Concentration-dependent Regulation of Neuronal Gene Expression by Nerve Growth Factor

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Abstract. NGF is a neurotrophic protein that promotes the survival, growth, and differentiation of developing sympathetic neurons. To directly determine the effects of different concentrations of NGF on neuronal gene expression, we examined mRNAs encoding the p75 low-affinity NGF (LNGF) receptor, Tα1 α-tubulin (Tα1), and tyrosine hydroxylase (TH) in pure cultures of rat sympathetic neurons from postnatal day 1 superior cervical ganglia. Studies of the timecourse of gene expression during 2 wk in culture indicated that a 5-d incubation period would be optimal for the concentration-effect studies. Analysis of RNA isolated from neurons cultured in 2–200 ng/ml NGF for 5 d revealed that, as the NGF concentration increased, neurons expressed correspondingly increased levels of all three mRNAs. Both LNGF receptor and TH mRNAs increased seven-fold, and Tα1 mRNA increased four-fold in neurons cultured in 200 versus 10 ng/ml NGF. In contrast, T26 α-tubulin mRNA, which is constitutively expressed, did not alter as a function of NGF concentration. When neurons were initially cultured in 10 ng/ml NGF for 5 d, and then 200 ng/ml NGF was added, LNGF receptor, Tα1, and TH mRNAs all increased within 48 h. The timecourse of induction differed: Tα1 mRNA was maximal by 5 h, whereas LNGF receptor and TH mRNAs first began to increase at 12 h after the NGF increase. These experiments show that NGF regulates expression of a subset of mRNAs important to neuronal growth and differentiation over a broad concentration range, suggesting that the effects of NGF may be mediated by more than just a single receptor operating at one fixed affinity. These results also suggest a mechanism for coupling neuronal synthesis of axonal proteins to increases in size of the innervated target territory during growth of the organism.

NGF is believed to mediate its actions by binding to a high-affinity membrane-bound NGF receptor (Green et al., 1986). A low-affinity NGF (LNGF) receptor has been cloned (Johnson et al., 1986; Radeke et al., 1987), and shown to be an 80–85-kD transmembraneglycoprotein. When this receptor is expressed in medulloblastoma (Pleasure et al., 1990) or LNGF receptor-negative PC12 cells (Hempstead et al., 1989) both low- and high-affinity NGF binding sites are produced, suggesting that the LNGF receptor is required for generation of high-affinity NGF binding sites. However, a unique 143-kD protein that binds NGF with high affinity (Hosang and Shooter, 1985) has recently been characterized in PC12 cells (Meakin and Shooter, 1991). It is likely that this molecule is the trk tyrosine kinase protooncogene product (Kaplan et al., 1991a,b; Klein et al., 1991). trk may require the LNGF receptor to bind NGF with high affinity (Hempstead et al., 1991). Thus, NGF appears to bind to multiple receptors, thereby potentially activating a variety of intracellular signalling pathways.

The growth of both developing and mature sympathetic neurons depends on the balance of various factors that promote survival and differentiation. NGF is one of the most important of these factors, acting through the LNGF receptor to promote cell survival and differentiation. The concentration-dependent regulation of mRNA expression for key proteins associated with neuronal growth and differentiation provides insights into the mechanisms by which NGF mediates its effects.

