Neurotrophin Regulation of Gene Expression

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ABSTRACT: The neurotrophins comprise a family of secreted proteins that elicit profound responses in cells of the developing and mature vertebrate nervous system including the regulation of neuronal survival and differentiation. The molecular mechanisms by which the neurotrophins exert their effects have been the subject of intense investigation. The neurotrophins elicit responses in neurons via members of the Trk family of receptors and the p75 neurotrophin receptor. Once activated, neurotrophin receptors trigger a large number of biochemical events that propagate the neurotrophin signal from the plasma membrane to the interior of the cell. An important target of the neurotrophin-induced signaling pathways is the nucleus, where neurotrophin-induced signals are coupled to alterations in gene expression. These neurotrophin-induced changes in gene expression are critical for many of the phenotypic effects of neurotrophins including the regulation of neuronal survival and differentiation.

RESUME: Regulation de l'expression génique par les neurotrophines. Les neurotrophines incluent une famille de protéines sécrétées qui provoquent des réponses intenses au niveau des cellules du système nerveux en développement et adulte chez les vertébrés, incluant la régulation de la survie et la différenciation neuronale. Les mécanismes moléculaires par lesquels les neurotrophines exercent leurs effets ont été le sujet d'investigations poussées. Les neurotrophines provoquent des réponses neuronales via des récepteurs qui sont membres de la famille Trk et le récepteur de la neurotrophine p75. Une fois activés, les récepteurs de la neurotrophine déclenchent plusieurs réactions biochimiques qui propagent le signal de la neurotrophine de la membrane plasmatique à l'intérieur de la cellule. Le noyau est une cible importante des voies de signalisation sensibles à la neurotrophine où les signaux induits par les neurotrophines sont couplés à des changements dans l'expression génique. Ces changements dans l'expression génique induits par les neurotrophines sont critiques pour plusieurs effets phénotypiques des neurotrophines, dont la régulation de la survie et de la différenciation neuronale.


The discovery of nerve growth factor over forty years ago and the extensive characterization of its trophic effects in the peripheral nervous system \(^1\) stimulated research into the identification of novel proteins that promote the survival of distinct populations of neurons including those that reside in the central nervous system. Over the past decade, four other neurotrophins have been characterized and cloned. These consist of brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), NT-4/5, and NT-6. Recent studies have revealed that in addition to their trophic effects, the neurotrophins elicit a wide variety of responses in the nervous system including the regulation of the proliferation and differentiation of neuronal precursor cells in the developing nervous system.

BIOLOGICAL ACTIVITIES OF THE NEUROTROPHINS

Current concepts of neurotrophin function stem principally from four types of experimental approaches. Studies of the expression pattern of the neurotrophins and their receptors have provided important clues regarding the site and nature of neurotrophin function. Extensive work characterizing the biological activities of the neurotrophins in primary neuronal cultures and the use of neutralizing antibodies in vivo have enhanced our understanding of the physiologic role of the neurotrophins. Finally, gene targeting of each neurotrophin and its cognate receptor has confirmed some of the functions of the neurotrophins that were predicted by the other three experimental approaches but has cast doubt on other functions. In addition to their functions during the development of the nervous system, the neurotrophins can promote the recovery of neurons in the adult nervous system following injury.\(^2\)\(^3\) These observations have generated interest in the therapeutic potential of the neurotrophins in degenerative disorders of the nervous system. An overview of the biological activities of the neurotrophins, which have been reviewed extensively,\(^4\)\(^6\) will be presented here.

Peripheral Nervous System

The biological activities of the neurotrophins have been examined most extensively in studies of the sensory and sympathetic neurons of the avian and rodent peripheral nervous system...
systems (PNS). These studies have revealed two principles of neurotrophin biology in the PNS. The development of each specific population of neurons appears to undergo sequential dependence upon distinct neurotrophins, and the various neurotrophins support the survival of distinct but overlapping populations of neurons of the PNS.

NT-3 appears to promote the proliferation of neural crest cells and of sensory and sympathetic neuroblasts as they come to reside in their respective ganglia. It has been suggested that NT-3 enhances the proliferation of these neuroblasts either by directly increasing their rate of proliferation or by promoting their survival. Following the early development of the sensory and sympathetic neurons in the PNS, these neurons undergo a subsequent period of target-dependent neuronal survival that depends on limiting amounts of NGF produced by the target tissue of these neurons. In early experiments carried out by Levi-Montalcini and Cohen, greater than 90% of the sympathetic neurons were found to disappear in mice injected at birth with an NGF-neutralizing antibody. The results of these experiments were recently confirmed in mice in which the NGF gene or the gene encoding the NGF receptor TrkA was disrupted by gene targeting. Mice that are NGF-/- or trkA-/- die within the first month of life. Their sympathetic ganglia are essentially absent by 10 days of life. In addition, they lack the small-sized sensory neurons within the dorsal root and trigeminal ganglia that are believed to respond to pain and temperature stimuli.

