically, large amounts of neurotrophins, including NGF, NT-3, and NT-4/5 are secreted by denervated muscle *(312),* which may shift the balance between primarily a trkB-mediated positive growth signal to a p75-growth inhibitory signal (Fig. 4B). It is important to note however, that NGF is also upregulated in the distal nerve stump during the first week of regeneration (*232; see* Fig. 2). In light of experiments which demonstrate the importance of NGF in Schwann cell migration *(241),* this transient upregulation of NGF may serve to promote Schwann cell organization into the linear Bands of Bungner which guide regenerating axons through the injury site and into the distal nerve stump (reviewed in ref. *24*). Additionally, this transient upregulation of NGF may correspond to the initial slow rate of regeneration after injury, as peak regeneration rates do not occur until 3–5 d postinjury *(313).* In other words, the early expression of NGF may bind to p75 receptors on regenerating motor axons and possibly mediate a slight inhibitory effect on axonal regeneration, accounting for the delayed regeneration across the injury site during the first week after injury.

Although we have provided strong evidence that the p75 receptors serve to inhibit motor axonal regeneration *(144,301),* the exact downstream mechanisms are unclear. Based on preliminary evidence presented in abstract form *(310),* ceramide is a key inhibitory signal, at least for motor axonal regeneration following immediate nerve repair. There are several possible mechanisms to explain the inhibitory actions of ceramide. Ceramide may inhibit the trkB receptor activation, as demonstrated for trkA receptors in PC12 cells *(126).* Ceramide has also been demonstrated to dephosphorylate Akt, thus inactivating the PI3K-Akt pathway *(314–316).* The latter possibility would provide a neurotrophic factor-dependent model by which neurotrophins can inhibit axonal regeneration via p75 without affecting motoneuronal regulation of ChAT activity which is maintained via the ras-erk pathway (Fig. 4B). Distinct pathways for regulation of motor axonal regeneration and motoneuronal expression of ChAT are consistent with the fact that doses of BDNF used to upregulate ChAT in axotomized motoneurons *(215,252)* potently inhibit motor axonal regeneration *(301).*

Regardless of the downstream mechanisms, it is clear from the data from our laboratory *(144,301),* and others *(143,148,153)* that the p75 receptor mediates an inhibitory signal for axonal growth and regeneration. The possible functional consequence of this inhibitory signal is that NGF and other neurotrophins derived from skeletal muscle may bind to p75 receptors on regenerating motor axons, and together with other muscle derived signals (e.g., s-laminin) serve as stop signals and initiate the formation of the neuromuscular junction (reviewed in Sanes and Lichtman, 1999). The models presented in Fig. 4 which describe differential regulation of motor axonal regeneration and muscle reinnervation provide a framework upon which we can critically examine the mechanisms involved in the neurotrophin regulation of motor axonal regeneration and functional recovery.

GDNF Family Members

Overexpression of GDNF in developing muscle by coupling the GDNF gene to the muscle-specific promoter myogenin causes hyperinnervation of motor endplates during development *(318).* This hyperinnervation is

associated with motor axonal sprouting and enlargement of motor units, and not increased motoneuronal survival. Thus it is suggested that GDNF may facilitate synapse formation, or act as a "synaptotrophin," for developing neuromuscular junctions *(318).* This effect is specific for GDNF, as overexpression of NT-3 or NT-4/5 did not cause hyperinnervation. In a subsequent series of experiements, Keller-Peck et al. *(319)* demonstrated that subcutaneous administration of exogenous GDNF during the first week of postnatal life significantly increased motor axon branching and muscle hyperinnervation. If GDNF treatment continued into adulthood (approx 6 wk), there was no additional change in the number of hyperinnervated muscle fibers, but the authors observed retraction bulbs at motor axon terminals *(319).* Thus, GDNF not only promotes the formation of neuromuscular junctions, but may also induce continued plasticity and/or remodelling of muscle innervation.