**Interactions** between a developing peripheral neuron and its target organ are believed to partially determine the phenotype of that neuron, and to play an important role in neuronal competition and cell death. Nerve growth factor (NGF) is a neurotrophic factor involved in the survival and differentiation of developing sympathetic and neural crest-derived sensory neurons. NGF, given systemically, promotes growth of nerve terminals and dendritic arborization of sympathetic neurons (Levi-Montalcini and Booker, 1960a; Snider, 1988; Ruit et al., 1990), and affects neurotransmitter phenotype and gene expression in both sensory and sympathetic neurons (Kessler and Black, 1980; Otten et al., 1980; Thoenen et al., 1971; Mathew and Miller, 1990; Miller et al., 1991). Conversely, antibodies to NGF lead to the death of embryonic sensory neurons (Johnson et al., 1980; Aloe et al., 1981) and neonatal or mature sympathetic neurons (Levi-Montalcini and Booker, 1960b; Angeletti et al., 1971; Gorin and Johnson, 1980), although cross-reactivity of these antibodies with other members of the neurotrophin family could have contributed to the observed effects (Acheson et al., 1991; Rosenthal et al., 1990). These studies suggest that NGF plays an important role in regulating neuronal survival and differentiation.
neurons in vivo is regulated largely as a function of the target tissue available for innervation (reviewed in Purves et al., 1988). Many of the effects of increased available target territory can be mimicked by exogenous NGF (Purves et al., 1988; Ruit et al., 1990), leading to the hypothesis that survival and growth of sympathetic neurons is dependent upon the availability of target-derived NGF, and that increased available NGF will lead to spatially regulated growth and terminal arborization. One assumption underlying this hypothesis is that sympathetic neurons respond to NGF in a concentration-dependent fashion.

To directly test the hypothesis that NGF regulates gene expression in cultured sympathetic neurons in a concentration-dependent fashion, we have examined the effect of different NGF concentrations on expression of p75 low-affinity NGF receptor, tyrosine hydroxylase, and Tα1 α-tubulin mRNAs, all of which are regulated by NGF in PC12 cells, and in sympathetic neurons in vivo (Miller et al., 1991). Our results demonstrate that the concentration of NGF affects the timecourse of expression of LNGF receptor and Tα1 mRNAs as they develop in culture and that all three mRNAs increase as a function of increasing 2.5S NGF over a range of 2 to 200 ng/ml. Furthermore, in sympathetic neurons that have been cultured in a low concentration of NGF, the addition of increased NGF upregulates all three mRNAs within 12 h. Thus, NGF does not regulate cellular phenotype in an all-or-none manner, but instead modulates gene expression over a broad range of concentrations. These data therefore suggest a molecular mechanism whereby the innervated target tissue may directly regulate the synthesis of a subset of neuronal proteins both during development and in the mature animal. Furthermore, these results are consistent with the hypothesis that NGF actions on sympathetic neurons may be mediated by receptor complexes that are capable of graded responses over a large concentration range.

Materials and Methods

Neuronal Cultures

Mass cultures of pure sympathetic neurons from newborn rat superior cervical ganglia (SCG) were prepared as previously described (Campenot and Draker, 1989). Briefly, the SCG were removed from postnatal day 1 (PI) Sprague-Dawley rats supplied by the University of Alberta (Edmonton, Alberta, Canada) farm. Ganglia were enzymatically dissociated in 0.1% trypsin (Calbiochem-Behring Corp., La Jolla, CA) for 17 min at 37°C, 0.01 mg/ml DNAase I (Sigma Chemical Co., St. Louis, MO) for 2 h to 7 d. To confirm that equivalent amounts of RNA were loaded in each lane, ethidium bromide was added to the sample buffer before electrophoresis, and gels were photographed under ultraviolet illumination. In addition, some of the nitrocellulose filters were stained with methylene blue (Moore, 1988) subsequent to hybridization to ensure that equivalent amounts of RNA were transferred. Northern blots were quantitated using a scanning laser densitometer (Ultrascan XL; LKB Instruments, Inc., Bromma, Sweden). Representative Northern blots from different experiments were chosen for quantitation after ensuring that the amounts of total RNA in the pertinent lanes were identical. Several different film exposures of the same data were analyzed to ensure reproducibility. Individual Northern blots were never reprobed more than once, and then only when the mRNAs were significantly different sizes.

Hybridization Probes

Probes to Tα1 α-tubulin mRNA were prepared as described (Miller et al., 1987). For NGF receptor studies, a subcloned 310 nucleotide EcoRI/BamHI fragment containing nucleotides 400-710 of the rat cDNA (Radeke et al., 1987) was used to generate riboprobes (Miller et al., 1991). Anti-sense RNA probes specific to tyrosine hydroxylase mRNA (plasmid K35) (Lewis et al., 1983) were generated as previously described (Miller et al., 1991). A riboprobe specific for the 3' untranslated region of Tα2 α-tubulin mRNA was prepared from a 133 nucleotide fragment that was PCRd from PC12 mRNA, and subcloned into pGEM4.