While NGF supports the survival of the small sensory neurons, in vitro and in vivo evidence indicates that NT-3 promotes the survival of the large sensory neurons of the dorsal root ganglia during the period of target innervation. The phenotype of mice with targeted mutation of the NT-3 or the gene encoding the NT-3 receptor, TrkC, is the neurological movement disorder of "pseudoathetosis" that reflects an impaired sense of position. A remarkable function of BDNF, as revealed in mice in which the BDNF gene was targeted, is to promote the survival of vestibular sensory neurons. BDNF and TrkB are also required for the survival of a group of visceral sensory neurons in the nodose and petrosal ganglia that innervate the carotid body and for the survival of dorsal root ganglia neurons.

NT-4 displays many of the same activities in vitro as BDNF. This is consistent with the idea that TrkB mediates the biological effects of both neurotrophins. However, examination of the NT-4-/- mice suggests that BDNF and NT-4 have distinct physiological roles. The NT-4-/- mice do not display the abnormalities that are found in the BDNF-/- or trkB-/- mice, except that they have reduced numbers of visceral sensory neurons within the nodose and petrosal ganglia.

Central Nervous System

The role that the neurotrophins play in the development of the mammalian CNS has been more difficult to ascertain. This has been attributed to the complexity of the innervation pattern of the various neuronal populations within the CNS, the presence of multiple neurotrophins and their receptors within the same region of the CNS, and the possibility that CNS neurons require additional factors for their development. The CNS neuronal populations that have been examined in detail with regard to their neurotrophin requirement have generally been those that degenerate in human diseases.

When injected into rodent brains, NGF is capable of preventing death of cholinergic neurons of the basal forebrain following their axotomy in the septohippocampal projection. Injected NGF can also improve the behavioral performance of aging rats, a finding that is correlated with the ability of NGF to reduce the atrophy of the septal cholinergic neurons in these rats. These data raised the possibility that NGF may control the survival of these neurons during the development of the mammalian brain. However, results from the NGF-/- and trkA-/- mice reveal that this population is normal in size.

Recent studies suggest that the neurotrophin NT-3 may promote the differentiation of rat embryonic cerebral cortical or hippocampal precursor cells into neurons. Although in conventional histologic examination the cerebral cortex and hippocampus in mice with targeted mutations of the NT-3 and trkC genes appear to be normal, a detailed examination of this area of the CNS remains to be carried out. BDNF also appears to regulate the differentiation of cerebral cortical neurons. Examination of the cerebral cortex in BDNF-/- mice has revealed a reduction in the expression of neupeptide Y (NP-Y) and the calcium binding protein parvalbumin, which are normally found in distinct subsets of cortical interneurons that also express the neurotransmitter y-aminobutyric acid (GABA). As the number of GABAergic neurons appears to be normal in these mice, these results suggest that BDNF regulates the phenotype of these neurons.

Prior to the gene knockout experiments, in vitro and in vivo experiments suggested that the neurotrophins BDNF, NT-3 and NT-4 play a role in the regulation of motor neuron survival during CNS development. Although initial examination of the trkB-/- and trkC-/- mice was consistent with this conclusion, reexamination of these animals and analysis of mice deficient in both BDNF and NT-4 has apparently failed to reveal such abnormalities, leaving the quest open for the motor neuron neurotrophic factor. Although the known neurotrophins may not be the physiological motor neuron trophic factor, their ability to reduce motor neuron loss in animal models of motor neuron degeneration has raised interest in the possibility that these agents may be used in the treatment of motor neuron diseases such as amyotrophic lateral sclerosis.

Molecular Mechanisms of Neurotrophin Action

The diversity of the biological effects that a given neurotrophic factor exerts on neurons raises questions about the nature of the intracellular mechanisms that transduce the neurotrophic factor signal and that ultimately lead to the multiple phenotypic effects of the neurotrophic factor. Neurotrophin receptor proteins have been identified on responsive neurons, and biochemical events that occur subsequent to receptor activation have been characterized. One set of biochemical changes that is believed to be critical for many of the neuronal responses to these agents consists of alterations in gene expression. Understanding the nature of the pathways by which the neurotrophic factor signal is transduced from its receptor to the nucleus may therefore provide insight into how the neurotrophins trigger specific cellular responses.

Early investigations into the binding properties of NGF on responsive peripheral neurons revealed that these cells express two receptor populations. NGF bound to some sites with high affinity ($K_d = 1x10^{-14}$) and slow dissociation rates and to other sites with low affinity ($K_d = 1x10^{-8}$) and fast dissociation rates.
Nearly a decade later, an NGF receptor of about 75 kDa that is currently termed p75 was molecularly cloned. At the time that p75 was cloned, this receptor defined a new family of proteins that is now known to include proteins, such as CD40, Fas and the tumor necrosis factor α (TNF-α) receptors, that are predominantly expressed by immune cells. In addition to NGF, other neurotrophins can also bind to p75 with similar affinity kinetics as NGF.

More recently, the trk proto-oncogene has been shown to encode a second receptor for NGF. Other proteins with structural similarity to Trk (currently referred to as TrkA) such as TrkB and TrkC have been characterized. The Trk proteins bind to the neurotrophins with relatively high affinity and overlapping specificity, such that TrkA binds to NGF, TrkB binds to BDNF and NT-4, and TrkC binds to NT-3. The three Trk receptors display extensive structural similarities with 66% identity in the primary amino acid sequence. They all have three immunoglobulin-like domains and a cysteine-rich domain in their extracellular regions. Within the intracellular region, the Trks all contain a tyrosine kinase domain which places these proteins in the superfamily of receptor tyrosine kinases (RTKs) that includes receptors for growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF).

