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Neurotrophic Factors

Hans Thoenen and David Edgar

The development and maintenance of function of the nervous system results from the concerted interaction of a great variety of genetic and epigenetic regulatory mechanisms. Transplantation and ablation experiments performed during this century have demonstrated that the survival of developing vertebrate neurons can be determined by their fields of innervation (*1*). So far, however, only one trophic factor has been shown to be responsible for this epigenetic determination of neuronal survival: the protein nerve growth factor (NGF) was demonstrated to be required for the survival of developing peripheral sympathetic and sensory neurons by showing that neutralization of endogenous NGF by antibodies to NGF (anti-NGF) resulted in the death of these neurons (*2*). More recently, numerous tissue culture experiments have been used to show that NGF is only one of a number of molecules able to maintain the survival of embryonic neurons in vitro, implying that such molecules might also function as trophic factors to support neuronal survival in vivo (*3*). The central thesis of this article is that in order to prove that putative trophic factors (detected by experiments in vitro) do have a physiological role, it is necessary to purify them to produce specific antibodies to them. Accordingly, the consequences of neutralization of the endogenous molecules in vivo—and hence their physiological role—can then be established.

Nerve Growth Factor

The detection of large amounts of NGF in the submandibular gland of the male mouse some 30 years ago was a prerequisite for its purification, necessary for the production of anti-NGF to delineate the neurotrophic actions of NGF in vivo (*2*). In addition, determination of the amino acid sequence of mouse NGF more than a decade ago (*4*) provided the information necessary for its recent molecular cloning (*5*). This work has now led to the elucidation of the structure of the NGF precursor and its genomic organization. Thus, the major part of the precursor sequence has been shown to be on the amino terminal side of β-NGF (the active subunit of the NGF molecule), whereas the carboxyl terminal arginine is followed only by two amino acids. The region coding for β-NGF represents about one-third of the total precursor messenger RNA (mRNA). Analysis of the organization of mouse and human genomes has shown that the NGF gene is present as a single copy, and that all the information for the β-NGF sequence is located in a single exon. Moreover, the amino acid sequence homology of mouse and human NGF is more than 90 percent, as deduced from the genomic DNA sequence (*5*).

A sensitive two-site enzyme immunoassay allowing determination of the NGF present in effector organs has only recently been developed, allowing a major gap in the NGF story to be closed (*6*). These investigations demonstrated a correlation between the density of sympathetic innervation and the levels of NGF in the corresponding peripheral target tissues; experiments with tissue culture have shown that the local concentration of NGF determines the extent of ramification of sympathetic nerve fibers in vitro (*7*), implying that the levels of NGF in target tissues may be responsible for the density of sympathetic innervation. Tissue culture experiments have also shown that target tissues can synthesize NGF in vitro (*8*), and recent work with nucleic acid probes to quantify the mRNA for NGF demonstrates that levels of NGF are correlated with the amounts of its mRNA (*9*). Thus, the rate of synthesis of NGF in target tissues is probably determined by regulation of

Hans Thoenen and David Edgar are at the Department of Neurochemistry of the Max-Planck-Institute for Psychiatry, D-8033 Martinsried/Munich, Federal Republic of Germany.

production of its mRNA. It is not yet clear, however, which cells of the target tissues actually synthesize NGF.

Although we know more about NGF than any other neurotrophic factor, our information on the mechanism (or mechanisms) that regulate its synthesis and release is fragmentary. It seems that NGF synthesis in peripheral effector organs is not controlled by androgens, in contrast to its synthesis in the mouse submandibular gland (*6*). Furthermore, the release of NGF from the iris in organ cultures is not dependent on calcium influx, suggesting a constitutive release pathway (*8*). The marked increase in NGF after sympathetic or sensory denervation (or both) may be due to a loss of inhibition of NGF synthesis exerted by the innervating neurons or, alternatively, it may simply reflect the lack of removal of NGF by retrograde transport; ligation experiments have shown that endogenous NGF is transported retrogradely from the periphery to the corresponding neuronal cell bodies (*10*). The relatively high levels of NGF in sympathetic ganglia (*6*) do not, therefore, result from local synthesis [only very small amounts of NGF mRNA are detectable in the ganglia (*9*)], but from accumulation by retrograde axonal transport. Experiments where ^{125}I-labeled exogenous NGF has been retrogradely transported indicate that the NGF arriving at the cell body is intact, as shown by immunoprecipitation and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (*11*). Furthermore, the endogenous NGF arriving at the ganglionic neuronal cell bodies is biologically active (*6*).

