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

Y. Ma, R. B. Campenot, and F. D. Miller

Department of Anatomy and Cell Biology, University of Alberta, Edmonton, Canada

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 2.5S 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.

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, 1960*a*; 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, 1960*b*; 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.

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)[1] receptor has been cloned (Johnson et al., 1986; Radeke et al., 1987), and shown to be an 80–85-kD transmembrane glycoprotein. When this receptor is expressed in medulloblastoma (Pleasure et al., 1990) or NGF 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., 1991*a*,*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

1. *Abbreviations used in this paper*: DIV, days in vitro; LNGF, low affinity NGF; P1, postnatal day 1; SCG, superior cervical ganglia.

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 (P1) 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 3 min at room temperature, and finally mechanically dissociated (Campenot, 1982). Dissociated cells were plated at a density of two ganglia per dish in 60-mm collagen-coated tissue culture dishes in L15-CO_2 medium (Gibco Laboratories, Grand Island, NY) as described by Hawrot and Patterson (1979). Medium was supplemented with methylcellulose (0.6%), rat serum (2.5%), ascorbic acid (1 mg/ml), and the mitotic inhibitor cytosine arabinoside (10 μM). This method produces neuronal cultures that are free of nonneuronal cells. 2.5S NGF was provided by Dr. Richard Murphy (University of Alberta) and was prepared by the method of Bocchini and Angeletti (1969) as modified by Mobley et al. (1976). For some experiments, we used the "A" chain of 2.5S NGF (50–200 ng/ml) highly purified by HPLC (Murphy et al., 1989). NGF was added to neuronal cultures at concentrations ranging from 2–200 ng/ml (details below). Cultures were maintained in a 5% CO_2 atmosphere at 37°C. Experiments were performed on the fifth day in vitro (DIV), after determining the timecourse of gene expression over 2–14 d.

RNA Isolation and Analysis

Total cytoplasmic RNA was prepared from cultured neurons as described by Sambrook et al. (1989). Total RNA (1–5 μg) was fractionated by electrophoresis on 1.2% agarose gels in the presence of 1 M formaldehyde (Rave et al., 1979) and transferred to nitrocellulose (Thomas, 1980). Antisense RNA probes (see below) were hybridized to the immobilized RNA as described (Miller et al., 1991). Nitrocellulose filters were subsequently exposed to XAR or XRP X-ray film (Eastman Kodak Co., Rochester, NY) 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 (Monroy, 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). Antisense 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 T26 α-tubulin mRNA was prepared from a 133 nucleotide fragment that was PCRed 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 P1 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

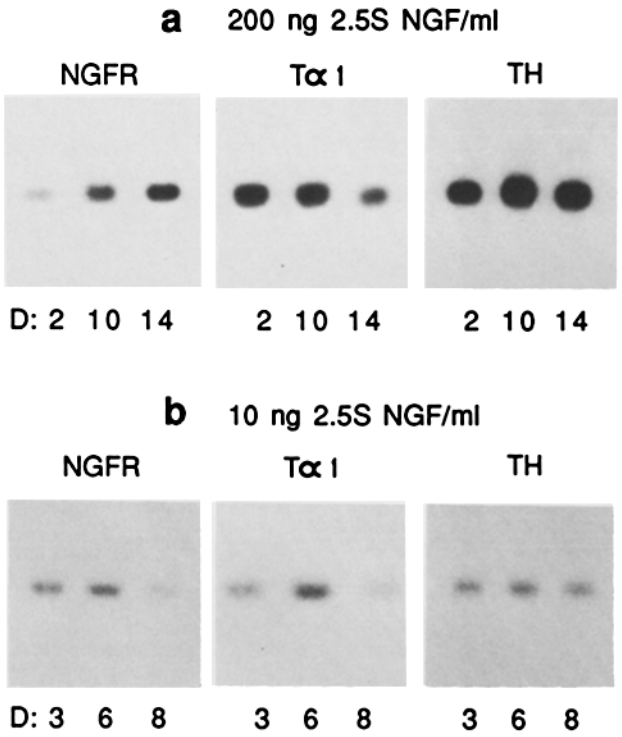

Figure 1. Expression of LNGF receptor (*NGFR*), Tα1 α-tubulin (*Tα1*), and tyrosine hydroxylase (*TH*) mRNAs in neonatal sympathetic neurons cultured from 2 to 14 d (*D*) in the presence of 200 (*a*) or 10 (*b*) ng/ml 2.5S NGF. Equal amounts of total RNA were analyzed by Northern blot analysis with probes specific for each of the three mRNAs.

10 ng/ml NGF, would be suitable for the concentration-effect studies.

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.

Neurons cultured in only 2 ng/ml NGF appeared healthy, and Northern blot analysis revealed that they expressed lower levels of LNGF receptor, tyrosine hydroxylase, and Tα1 α-tubulin mRNAs than those cultured in 10 ng/ml (for example, see Fig. 3 *b*). However, this NGF concentration was not used in further experiments due to concerns about possible survival effects.

To determine whether the observed increases in neuronal gene expression were specific, Northern blots were probed for T26 α-tubulin mRNA, which is constitutively expressed in all cell types, and is not increased by NGF in PC12 cells (Miller et al., 1987). T26 α-tubulin mRNA levels were similar in 10 (data not shown), 50, 100, and 200 ng/ml (Fig. 3 *d*) 2.5S NGF. Thus, NGF receptor, Tα1 α-tubulin, and tyrosine hydroxylase mRNAs were specifically increased in sympathetic neurons as a function of increasing NGF concentration.