Current understanding of the mechanisms by which neurotrophins elicit responses in neurons has come in large part from studies of NGF action on the pheochromocytoma cell line PC12. Upon exposure to NGF, PC12 cells differentiate into sympathetic neuron-like cells. The NGF-induced differentiation of PC12 cells results from the interaction of NGF with its receptor, TrkA, and from the intracellular biochemical events that occur subsequent to receptor activation. The PC12 cell system also serves to highlight the contrast in the biological outcomes that can result from the activation of different RTKs. Whereas NGF triggers the differentiation of PC12 cells into sympathetic neuron-like cells, the growth factor EGF, whose receptor also contains intrinsic tyrosine kinase activity, promotes the proliferation of PC12 cells. It is likely that the differences between the NGF- and EGF-induced signaling pathways become apparent in the nucleus, since NGF-induced changes in gene expression are critical for the differentiation response of PC12 cells.

**Trk Receptor Activation**

The TrkA receptor appears to be necessary and sufficient to mediate the biological effects of NGF. PC12 cells that lack functional TrkA (nnr5 cells) do not undergo a differentiation response when exposed to NGF. However, TrkA when expressed in nnr5 cells restores NGF responsiveness to these cells. In addition, the similar phenotype of trkA-/- and NGF-/- mice suggests that TrkA is likely to be the functional receptor for NGF in vivo.

The first step in Trk receptor activation is its dimerization, which is effected by the binding of its cognate neurotrophin (Figure 1). The solved structure of the NGF crystal together with the identification of amino acids in the NGF molecule that are required for efficient and specific binding to TrkA have led to a model of NGF-TrkA interaction. Within the NGF dimer, each NGF protomer associates with the other along its long axis. Therefore, the NGF dimer exhibits two-fold symmetry. The amino acids that contribute to TrkA binding appear to be grouped along one side of the NGF dimer and to be derived from both protomers. The binding region of NGF is parallel to the axis of symmetry of the NGF dimer. Therefore, each NGF dimer contains two identical TrkA binding regions that are on opposite sides of the dimer. This may promote the binding of an NGF dimer to two TrkA molecules, thereby effecting receptor dimerization and activation.

The activation of the TrkA receptor protein is manifest in the induction of its kinase activity resulting in TrkA autophosphorylation on tyrosine residues. It appears that dimerization of the receptor triggers the kinase within each TrkA molecule to transphosphorylate tyrosine residues on the other TrkA molecule within the receptor dimer.

Once the TrkA receptor becomes autophosphorylated on tyrosine residues, the phosphorylated tyrosines act as docking sites for intracellular signaling proteins. A characteristic of these signaling proteins is that they contain modular domains that are capable of interacting with proteins that contain a phosphorylated tyrosine. Two types of phosphotyrosines interacting protein modules have been defined, the Src homology 2 module (SH2) and the protein tyrosine binding domain (PTB). The SH2 domain is about one hundred amino acids in length and is found in a variety of signaling proteins that include protein and lipid kinases, phospholipases, phosphatases, and adaptor proteins that lack enzymatic activity. Different SH2 domains have been found to bind to distinct phosphotyrosine peptides. The specificity of these interactions appears to be determined by the sequence of the amino acids that are found immediately C-terminal to the phosphotyrosine residue.

Phosphorylated TrkA can interact with the proteins phospholipase Cγ (PLCγ) and the regulatory (p85) subunit of the phosphatidylinositol 3 kinase (PI-3K) via their SH2 domains. The adaptor protein Shc appears to interact with phosphorylated TrkA via its PTB domain. Once these proteins bind to the TrkA receptor, they become activated either by phosphorylation or due to a conformational change. Once activated the TrkA-associated proteins transmit signals to effector proteins and thus propagate the NGF signal. What follows these initial signaling events is an array of partly parallel and partly interconnected signaling pathways that we are now beginning to understand in some detail.
of Shc and PLC\(\gamma\) to TrkA appears to be critical for NGF activation of Ras.\(^{42,46}\)

Once Shc associates with TrkA via phosphorylated Tyrosine 490 of TrkA, Shc is believed to become a substrate for the TrkA tyrosine kinase. Thus, NGF induces the tyrosine phosphorylation of Shc. That the Shc protein is important in mediating the biological activities of NGF is suggested by the finding that overexpressed Shc protein promotes neurite outgrowth in PC12 cells in the absence of NGF treatment.\(^{49}\) The phosphorylation of Shc on tyrosine is critical in this process and is believed to promote neurite outgrowth.\(^{55}\)

MAPK translocates to the nucleus and catalyzes the phosphorylation of Elk at sites that enhance the ability of Elk to activate transcription. Activated MAPK also stimulates the activity of p70 and p90, which leads to the induction of c-fos, c-jun, and other cAMP-responsive genes.\(^{56}\)

**NGF-induced Changes in Gene Expression**

A target of the NGF-induced signaling pathways that are activated downstream of TrkA is the nucleus. NGF-induced changes in gene expression appear to be critical for many of its biological activities including neurite outgrowth in PC12 cells.\(^{60}\) It has long been expected that the investigation of the mechanism by which NGF-induced changes in gene expression will help our understanding of how the specificity of the NGF signal is maintained as it propagated from the plasma membrane across the cytoplasm. In this regard, it is particularly important...
to compare the effects of TrkA activation on gene expression with those of another RTK, the mitogenic EGF receptor. EGF receptor activation in PC12 cells triggers many of the same signaling events as TrkA, including the activation of the Ras-MAP kinase pathway, but leads to a mitogenic cellular response rather than a differentiation response.