There is compelling evidence that the receptor binding domain of the NGF molecule has remained highly conserved during evolution; the biological activities of mouse and bovine NGF are identical although they show little immunological cross-reactivity (*12*). Tryptophan residues, in particular residue 21, are essential for the biological activity of NGF (*13*). However, an unambiguous identification of the receptor binding domain has not yet been accomplished. A report that a peptide fragment consisting of residues 10 to 25 and 75 to 88, linked by a disulfide bridge (cysteines 15 and 80), is 100 times more active than native NGF (*14*) appears not to have been confirmed, and the corresponding synthetic fragment was reported to be inactive (*15*). Knowledge of the sequences of mouse and human NGF along with the sequences of NGF's from other sources should tell us which regions of the NGF molecule are most highly conserved. This information together with the possibility of producing such molecules by expression vectors and subjecting them to site-directed mutagenesis could lead to the resolution of the receptor binding domain of NGF.

The molecular mechanism of action of NGF on its target cells is still unresolved, although the kinetics of the interaction of NGF with its receptors have been established (*2*). The NGF receptors have been identified recently by affinity-labeling and partially purified by affinity chromatography (*16*). The binding of NGF induces a change in molecular weight of the receptor from 100,000 to 158,000 in PC12 cells. This change is accompanied by a decreased off rate for NGF, that is, a transformation from low- to high-affinity receptors (*17*); it also corresponds to a change in receptor extractability by Triton X-100 (*18*). Whether the detergent-resistant association of the high-affinity receptor with the cytoskeleton (*18*) reflects a mechanism whereby information might be transferred from the receptor via the cytoskeleton to regulatory sites inside the cell remains to be determined.

Summary. In addition to nerve growth factor (NGF), many proteins present in soluble tissue extracts and in the extracellular matrix influence the survival and development of cultured neurons. The structure, synthesis, and mechanism of action of NGF as a neurotrophic factor are considered along with the experiments on the new putative trophic molecules.

Second Messenger Question

The information available seems not to support postulated second messenger mechanisms. NGF does not act as its own second messenger after binding to the cell surface receptors by being transferred into the cytosol. When injected into the cytoplasm of PC12 cells (*19, 20*) or directly into the nuclear chromatin (*20*), NGF did not induce either fiber outgrowth (*19, 20*) or enzymes typical of NGF action via membrane receptors (*21*). Conversely, the injection of antibodies to NGF into the cytoplasm did not abolish the membrane-mediated effects of NGF (*19–21*). The possibility that proteolytic (nonantigenic) degradation products of NGF act as second messengers has been shown to be unlikely: inhibition of the rate of degradation of internalized NGF in PC12 cells did not interfere with its effects on enzyme induction or fiber outgrowth (*21*).

The role of calcium influx as a potential "second messenger" has been discounted in experiments with cultured sympathetic neurons of the newborn rat; in these experiments NGF-mediated induction of tyrosine hydroxylase (TH) was not inhibited by EGTA, calcium channel blockers, or calmodulin antagonists, although the antagonists did block TH induction resulting from high concentrations of potassium (*22*). Similarly, the possibility that cyclic adenosine monophosphate (cyclic AMP) acts as second messenger is unlikely because NGF-mediated selective enzyme induction in calf adrenal medullary cells is distinctly different from that initiated by cyclic AMP, with respect to both the regulation and the pattern of enzyme induction (*23*). The NGF-mediated induction of enzymes involved in the synthesis of adrenergic transmitter is restricted to tyrosine hydroxylase, dopamine β-hydroxylase and phenylethanolamine *N*-methyltransferase. In contrast, cyclic AMP induces not only these enzymes but also dopa decarboxylase. Moreover, the cyclic AMP–mediated enzyme induction can be blocked by the mRNA transcription inhibitor α-amanitin, whereas NGF-mediated enzyme induction is unaffected (*23*). The adenosine derivative 9-β-arabinofuranosyladenine does block enzyme induction by NGF (*23*), suggesting that the regulatory action of NGF may be at the level of mRNA processing, although other actions of this derivative, such as inhibition of transmethylation reactions (*24*), have yet to be discounted.

Closely related to the unresolved question of second messenger mechanisms are the "early effects" induced by the presentation of NGF to neurons that depend on it for survival. After chick sensory and sympathetic neurons are cultured for several hours in the absence of NGF, subsequent addition of NGF to the culture medium has been reported to result in a rapid activation of the sodium-potassium pump and subsequent restoration of sodium-dependent uptake mechanisms of the neurons such as those for glucose and uridine (*25*). However, such experiments cannot determine whether the activation of the sodium pump is the primary action of NGF, or whether it is a secondary effect resulting from restoration of general cell functions by other, still unknown, mechanisms; in the absence of NGF the neurons are dying.