Results

Timecourse of Expression of LNGF Receptor, Tyrosine Hydroxylase, and Tα1 α-Tubulin mRNAs in Cultured Sympathetic Neurons

To determine a suitable timeperiod for investigating the effects of different NGF concentrations on the levels of NGF receptor, tyrosine hydroxylase and Tα1 α-tubulin mRNAs, pure cultures of PI SCG neurons were maintained in either 10 or 200 ng/ml 2.5S NGF, and total RNA was isolated at different times up to 2 wk after plating. Northern blot analysis revealed that levels of LNGF receptor and Tα1 mRNAs varied over time in culture (see Figs. 1 and 4 a). In the presence of 200 ng/ml 2.5S NGF, the largest change was observed with LNGF receptor mRNA, which increased sixfold from 2 to 14 DIV (see Figs. 1 a and 4 a). In contrast, levels of Tα1 α-tubulin mRNA (see Figs. 1 a and 4 a) did not vary significantly from 2 to 10 DIV, but decreased approximately twofold from 10 to 14 DIV. Tyrosine hydroxylase mRNA levels did not change more than 1.5-fold throughout the timecourse (see Figs. 1 a and 4 a). Similar results were obtained in 3 different experiments.

In the presence of a 20-fold lower concentration of 2.5S NGF, 10 ng/ml, a different pattern of expression was observed. LNGF receptor mRNA levels were approximately equal from 3 to 6 DIV, and subsequently decreased approximately twofold by 8 DIV (Fig. 1 b), even though the neurons appeared healthy at the low NGF concentration (data not shown). Levels of Tα1 α-tubulin mRNA increased slightly from 3 to 6 DIV, and subsequently decreased approximately twofold by 8 DIV. In contrast, tyrosine hydroxylase mRNA levels were approximately equal from 3 to 8 DIV (Fig. 1 b). Similar results were obtained in three independent experiments. We concluded from these results that a 5-d incubation period, before the decrease in mRNA levels that occurred at
Expression of LNGF Receptor, Tα1 α-Tubulin, and Tyrosine Hydroxylase mRNAs in Sympathetic Neurons as a Function of NGF Concentration

To determine the effect of different concentrations of NGF, cultured neurons were maintained for 5 DIV in the presence of 2, 10, 50, 100, or 200 ng/ml 2.5S NGF, and total cytoplasmic RNA was isolated. Neurons appeared healthy in all NGF concentrations, although the neurite density increased with increasing NGF (Fig. 2, a-d).

Northern blot analysis of equal amounts of total RNA revealed that expression of all three mRNAs was increased as a function of increasing NGF concentration (Figs. 3 and 4 b). Both LNGF receptor and tyrosine hydroxylase mRNA levels were approximately sevenfold higher in 200 ng/ml versus 10 ng/ml 2.5S NGF (Fig. 3, a and c, Fig. 4 b). Within that range, the steepest increase in both occurred between 10 and 50 ng/ml 2.5S NGF. Tα1 α-tubulin mRNA increased somewhat less, approximately fourfold, between 1 and 200 ng/ml 2.5S NGF (Figs. 3, b and 4 b). Similar results were obtained in three independent experiments.

To ensure that NGF itself, and not some undetected contaminant, was responsible for the observed induction in mRNA levels at higher concentrations, we cultured neurons for 5 d in the presence of 50 or 200 ng/ml of the purified, intact A chain of 2.5S NGF. Northern blot analysis of the total cytoplasmic RNA from these cultures confirmed that all three mRNAs were elevated in 200 versus 50 ng/ml of A chain NGF (data not shown).