Immediate Early Genes and Late Response Genes

NGF is known to induce the expression of a large number of genes in PC12 cells. These genes have been classified in two major groups, termed the immediate early genes (IEGs) and the late response genes (LRGs). The induction of IEG expression occurs within minutes of NGF treatment. Many of these IEGs encode transcription factors that include the Fos, Jun, Zif268 (or NGFIA), and Nur77 (or NGFIB) families of proteins. The products of these NGF-induced IEGs bind to promoter sequences within LRGs and consequently regulate LRG expression. The induction of LRG expression occurs typically within hours of NGF treatment.

A variety of NGF-induced LRGs have been identified in PC12 cells. NGF-induced LRGs encode proteins that contribute to the neuronal properties PC12 cells acquire upon exposure to NGF including the extension of neurites, neurotransmitter synthesis, and the ability to generate Na+-based action potentials. For example, the induction of the protease transin and the intermediate filament protein peripherin might play a role in neurite outgrowth. The tyrosine hydroxylase gene encoding the rate limiting enzyme in noradrenergic transmitter biosynthesis is also induced with delayed kinetics by NGF. NGF also induces the expression of the peripheral sodium channel proteins PNI and PNII, which endow PC12 cells with the ability to respond to depolarizing stimuli with the generation of action potentials.

Although a common set of IEGs appears to be activated by NGF and EGF, the induction of some LRGs appears to be ligand-specific. Two of these proteins, the serum response factor (SRF) and p62 ternary complex factor (p62TCF), have been studied extensively. The SRF protein binds as a dimer to the consensus sequence CC(A/T)GG that is found within the inner core of the SRE. The SRF binding site has been shown to be critical for the ability of the SRE to mediate c-fos transcription in cells exposed to serum, growth factors and NGF.

The c-fos Proto-oncogene

The best characterized IEG encodes the transcription factor Fos. The Fos protein is a member of the superfamily of transcription factors that contain the basic leucine zipper (bZIP) motif. The basic region of the protein mediates binding to DNA; the leucine zipper mediates dimerization with other proteins. A heterodimer containing Fos and another leucine zipper containing transcription factor Jun, binds to the consensus sequence TGGAGTC that is found within 5' regulatory regions of many genes including the LRGs. Whether the Fos protein contributes to the biological activities of NGF and other neurotrophins in the developing or mature nervous system is unknown. The c-fos gene has been disrupted in mice by gene targeting. However, a detailed examination of the nervous system with a view toward the actions of the neurotrophins remains to be carried out.

A wide variety of extracellular stimuli have been shown to induce the expression of the c-fos gene in neuronal and nonneuronal cells. Consequently, the regulation of c-fos expression has been studied in some detail and has provided a useful model for investigating the mechanisms by which a particular signal, such as the NGF signal, is coupled to changes in gene expression. Although extracellular ligand regulation of c-fos expression could occur at several levels from transcriptional initiation to the regulation of Fos protein activity, the regulation of transcriptional activation has been studied in greatest detail. NGF induces the expression of the c-fos gene at the transcriptional level in a rapid, robust and transient manner. Importantly, it appears that NGF induction of c-fos transcription occurs in the presence of protein synthesis inhibitors suggesting that this induction occurs independently of new protein synthesis.

The implication of this finding is that a preexisting cellular machinery mediates the NGF-induced expression of c-fos and that this might involve posttranslational modifications of proteins that bind to the 5' regulatory sequences of the c-fos gene.

The c-fos promoter has been dissected in many studies over the past decade and a number of major regulatory sequences have been characterized. These include the serum response element (SRE), the calcium response element (CaRE), the FosAPI site (FAP) and the sis-inducible element (SIE) that are found respectively at -300, -60, -290, and -340 nucleotides upstream of the c-fos transcriptional start site. The CaRE is also known as a cyclic AMP response element (CRE).

The Role of SRE Binding Proteins in Mediating NGF-induced Transcription

The SRE was first identified as a promoter element that is critical for serum and growth factor induction of c-fos transcription in fibroblast cell lines. Deletion analysis of the c-fos promoter revealed that the SRE is also required for NGF induction of c-fos transcription. The SRE is a 20 bp sequence with dyad symmetry that has been shown to bind to many proteins. Two of these proteins, the serum response factor (SRF) and p62 ternary complex factor (p62TCF), have been studied extensively. The SRF protein binds as a dimer to the consensus sequence CC(A/T)GG that is found within the inner core of the SRE. The SRF binding site has been shown to be critical for the ability of the SRE to mediate c-fos transcription in cells exposed to serum, growth factors and NGF.