Although the rapidity of the response of the sodium pump to NGF favored the assumption of a direct activation, NGF did not directly stimulate the sodium, potassium adenosine triphosphatase (ATPase) in membrane preparations from the same neurons (*26*). Thus these early effects most probably reflect a rapid but indirect restoration of general cell functions. Similarly, although the rapid activation of *N*-methylation of phospholipids by NGF may be causally related to fiber outgrowth (*27*), many other ligand-receptor interactions in other systems also result in the enhanced *N*-methylation of phospholipids (*28*). Thus, it is essential to establish whether other ligand-receptor interactions—such as tetanus toxin– or lectin-binding, which have no neurite outgrowth–promoting activity—activate *N*-methylation before the significance of this observation can be evaluated.

Not only NGF but also high potassium concentrations lead to the survival of chick sympathetic neurons, and both induce dephosphorylation of a 70-kilodalton protein (*29*). Again, however, it remains to be established whether this dephosphorylation of the 70-kilodalton molecule is causally related to survival, or if it is merely a correlated effect.

Purification of New Neurotrophic Molecules

A fundamental requirement for the purification and characterization of new neurotrophic factors is the availability of defined neuronal culture systems (*3*). These are necessary so that the activity of preparations containing putative trophic agents can be quantified from data on the ability to support neuronal survival. When such an assay based on the survival of cultured sensory neurons dissociated from embryonic chick dorsal root ganglia was used, a neurotrophic activity in mammalian brain could be detected and quantitatively assessed (*30*). A purification factor estimated to be more than 10^6 was necessary to achieve homogeneity of the active molecule, as judged by two-dimensional gel electrophoresis (*31*). Although this molecule exhibits some physicochemical properties (molecular weight, 12,300; isoelectric point, >10.1) similar to those of the monomer of β-NGF (molecular weight, 13,259; isoelectric point, 9.3), the immunological and biological properties are distinctly different from those of NGF. (i) There is no immunological cross-reactivity between NGF and the new brain factor (*31*). (ii) The two molecules act maximally on sensory neurons of different developmental ages (*30*). (iii) In contrast to NGF, the brain factor does not support the survival of sympathetic neurons but may support retinal neurons, which do not respond to NGF (*32*). Thus, the brain-derived putative neurotrophic factor seems to be able to exert a survival-promoting activity on neurons that have a projection in the central nervous system.

Manthorpe and co-workers have reported the purification of a neurotrophic factor, from embryonic chick eye tissue (CNTF) that supports the survival of cultured chick parasympathetic neurons (*33*). This molecule, with a molecular weight 20,600 and an isoelectric point of 5.0, did not cross-react with antibodies to mouse NGF and was also clearly different from the factor derived from mammalian brain. The eye-derived protein has a comparatively unspecific spectrum of action in that it supports the survival of both sympathetic and parasympathetic neurons (that do not respond to the brain factor), in addition to its ability to maintain sensory neurons for at least 24 hours in culture (*33*). Elucidation of the physiological roles of both of these putative neurotrophic proteins awaits the production of antibodies that could be used to determine their cellular location and to observe the consequences of their neutralization in vivo.

"Instructive" and Neurite Growth-Promoting Molecules

In addition to the identification and purification of neuronal survival factors, the purification of molecule(s) responsible for the induction of cholinergic properties in cultured adrenergic neurons of newborn rat is relatively far advanced (*34*). The main cholinergic-inducing activity found in heart cell–conditioned medium migrates as a molecule of 40 to 45 kilodaltons on SDS gel electrophoresis, and has been purified some 10,000-fold (*35*). It seems that the same molecule also increases choline acetyltransferase activity in spinal cholinergic neurons (*36*) and possibly also in chick parasympathetic neurons (*37*).

Progress is also being made in the identification and isolation of molecules that affect the morphological phenotypes of neurons rather than affecting their biochemical properties or survival. Kligman has reported that a soluble brain extract contains an activity that stimulates neurite outgrowth from cultured cerebral cortex neurons (*38*). This activity was purified and shown to be associated with a dimeric protein with subunits with a molecular weight of 37,000 upon reduction. Similarly, Davis and co-workers have partially purified a neurite-promoting activity of RN 22 schwannoma cells that acts by adhesion to polycationic culture substrates to stimulate the rate of neurite growth (*39*). They showed that their most pure preparation contains two proteins with apparent molecular weights 200,000 and 190,000 on SDS–polyacrylamide gel electrophoresis. It is not yet clear which of these proteins possess neurite outgrowth–promoting activity, although the larger cross-reacted with antibodies to the basement membrane protein laminin, which stimulates neurite outgrowth (see below). The antibodies to laminin failed, however, to block the neurite outgrowth–promoting activity of the schwannoma factor; but they could be used to immunoprecipitate it, and they do block the effect of laminin, which indicates that the molecules are not identical (*39*) even though there may be antigenic similarity between the factor and laminin.