Induction of LNGF Receptor, Tyrosine Hydroxylase, and Tα1 α-Tubulin mRNAs by Increased NGF

To ensure that the observed effects were not because of preferential survival of subpopulations of sympathetic neurons, and to determine if neurons initially cultured in a low NGF concentration could upregulate gene expression in response to increased NGF, neurons were maintained for 5 d in 10 ng/ml NGF, and in some dishes, the NGF concentration was subsequently raised to 200 ng/ml. At various times following NGF addition, total cytoplasmic RNA was isolated and Northern blots probed for LNGF receptor, Tα1 α-tubulin, and tyrosine hydroxylase mRNAs. Increases in both LNGF receptor and tyrosine hydroxylase mRNA levels were first evident 12 h after NGF addition, and were still increasing at 48 h, the latest timepoint examined (Fig. 5, a and c). At 48 h, the increase was approximately fourfold, as compared to control neurons maintained for the same length of time in 10 ng/ml NGF. In contrast, Tα1 α-tubulin mRNA levels increased faster, but to a lower relative degree, displaying a twofold increase 5 h after NGF addition, and maintaining that increase throughout the timecourse (Fig. 5 b). Similar results for each of the three mRNAs were obtained in at least three independent experiments.

Discussion

NGF Regulates Neuronal Expression of LNGF Receptor, Tα1 α-Tubulin, and Tyrosine Hydroxylase mRNAs in a Graded Fashion

Administration of NGF to neonates has dramatic effects on sympathetic neurons, causing increased terminal sprouting (Levi-Montalcini and Booker, 1960a), increased dendritic arborization (Snider, 1988), increased activity of enzymes involved in catecholamine biosynthesis (Thoenen et al., 1971), and increased expression of LNGF receptor, Tα1 α-tubulin, and tyrosine hydroxylase mRNAs (Mathew and Miller, 1990; Miller et al., 1991). Increased NGF also supports the survival of increased numbers of sympathetic neurons in vivo (Hendry and Campbell, 1976), and in culture (Chun and Patterson, 1977a).

The data presented here extend these observations and demonstrate that, independent of survival effects, different concentrations of NGF differentially regulate the develop-
ment of sympathetic neurons in culture. In high NGF concentrations, the patterns of expression of LNGF receptor, Tα1 α-tubulin, and tyrosine hydroxylase were qualitatively similar to that previously observed in the neonatal SCG in vivo (Buck et al., 1987; Mathew and Miller, 1990; Miller et al., 1991). This is not the case in low concentrations of NGF, where LNGF receptor mRNA was downregulated from 2 to 8 DIV as opposed to the sixfold upregulation observed in high NGF. Furthermore, neurons cultured in high versus low NGF express many times higher levels of LNGF receptor, Tα1 α-tubulin, and tyrosine hydroxylase mRNAs. Thus, differences in NGF concentration, over a range sufficient for survival, may differentially regulate the development of sympathetic neurons.

These results may have implications for the role target-derived NGF plays in neuronal competition and cell death. Initial exposure of a developing sympathetic neuron to

Figure 2. Pure cultured neonatal sympathetic neurons grown in the presence of 10 (a), 50 (b), 100 (c), or 200 (d) ng/ml 2.5S NGF for 5 DIV. Note the increasing neurite density with increasing NGF concentration.

Figure 3. Expression of LNGF receptor (a), Tα1 α-tubulin (b), tyrosine hydroxylase (c), and T26 α-tubulin (d) mRNAs in equal amounts of total cytoplasmic RNA isolated from neonatal sympathetic neurons cultured for 5 d in the presence of 2–200 ng/ml 2.5S NGF (denoted on the bottom of each panel). Note that the faint upper band in (c) represents LNGF receptor mRNA, since this blot was probed first for LNGF receptor, and secondly for tyrosine hydroxylase.
ability to compete effectively for NGF could, in turn, lead to a downregulation of receptor mRNA levels, as observed in culture in the present experiments, and an inability to synthesize the proteins necessary for further terminal arborization.