The mechanism by which SRF activates transcription has been studied most extensively in serum stimulated fibroblast cell lines. SRF has been shown to undergo a rapid phosphorylation event in response to serum that appears to enhance the ability of SRF to bind to the SRE in vitro. However, an in vivo role for this phosphorylation event remains to be determined. Interestingly, one of the kinases that may potentially mediate this event in vivo is pp90RSK, which would make SRF a direct target of the Ras-MAP kinase pathway. Although this might be the case for serum in fibroblast cells, it appears that SRF does not undergo the Serine 103 phosphorylation in PC12 cells upon exposure to the purified polypeptide factors NGF and EGF.

In addition to activating transcription directly, it is now widely accepted that a primary function of SRF is to recruit other transcription factors to the SRE that in turn activate transcription. p62TCF is a protein that is recruited to the SRE by SRF and binds to the sequence CAGGAT within the 5' region of the SRE. The p62TCF binding site is critical for growth factor and NGF induction of c-fos transcription. Several proteins have been cloned that exhibit the properties of p62TCF. These proteins include the SRF accessory factors SAP 1 and 2, and Elk.

Accumulating evidence suggests that p62TCF constitutes an important target of the Ras-MAP kinase pathway. Growth factor or NGF treatment of responsive cells triggers the phosphorylation of Elk, an event that is mediated by MAPK.
Although the phosphorylation of Elk occurs on multiple serines within the C-terminal domain of Elk, the phosphorylation of Serine 383 and 389 is particularly critical for the ability of Elk to mediate growth factor and NGF-induced transcription.\textsuperscript{82,86} The ability of Elk to bind to the CRE and form a ternary complex with SRF might be enhanced by the phosphorylation of Elk.\textsuperscript{83} However, the effect on DNA binding has not been reproduced in other studies.\textsuperscript{86} Elk phosphorylation stimulates the transactivation potential of Elk when it is bound to DNA via the DNA binding domain of a heterologous protein suggesting that the phosphorylation event affects the transactivation potential of Elk directly.

Several other proteins have been identified that bind to the SRE independently of SRF but their roles in mediating SRE-mediated transcription have not been fully characterized. Proteins that bind to the 3' region of the SRE do not appear to play a critical role in mediating NGF-induced transcription via the SRE.\textsuperscript{80} The role of other SRE-binding proteins in NGF-induced transcription remains to be explored. In addition to p62TCF, the transcription factor YY1 binds to the 5' region of the SRE.\textsuperscript{88-90} The SRF and YY1 binding sites overlap. Although initial evidence suggested that SRF and YY1 bind to the SRE antagonistically,\textsuperscript{91} a more recent study suggested that YY1 facilitates the binding of SRF to the SRE.\textsuperscript{92} Thus, YY1 might enhance SRE mediated transcription. The homeodomain protein Phox1 has been found to bind to the inner core of the SRE.\textsuperscript{93} The sequence requirements within the SRE for Phox1, however, are distinct but overlapping with those for SRF and comprise the AR rich inner core. Phox1 enhances the binding of SRF to the SRE \textit{in vitro}. This has been correlated with the ability of Phox1 to enhance SRF mediated transcription \textit{in vivo}.

**Cyclic AMP Response Element Binding Protein**

Although the SRE is essential for growth factor and NGF induction of \textit{c-fos} transcription, other sites within the \textit{c-fos} promoter contribute to the growth factor and NGF response.\textsuperscript{74,80,94} These sites include the CaRE, FAP and SIE promoter elements, each of which can function as a cyclic AMP response element (CRE) by activating transcription in response to stimuli that elevate the intracellular levels of cyclic AMP.\textsuperscript{74,95} The CaRE can also induce \textit{c-fos} transcription in response to stimuli, such as membrane depolarization, that elevate the intracellular levels of calcium.\textsuperscript{71}

The CREs mediate NGF-induced \textit{c-fos} transcription at least in part by their ability to bind the transcription factor cyclic AMP response element binding protein (CREB).\textsuperscript{80,94} Like Fos, CREB is a member of the bZIP superfamily of transcription factors.\textsuperscript{96,97} CREB was first identified as a transcription factor that mediates cyclic AMP-induced transcription. However, subsequent experiments revealed that CREB can also mediate calcium-induced transcription.\textsuperscript{98} Agents that stimulate a rise in the intracellular levels of cyclic AMP or calcium induce the phosphorylation of CREB at Serine 133. The inducible Serine 133 phosphorylation is critical for the ability of CREB to mediate NGF-induced \textit{c-fos} transcription.\textsuperscript{94} However, the NGF-induced CREB phosphorylation appears to be catalyzed by Rsk2, a member of the pp90\textsuperscript{RSK} family.\textsuperscript{100} NGF activation of Rsk2 occurs via the Ras-MAPK signaling pathway. Thus, distinct signaling pathways mediate CREB activation in response to NGF, growth factors, cyclic AMP and calcium signals.

Once phosphorylated in response to NGF, growth factors, calcium or cyclic AMP signals, CREB appears to mediate transcription via distinct mechanisms depending on the nature of the initial stimulus.\textsuperscript{99} In response to calcium or cyclic AMP signals, CREB can activate transcription independently of other promoter-bound transcription factors. By contrast, to confer NGF-induced transcription, CREB activates transcription via a mechanism that requires a cooperative interaction with other promoter-bound transcription factors, such as SRF, bound at the \textit{c-fos} promoter. The presence of CREs within the promoters of other NGF-induced IEGs suggests that CREB cooperativity with other transcription factors is a mechanism that might be generally employed by cells to couple the NGF signal to IEG transcription.