Adopting a different approach, Gurney has shown that antibodies to the proteins secreted by denervated muscle can block neuronal sprouting in vivo (*40*). Thus, it appears that denervated muscle cells produce a sprouting factor, and an antiserum that blocks its activity recognizes a protein of apparent molecular weight 56,000. It will be interesting to see if this molecule, which is apparently responsible for neuronal sprouting in vivo, is also able to act as a neurite-promoting factor in vitro. Tissue culture experiments have indicated that this molecule may be able to support the survival or stimulate (or both) neurite outgrowth of spinal neurons from young (E4-5) chick embryos for short periods of time (24 hours) in vitro (*40*).

Influence of Extracellular Matrix Molecules on Neuronal Development

Not only the direction and rate of growth of neurites is dependent on the presence of appropriate substrates (*41*), but substrate-associated molecules can also affect neuronal differentiation (*42*) and can modulate the survival effect of neurotrophic factors (*43*). For example, fixed rat heart cells when used as culture substrates are able to induce cholinergic properties in sympathetic neurons, which otherwise would remain adrenergic (*42*). It is not yet established, however, what relation exists between the molecules released into the medium that also have this effect (*34–36*) and those present

on the fixed heart cell membranes (*42*). Moreover, molecules of the extracellular matrix produced by embryonic chick heart cells modulate the NGF-dependent survival of sympathetic neurons (*43*), although they cannot promote survival themselves (*43, 44*). The maximal survival of chick sympathetic neurons resulting from supramaximal concentrations of NGF is 40 to 50 percent when the neurons are cultured on a polycationic substrate; however, when this substrate was first treated with heart cell–conditioned medium, virtually all of the neurons could be induced to survive by NGF. A distinct subpopulation of sympathetic neurons was subsequently shown to require the presence of the heart cell matrix deposited from the conditioned medium in order to survive. Neurons with adrenergic properties survived in response to NGF alone whereas those neurons present in chick sympathetic ganglia with nonadrenergic (presumably cholinergic) properties required both NGF and the heart cell matrix (*45*).

The neurite outgrowth–promoting effects of the substrate-attached materials from heart-conditioned medium are shared by those of conditioned medium produced by a rat schwannoma cell line RN 22 and various other conditioned media (*46*). These agents also potentiate the survival effects of NGF (and also potassium) on sympathetic neurons (*43, 46*), and the brain-derived growth factor on sensory neurons (*47*). From the experiments with conditioned media, however, it cannot be decided whether the potentiation of the survival effect and the neurite–promoting activity are due to the same or to different molecules. The fact that the basement membrane protein laminin has both a strong neurite promoting activity (*48, 49*) and can potentiate the survival effect of NGF (*49*) shows that these two properties can belong to the same molecule.

Laminin has proved to be an excellent model to analyze the mechanism of interaction of substrate molecules with cell membranes (*50*). Laminin can be cleaved proteolytically into fragments to which antibodies can be produced (*51*). Antibodies to parts of the three short arms of laminin in which the binding domains to tumor cells and hepatocytes reside did not block the fiber outgrowth promoting activity and the survival potentiating effects of laminin (*49*). However, antibodies to the globular domain at the end of the long arm of laminin abolished both the neurite outgrowth promoting activity and the enhancement of the survival effect of NGF (*49*). Previously, the only functional property ascribed to the globular domain at the end of the long arm of laminin was that it contains a heparin binding site (*51*). Thus, neurons may interact with laminin via the heparan sulfate of the neuronal membrane (*52*). Furthermore, the fact that the laminin molecule has two distinct binding sites for neurons and nonneuronal cells points to the possibility that laminin mediates intercellular interactions within the developing nervous system. In the adult, laminin is located in the basal laminae of peripheral nerves and is apparently synthesized by Schwann cells (*53*). Although such laminae are generally present on the outside of the Schwann cell-axon "unit," away from the axons, during axonal regeneration in injured peripheral nerves the axons have been seen to grow along the inside of the remaining basal laminae (*54*). This indicates that the laminin of the peripheral nervous system may be necessary for the ability of these neurons to regenerate their projections.