Data presented here indicate that, in sympathetic neurons cultured for 5 d in a broad range of increasing NGF concentrations, LNGFR receptor, Tά α-tubulin, and tyrosine hydroxylase mRNA levels all increased in a graded fashion. This graded response occurred over a surprisingly large concentration range from 2–200 ng/ml 2.5S NGF. These results are not due to preferential neuronal survival in different concentrations of NGF, since neurons maintained in 10 ng/ml NGF for 5 d upregulated all three mRNAs within 12 h of treatment with 200 ng/ml NGF. It is also unlikely that the increases are simply a result of neuronal hypertrophy, since all comparisons were normalized for equal amounts of total ribosomal RNA, and Tά α-tubulin mRNA, which is constitutively expressed in neurons (Miller et al., 1987), did not change. Thus, NGF is capable of modulating the expression of a specific subset of neuronal genes over a broad concentration range. Precedent for this surprisingly large NGF response range arises from previous work demonstrating that neurite density (Chun and Patterson, 1977b; Campenot, 1982) and tyrosine hydroxylase (Raynaud et al., 1988) are regulated as a function of NGF concentration.

It is possible that, at higher concentrations, NGF may be acting pharmacologically rather than physiologically by binding to receptors for other neurotrophins such as BDNF (Barde et al., 1982; Leibrock et al., 1989) and NT-3 (Hohn et al., 1990; Maisonpierre et al., 1990). For example, at high concentrations, NGF can support the survival of BDNF-responsive sensory neurons (Rodriguez-Tebar et al., 1990). If, however, NGF is acting pharmacologically at high concentrations, our data infer that the mechanisms of action for the different neurotrophins converge at the genetic level.

The graded genetic response of sympathetic neurons to NGF provides a mechanism for regulating neuronal phenotype over a wide range of target sizes in both normal and perturbed states. During normal development and growth of the animal, target tissues that are innervated by the sympathetic nervous system, such as the vasculature, grow severalfold. Since the number of sympathetic neurons in, for example, the superior cervical ganglion, remain constant after postnatal day 20 (Wright et al., 1983), then any individual neuron is faced with innervating an increasingly large target area. It is, therefore, a biological necessity that these neurons are capable of responding in a graded fashion to the increased target tissue by synthesizing the protein necessary for the increased innervation.

A similar mechanism can be invoked to explain phenomena such as collateral sprouting. Partial denervation of target or-

Figure 4. Quantitation of the representative Northern blots using scanning laser densitometry. Results are expressed relative to the scanning density at day 2 (a) or at 10 ng/ml 2.5S NGF (b). (a) Timecourse of expression of LNGFR receptor (ό), Tά α-tubulin (ό), and tyrosine hydroxylase (ό) mRNAs in equal amounts of total cytoplasmic RNA isolated from neonatal sympathetic neurons cultured in 200 ng/ml 2.5S NGF for 2–14 DIV. (b) Expression of LNGFR receptor, Tά α-tubulin, and tyrosine hydroxylase mRNAs in equal amounts of total cytoplasmic RNA isolated from neonatal sympathetic neurons cultured for 5 DIV in the presence of 10–200 ng/ml 2.5S NGF. Symbols are identical to those used in a.

Figure 5. Expression of LNGFR receptor (a), Tά α-tubulin (b), and tyrosine hydroxylase (c) mRNAs in neonatal sympathetic neurons cultured in the presence of 10 ng/ml 2.5S NGF for 5 d, followed by the addition of 200 ng/ml 2.5S NGF for 5, 12, or 48 h. The upper panels are photographs of autoradiographs produced by hybridizing Northern blots with radiolabelled probes specific for each mRNA. The lower panels are photographs of the original agarose gels with the samples electrophoresed in the presence of ethidium bromide to demonstrate that equivalent amounts of total RNA were loaded in each lane.
gans by unilateral ganglionectiontomy (Lingappa and Zigmond, 1987; Dornay et al., 1985; Smith et al., 1990) or of neural structures that produce NGF, like the hippocampus (Crutcher and Chandler, 1985) leads to sprouting and hypertrophy (Smith et al., 1990) of sympathetic neurons that subsequently innervate the denervated territory. NGF is probably at least partially responsible for these observations, since antibodies to NGF inhibit the collateral sprouting of sensory neurons (Diamond et al., 1987), and locally increased NGF leads to spatially regulated growth of sympathetic neurons both in vivo (Edwards and Hanahan, 1989) and in vitro (Campenot, 1982). Furthermore, collateral sprouting of sympathetic neurons is accompanied by elevation of Tαα α-tubulin mRNA (Mathew and Miller, 1990), supporting the idea that, in vivo, neurons are responding to increased available NGF with a graded response at the genetic level.