The mechanism by which the Serine 133 phosphorylation facilitates the ability of CREB to activate transcription appears to be an increase in the transactivation potential of CREB. CREB appears to mediate transcription via distinct mechanisms depending on the nature of the initial stimulus.\textsuperscript{99} In response to calcium or cyclic AMP signals, CREB can activate transcription independently of other promoter-bound transcription factors. By contrast, to confer NGF-induced transcription, CREB activates transcription via a mechanism that requires a cooperative interaction with other promoter-bound transcription factors, such as SRF, bound at the \textit{c-fos} promoter. The presence of CREs within the promoters of other NGF-induced IEGs suggests that CREB cooperativity with other transcription factors is a mechanism that might be generally employed by cells to couple the NGF signal to IEG transcription.

The Generation of Signal Specificity Within the Ras-MAP Kinase Pathway

The identification of the Ras-MAP kinase signaling pathway as a major route for the NGF signal to proceed from the plasma membrane to the nucleus raised the question of how the cell deciphers signals that arise from similarly structured RTKs that all activate the Ras-MAP kinase pathway. For example, in PC12 cells the Ras-MAP kinase pathway is activated in response to both NGF and EGF. However, the phenotypic responses of PC12 cells to these agents are quite distinct: NGF enhances the binding of CREB to the SRE \textit{in vivo}, whereas EGF promotes their continued proliferation.

It is now believed that the kinetics of the activation of the Ras-MAP kinase pathway may be important in determining the type of biological response that results.\textsuperscript{58} Specifically, in PC12 cells the rapid but transient activation of this signaling pathway in response to EGF contrasts with the rapid but prolonged kinetics of activation upon exposure to NGF. How this difference in the kinetics of the Ras-MAP kinase cascade is generated is unclear but it appears to arise at the level of receptor phosphorylation. Recent experiments suggest that CREB contributes to the readout of differences in the kinetics of activation of the Ras-MAP kinase signaling pathway (Figure 2). CREB transforms
the differences in the kinetics of the activation of the Ras-MAP kinase pathway into qualitative differences in gene expression owing to the requirement for cooperativity in its transcriptional response to growth factor and NGF signals. The presence of CREs within the promoters of NGF-responsive LRGs suggests that CREB contributes directly to the expression of NGF-induced LRGs. The prolonged activation of CREB in NGF treated cells might facilitate its interaction with the products of the IEGs at the promoter of NGF-responsive LRGs leading to their induction. Although EGF and NGF might induce identical IEGs, at later time points CREB is no longer activated in EGF treated cells leading to a failure of induction of NGF-specific LRGs. At earlier time points, the absence of IEG products results similarly in the failure of EGF activated CREB to stimulate transcription of the LRGs.

Recent results from a number of laboratories support the model wherein quantitative differences in the Ras-MAP kinase pathway account for qualitative differences in transcriptional responses to distinct agents. For example, increasing the amplitude of EGF receptor tyrosine kinase activity by overexpression of the EGF receptor in PC12 cells induces a differentiation response to EGF. This correlates with the sustained activation of MAP kinase in these cells in response to EGF. Still, more experiments are required to validate the model that is proposed in Figure 2. For example, it will be important to demonstrate that the prolonged activation of CREB in NGF treated cells facilitates its cooperation with products of the IEGs at promoters of the NGF-responsive LRGs.

**Ras-MAP Kinase Independent Mechanisms of NGF-activated Transcription**

Although our studies have focused on mechanisms of NGF-activated c-fos transcription that occur via the Ras-MAP kinase pathway, recent evidence suggests that other signaling pathways will also be significant in transducing the NGF signal to the nucleus.

**PI-3K Signaling**

As described earlier, the induction of TrkA autophosphorylation by NGF triggers the association of a number of proteins with the receptor. The association of two proteins, the adaptor protein Shc and the enzyme PLCγ, appears to be important for the activation of Ras. In contrast, despite evidence that PI-3K might be capable of activating the Ras protein, the association and activation of PI-3K does not appear to be critical for NGF activation of Ras. This raises the question of the role PI-3K activation in NGF signaling. Recent findings suggest that PI-3K activation might be particularly important for the survival promoting actions of NGF.

Although the association of the regulatory subunit of PI-3K, p85, with TrkA is debated, NGF activation of PI-3K activity has been a reproducible finding. Association of p85 with TrkA triggers the tyrosine phosphorylation of p85 but the mechanism by which this phosphorylation stimulates the activity of the PI-3K enzyme is unclear. It might induce a conformational alteration in the p110 catalytic subunit. Once activated, PI-3K induces the phosphorylation of phosphatidylinositol leading to the production of PI-3,4-P2. PI-3,4-P2 has been shown to stimulate the activity of a kinase that is the product of the proto-oncogene Akt. Recent results suggest that Akt mediates the survival promoting effects of PI3K activation. The physiological substrates of Akt that promote neuronal survival remain to be determined.