Future Developments

Even if the purification of a putative trophic factor has been accomplished, this does not guarantee the determination of its physiological significance. The small quantities available are not only inadequate for pharmacological studies but also hamper the production of antibodies: although the principle of monoclonal antibody production allows immunization with impure preparations, if the molecule is a poor antigen then it may prove exceedingly difficult to obtain suitable antibodies. Indeed, the production of antibodies to neither the brain factor nor CNTF has been reported. However, recent advances in peptide chemistry and molecular genetics offer a way around these problems (*55*). Thus, appropriate oligopeptide sequences from such molecules may be determined and subsequently synthesized in sufficient quantities to produce antibodies against the putative trophic factors. Furthermore the cloning of these molecules and subsequent production in prokaryotic or eukaryotic systems by expression vectors may be the only way to produce them in quantities large enough to test the possibility of their therapeutic applications.

That neurotrophic molecules might be useful for promoting regeneration can be deduced from the observation that the regeneration of lesioned adult sympathetic nerve fibers is enhanced by NGF and delayed by antibodies to NGF (*56*). The local application of neurotrophic and appropriate substrate molecules therefore may aid regeneration, although mere regrowth is no guarantee for success since the stimulation of regeneration has to be followed by the formation of the correct corrections (*57*). Whether the promotion of the regeneration of nerve fibers is followed by the "correct wiring" is not known.

The lack of regeneration in the central nervous system is a multifactorial problem including glial scar formation and insufficient production of neurotrophic molecules or matrix molecules. In cases of more general processes of degeneration or atrophy the local application of neurotrophic molecules does not seem to be appropriate. Better understanding of the physiology of these molecules may make it possible to influence pharmacologically their production and release. For example, an atrophy of central cholinergic neurons is found in Alzheimer's disease (*58*). Although these neurons do not appear to depend on endogenous NGF for their maintenance, they do respond to exogenous NGF with increased levels of the enzyme choline acetyltransferase (*59*). Thus, neurotrophic factors may eventually be used either by direct local administration or by pharmacological modifications of their synthesis in vivo.

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RESEARCH ARTICLE

A General Method for Saturation Mutagenesis of Cloned DNA Fragments

Richard M. Myers, Leonard S. Lerman, Tom Maniatis

The development of procedures for introducing single-base substitutions into specific cloned DNA sequences has provided important tools for studying the molecular genetics of eukaryotes (*1, 2*) and prokaryotes (*3*). Two general strategies have been used for site-directed mutagenesis. First, single-base substitutions have been introduced into cloned DNA at specific nucleotide positions by oligonucleotide-directed mutagenesis procedures (*2, 4, 5*). Second, a variety of approaches have been developed for introducing random base substitutions into specific DNA sequences. One approach involves the use of various types of nucleotide misincorporation procedures (*6*). Alternatively, random mutations have been generated by treatment of single-stranded DNA with chemical mutagens followed by enzymatic synthesis of the complementary DNA strand (*7, 8*). For example, sodium bisulfite treatment of DNA molecules containing a single-stranded gap generates C to T transitions (*7*). However, unlike sodium bisulfite, chemicals that generate other transitions and transversions do not react preferentially with single-stranded DNA and therefore cannot be used to introduce mutations specifically at gaps. To overcome this problem the entire DNA molecule can be treated with these chemicals under conditions that minimize the frequency of multiple base substitutions in the sequence of interest. However, under these conditions, only a fraction of the target DNA fragments will contain a mutation. Therefore this approach is limited to situations where DNA molecules carrying a mutation can be identified by a genetic screen or selection. In particular, it is difficult to use these procedures to study DNA sequences involved in developmental or tissue-specific gene regulation, or to study structure-function relationships in proteins for which a genetic selection is not available. We have circumvented this difficulty by making use of a denaturing gradient gel electrophoresis procedure to identify and purify mutant DNA molecules in the absence of a phenotypic selection.

DNA fragments differing by single-base substitutions can be separated from each other by electrophoresis in polyacrylamide gels containing an ascending gradient of the DNA denaturants urea and formamide (*9–14*). DNA fragments of identical size, but differing by a single-base change, will initially move through the polyacrylamide gel at a constant rate. As they migrate into a critical concentration of denaturant, specific regions or "domains" within the fragment melt to produce partially denatured DNA. Melting of a domain is accompanied by an abrupt decrease in mobility, which is a consequence of the entanglement of

Richard M. Myers is a postdoctoral fellow and Tom Maniatis is a professor in the Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138. Leonard S. Lerman is the director of diagnostics, Genetics Institute, 87 Cambridgepark Drive, Cambridge, Massachusetts 02140.