Following nerve crush injury, adenoviral gene transefection of axotomized neonatal motoneurons with GDNF increases the numbers of myelinated axons in the facial nerve, and this increased number of myelinated axons was associated with significantly improved whisker function compared to nontransfected controls *(272,273).* We have recently examined the effects of exogenous GDNF on the number of motoneurons which regenerate their axons following immediate and delayed nerve repair after chronic axotomy *(309).* As observed for BDNF *(301),* exogenous GDNF did not promote axonal regeneration after immediate nerve repair at any of the doses tested. However, in sharp contrast to the strong dose-dependent effects of BDNF (*301; see* above), exogenous GDNF showed only facilitatory effects on promoting the axonal regeneration of chronically axotomized motoneurons. A role for other GDNF family members in mediating axonal regeneration after injury remains to be determined.

Neuropoeitic Cytokines

In line with the potent survival effects of CNTF on axotomized motoneurons, there are several lines of evidence which suggest that CNTF and other neuropoetic cytokines, either alone or in combination with other neurotrophic factors, play important roles in mediating motor axonal sprouting and functional recovery after peripheral nerve injury.

Based on the ability of exogenous CNTF to induce motor axonal sprouting CNTF at motor endplates *(320),* Siegel et al. *(321)* investigated the role of CNTF in axonal sprouting following partial denervation. Partial denervation normally induces a situation in which intact motor axons sprout axon collaterals to innervate denervated motor endplates *(322).* Sprouting does not occur in CNTF homozygous knockout mice following partial denervation, however, this sprouting can occur if exogneous CNTF is applied to the denervated muscle of CNTF knockout mice *(321).*

Exogenous CNTF and BDNF *(295),* or CNTF and BDNF covalently linked to collagen tubules *(323),* have been shown to slightly improve sciatic functional recovery after transection and repair as measured using walking track analysis. However, this modest improvement was not associated with increases in axonal conduction velocity *(323)* or axon diameter *(295,323).*

IL-6 and IL-6R have been shown to be important in motor axonal regeneration *(194).* Intraperitoneal injection of an antibody which prevents IL-6 binding to IL-6R, and thus restricts retrograde transport of IL-6 to the injured motoneurons, significantly reduces the number of hypoglossal motoneurons which regenerate their axons. In the same study, transgenic mice overexpressing IL-6, IL-6R, or both, showed accelerated motor axonal regeneration compared to nontransgenic controls.

The role of neuropoetic cytokines in peripheral nerve regeneration has also been evaluated using knockout mice for CNTF *(324)* and IL-6 *(325,326).* IL-6 knockout mice showed delayed regeneration after nerve crush injury

compared to wild-type controls *(325,326),* but recovered to the same extent at longer periods of regeneration *(326).* IL-6 knockout mice also demonstrated deficits in sensory, but not motor, compound action potentials after sciatic nerve crush *(325).* In contrast to the IL-6 knockout mice which eventually demonstrated functional recovery after nerve crush injury *(326),* CNTF knockout mice did not show this recovery after nerve crush even after long periods of regeneration *(324).* There was no difference in recovery between IL-6 knockout mice and controls, or CNTF knockout mice and controls, after nerve transection and repair *(324,326).*

In summary, these studies support a role for neuropoetic cytokines, such as IL-6 and CNTF, in recovery of denervated muscles after nerve injury, but the exact nature of this role is unclear. By relying primarily on functional analysis, it is unknown whether neuropoetic cytokines are important in increasing the number of motoneurons which regenerate their axons, increasing the rate of motor axonal regeneration, or increasing either regenerative or terminal sprouting of motor axons. Further investigation is required to provide clear and well-delineated mechanisms by which neuropoeitic cytokines mediate their effects on motoneurons during axonal regeneration and muscle reinnervation.

Conclusions

Despite the clear evidence that neurotrophic factors promote motoneuronal survival after injury in vivo, and their well-delineated intracellular pathways in vitro, clear quantitative evidence which describes their role in motor axonal regeneration is only beginning to emerge. Evidence presented from our laboratory and others suggests that successful axonal regeneration and functional recovery depend on a delicate balance between both positive and negative growth signals. Future experiments which are designed to translate our knowledge of neurotrophic factor signal transduction in vitro to in vivo models of nerve injury and repair, may allow for the design of more specific therapeutic interventions for the treatment of the poor functional recovery which often follows peripheral nerve injuries.

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