Another kinase that appears to be activated downstream of PI-3K is the kinase pp70S6K. Inhibition of PI-3K by the drug wortmannin inhibits the ability of growth factors to activate pp70S6K. Importantly, the activation of this kinase might be one way by which the PI-3K signal is transduced to the nucleus. In fibroblasts, serum can trigger the phosphorylation of the protein CREM, a CREB-related protein, at a site that is equivalent to CREB Serine 133 via the kinase pp70S6K. Although this kinase is not likely to mediate the NGF-induced phosphorylation of CREB Serine 133 in PC12 cells, it will be interesting to determine if pp70S6K stimulates CREB phosphorylation in other cells or in response to other neurotrophins and purified polypeptide growth factors such as PDGF.

![Figure 2: A model of how specificity of neurotrophin responses is generated by the Ras-MAPK signaling pathway.](https://www.cambridge.org/core/diagram.png)
NGF Activation of SNT

A mechanism by which specificity in signaling is imparted to the TrkA differentiation signal, as compared to RTK signals that are mitogenic, might result from the generation of a specific signal downstream of TrkA. In this regard, NGF but not EGF triggers the phosphorylation of a protein, termed SNT, that was identified based on its ability to associate with the protein p13sun1.\textsuperscript{113} SNT has been purified, and peptide sequences suggest that it is a novel protein. The tyrosine phosphorylation of SNT has been shown to occur in response to neurotrophins but not EGF. Interestingly, in PC12 cells basic FGF also induces the tyrosine phosphorylation of SNT which correlates with the ability of this agent to trigger a differentiation response in these cells.

The mechanism by which SNT becomes activated in response to NGF has been recently investigated. A sequence within the membrane proximal region of TrkA appears to be critical for the NGF activation of SNT.\textsuperscript{114} This region of TrkA contains the sequence KFG that is also found in other Trks but not in the EGF receptor. Importantly, deletion of this region within TrkA not only blocked the activation of SNT but also impaired the ability of NGF to induce a differentiation response. However, the ability of NGF to enhance the survival of PC12 cells was not affected. Moreover, NGF activation of the Ras-MAP kinase pathway was not affected by the KFG mutation of TrkA. Interestingly, the activation of c-fos transcription was reduced significantly, suggesting the presence of a Ras-MAP kinase independent mechanism that is critical in the activation of c-fos transcription in response to NGF. The transcription factors that couple the KFG-SNT signal to the activation of transcription remain to be determined. Another open question is the mechanism by which the membrane proximal region of TrkA activates SNT. It will also be important to determine how the FGF receptor triggers the tyrosine phosphorylation of SNT.

The Jun N-terminal Kinase Signaling Pathway

The proline-dependent kinase Jun N-terminal kinase (JNK) was identified based on its ability to phosphorylate Jun on Serines 63 and 73 upon exposure of cells to various stress inducers such as UV radiation.\textsuperscript{115} These phosphorylation events were shown to be important for the ability of Jun to activate transcription in a sequence specific manner. Other targets of JNK include the transcription factors ATF2 and Elk.\textsuperscript{116,117} JNK appears to be activated by a protein kinase cascade that resembles the Ras-MAP kinase cascade.\textsuperscript{118} Thus, current evidence suggests that the Ras-related GTPase proteins Rac and CDC42 activate the protein kinases MEKK 1 and 2. These kinases in turn activate the kinase MKK (or MEK) 3 and 4, which in turn activate the kinase JNK. Another kinase that is activated by MKK 3 is the p38 kinase. The magnitude of growth factor activation of components of these pathways appears to be much less than that in response to stress-inducing stimuli. Although evidence has been presented to suggest that NGF might activate this signaling pathway, recent work reveals that the withdrawal of NGF from cells that are dependent on NGF for survival also appears to activate the Jun kinase signaling pathway and that components of this pathway mediate the apoptosis that results from NGF withdrawal.\textsuperscript{119}

A question that remains to be addressed is the mechanism by which this pathway is activated upstream of the Ras-related pro-
neurotrophins, activates NFkB signaling in Schwann cells in a p75-dependent manner. The p75-activated NFkB signaling pathway does not lead to cell death and might therefore exert trophic or differentiative effects.129

PERSPECTIVES

The pace of research into the mechanisms by which neurotrophins mediate their effects has accelerated over the past few years. The intracellular biochemical pathways that propagate the neurotrophin signals from the plasma membrane to the nucleus in cell lines have thus been increasingly delineated. Much of our understanding about the mechanisms of neurotrophin action has come from studies of NGF-induced signaling. However, it is likely that as the mechanisms by which other neurotrophins mediate their effects are studied in greater detail differences between the intracellular signaling events that are activated by NGF and the other neurotrophins will become apparent. As these signaling pathways are characterized in greater detail, it will be important to determine the mechanisms by which specificity is conferred leading to a particular cellular response. It will also be important to determine the physiologic role of each neurotrophin-induced signaling pathway in the development of the nervous system. This will require the study of signaling pathways in vivo and the use of mice in which genes encoding the various signaling proteins have been disrupted.