NGF May Regulate Neuronal Gene Expression Via Multiple Mechanisms

Exposing sympathetic neurons initially cultured in low NGF to high NGF increases levels of Tαα α-tubulin, tyrosine hydroxylase, and LNGF receptor mRNAs within 48 hours, indicating that the concentration-dependent effects of NGF on gene expression were not a result of differential survival of subpopulations of sympathetic neurons. In addition, the time-courses of induction of these three mRNAs differed, suggesting that NGF regulates gene expression in sympathetic neurons via not one, but multiple molecular mechanisms.

Further support for these conclusions arises from studies in PC12 cells, where the timecourse of induction of these three mRNAs is similar to that reported here for sympathetic neurons (Miller et al., 1987, 1991). In PC12 cells, these increases are mediated transcriptionally for both LNGF receptor and total α-tubulin mRNAs (Miller et al., 1991), while tyrosine hydroxylase gene expression appears to be regulated at both the transcriptional (Gizang-Ginsberg and Ziff, 1990) and posttranscriptional (Miller et al., 1991) levels. The fact that tyrosine hydroxylase is regulated posttranscriptionally in sympathetic neurons (Hefti et al., 1982; Rohrer et al., 1978; Raynaud et al., 1988) and that inhibition of protein synthesis in either PC12 cells (Miller et al., 1991) or in sympathetic neurons (unpublished observations) prevents the NGF-mediated increase in LNGF receptor mRNA suggests that the genetic mechanisms may be similar in both cell types.

The molecular mechanisms underlying the graded changes in mRNA levels as described here remain undefined, but occur presumably at a number of cellular levels. Sympathetic neurons must have the cellular machinery to "detect" and respond to NGF over a 100-fold difference in concentration. This could occur via ligand/receptor interactions of differing affinities, and/or via a ligand/receptor complex(es) that activates multiple signal transduction pathways. It is unlikely that activation of the trk tyrosine kinase receptor alone is sufficient to explain these observations: in PC12 cells, tyrosine phosphorylation of trk by NGF is maximal at 1-10 ng/ml (Kaplan et al., 1991b). However, Hemptstead et al. (1991) have hypothesized that the high-affinity NGF receptor is a complex composed of p75 LNGF receptor and the trk proteincogene, both of which bind NGF with lower affinity than the complex (Kaplan et al., 1991a; Hemptstead et al., 1991). Furthermore, the p75 LNGF receptor has been hypothesized to interact with G-proteins via a mastoparan-like domain (Feinstein and Larhammar, 1990). These two different receptors, either acting alone, or as a complex, could therefore provide the neuron with the ability to bind NGF over a wide range of concentrations, potentially activating a number of different signal transduction pathways.

Further complexity arises from the fact that, as described here, continuous exposure to high NGF leads to a gradual increase in the synthesis of LNGF receptor mRNA. Although we have not demonstrated that this results in increased NGF binding, Bernd and Greene (1984) have shown that NGF increases both low- and high-affinity NGF binding on PC12 cells, potentially as a consequence of increased LNGF receptor mRNA levels (Miller et al., 1991). It is possible that increased levels of the p75 LNGF receptor on the surface of sympathetic neurons may serve to attenuate the effects of increasing NGF by sequestering NGF from a productive, high-affinity receptor and/or complex. The net effect could be that occupancy of the productive complex would occur over a much broader concentration range than predicted by the number of high-affinity binding sites alone.

In summary, NGF directly induces a graded increase in expression of a subset of mRNAs important for neuronal growth and differentiation over a very broad concentration range. This could provide a molecular mechanism for tightly regulating the growth of sympathetic neurons as a function of the size of the innervated target territory, and suggests that NGF actions may be mediated by several signal transduction pathways activated at different NGF concentrations.

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