In addition to learning about the role of neurotrophin signaling pathways in the normal development of the nervous system, characterization of the neurotrophin-activated signaling mechanisms should provide insights into the pathogenesis of degenerative disorders of the nervous system. Mutation of the CBP and rsk-2 genes have been found to be linked to two hereditary disorders, the Rubinstein-Taybi30 and the Coffin-Lowry131 syndromes respectively. Mental retardation and facial and digital dysmorphisms are important features of both of these disorders. It will be important to determine if disruption of neurotrophin-activated signaling contributes to the abnormalities in brain development that are manifest in these disorders. It will also be important to determine if inactivation of genes that encode other proteins that transduce neurotrophin signals to the nucleus underlie other diseases of the nervous system.

The elucidation of the intracellular mechanisms by which neurotrophins promote neuronal survival should also provide a basis for the potential development of novel strategies in the treatment of degenerative disorders of the nervous system. The therapeutic potential of the neurotrophins has been demonstrated in animal models of degenerative and metabolic disorders of the nervous system including models of motor neuron disease and polyneuropathies. However, the neurotrophins have only recently entered the clinical arena.1 Controlled clinical trials of the neurotrophins in diseases such as amyotrophic lateral sclerosis and diabetic neuropathy have revealed that the administration of the neurotrophins is often accompanied by adverse effects. To improve the therapeutic index of the neurotrophins, it will be important to gain further understanding of the pharmacokinetics and toxicity of the neurotrophins. In addition, an important goal of the investigations of the neurotrophin-activated signaling mechanisms that enhance neuronal survival is that increased understanding of these mechanisms will provide the rationale for the development of additional therapeutic agents that exhibit the trophic effects of the neurotrophins but that have few adverse effects.

GLOSSARY

Trk: A family of receptor proteins that contain intrinsic tyrosine kinase activity within their intracellular domain. The Trk family of proteins mediate many of the biological effects of the neurotrophins. Neurotrophin binding to a Trk protein induces the tyrosine kinase activity of the Trk protein leading to autophosphorylation of Trk on tyrosine residues.

SH2 domain: A phosphotyrosine-binding protein domain that is contained within many intracellular signaling proteins. The SH2 domain mediates the interaction of a particular signaling protein with a second protein via an interaction with a tyrosine-phosphorylated peptide motif within the second protein. The specificity of interaction is achieved by the type of SH2 domain and by the structure of the phosphopeptide motif.

PTB domain: A protein domain that is contained within intracellular signaling proteins and that mediates interactions with proteins containing a specific phosphotyrosine peptide motif.

She: An adaptor signaling protein that contains an SH2 domain and a PTB domain. Once TrkA becomes activated, She binds to TrkA via the PTB domain of She and phosphorylated Tyrosine 490 of TrkA. Once bound to TrkA, She becomes tyrosine phosphorylated.

Grb2: An adaptor signaling protein that contains an SH2 domain and two SH3 domains. Grb2 is constitutively associated with Sos. Tyrosine phosphorylation of She by an activated TrkA allows Grb2 to bind She and thus leads to the recruitment of a Grb2-Sos complex to the receptor.

Sos: A GDP/GTP exchange protein for the small GTP binding protein Ras.

PLCγ: A phospholipase that hydrolyzes phosphoinositides leading to the production of diacylglycerol and inositol phosphates such as inositol-1,4,5-trisphosphate (IP3).

PI-3K: A lipid kinase that catalyzes the phosphorylation of phosphatidylinositol leading to the production of PI-3,4,5-P3.

Ras: A small GTP-binding protein. GTP-bound Ras binds to and activates the protein kinase Raf.

Raf: A serine/threonine protein kinase. Once activated by Ras, it activates MAPKK or MEK.

MAPKK: A protein kinase with dual specificity. Once activated by Raf, it catalyzes the phosphorylation of MAPK on threonine and tyrosine.

MAPK: Mitogen-activated protein kinase is also referred to as extracellular activated kinase (ERK). Once activated, MAPK translocates to the nucleus where it catalyzes the phosphorylation of transcription factors.

Rsk2: A serine/threonine protein kinase that is activated by MAPK. One of the substrates of Rsk2 is the transcription factor CREB.

SRF: Serum response factor is a transcription factor that binds to the inner core of the serum response element (SRE), a DNA sequence that is located within the promoters of many immediate early genes such as the c-fos proto-oncogene.

Elk: A transcription factor that binds to the 5' region of the SRE only when SRF is bound to the SRE. Elk is a substrate for MAPK. Once phosphorylated by MAPK, Elk activates transcription.

CREB: Cyclic AMP response element binding protein is a transcription factor that activates transcription in response to growth factors, neurotrophins, and neurotransmitters.

CBP: CREB binding protein associates with Serine 133-phosphorylated CREB and recruits the RNA polymerase complex to the promoter of a gene leading to activation of transcription. CBP acts as a coactivator for other transcription factors in addition to CREB.

c-fos: The c-fos proto-oncogene is an immediate early gene whose...
protein product encodes a transcription factor. The Fos protein dimerizes with another transcription factor Jun to form the API transcription factor complex, which activates transcription of genes that contain API binding sites within their promoters. SNT: A tyrosine phosphorylated protein that is activated by NGF but not EGF. JNK: The Jun N-terminal kinase is a protein kinase that is activated by a variety of stress-inducing agents including UV irradiation and withdrawal of NGF. The c-Jun protein is a substrate of JNK.

p75: The low affinity neurotrophin receptor. The p75 protein is a member of a family of receptor proteins that includes the TNP receptors.

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REFERENCES