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, 1960*a*), 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, 1977*a*).

The data presented here extend these observations and demonstrate that, independent of survival effects, different concentrations of NGF differentially regulate the develop-

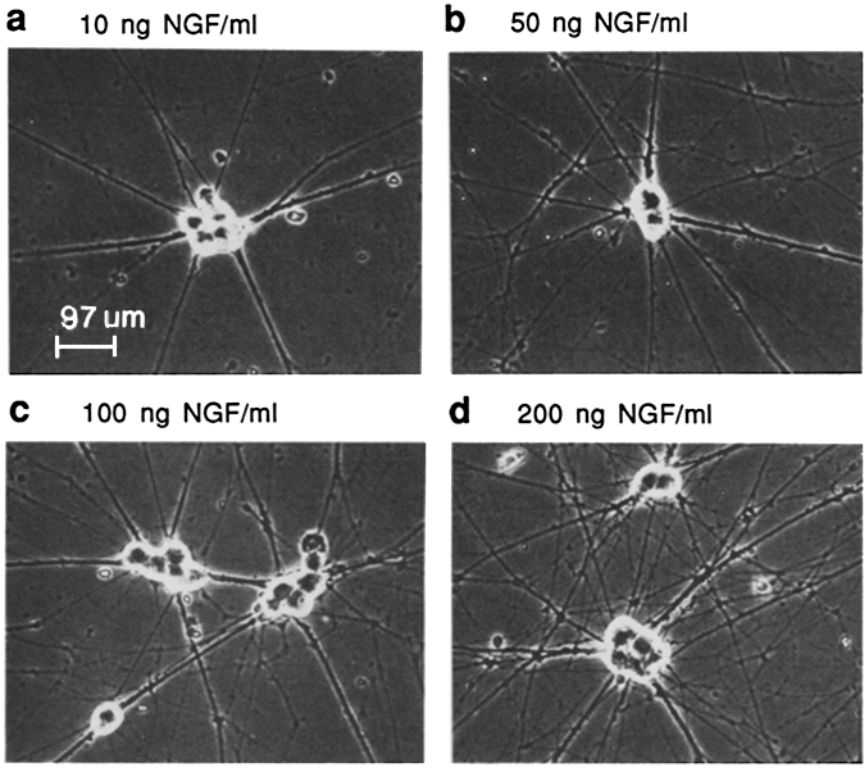

Figure 2. Pure cultured neonatal sympathetic neurons grown in the presence of 10 (*a*), 50 (*b*), 100 (*c*), or 200 (*d*) ng/ml 2.5S NGF for 5 DIV. Note the increasing neurite density with increasing NGF concentration.

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 target-derived NGF could increase LNGF receptor and tyrosine hydroxylase mRNAs and maintain elevated levels of Tα1 α-tubulin mRNA as seen with systemic NGF (Miller et al., 1991). The increased mRNA levels could provide proteins essential for expression of the terminal arbor, and/or for neuronal maturation. In addition, increased LNGF receptor mRNA could produce an increase in the number and density of high- and low-affinity neuronal receptors, as it does in PC12 cells (Bernd and Greene, 1984). This NGF-induced increase in receptor levels could increase the binding capacity of axons which first encounter the high NGF concentration, allowing them to function as an NGF "sink." This would deprive later-arriving axons of access to high levels of NGF. In this way, early-arriving neurons, which have already innervated target territory, would be more "fit," and would compete more effectively than later-arriving neurons for limiting concentrations of target-derived NGF. This in-

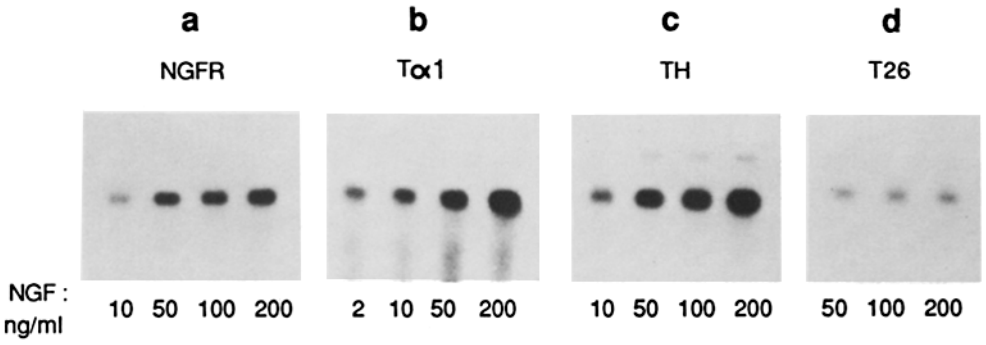

Figure 3. Expression of LNGF receptor (*a*), Tα1 α-tubulin (*b*), tyrosine hydroxylase (*c*), and T26 α-tubulin (*d*) mRNAs in equal amounts of total cytoplasmic RNA isolated from neonatal sympathetic neurons cultured for 5 d in the presence of 2–200 ng/ml 2.5S NGF (denoted on the bottom of each panel). 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.

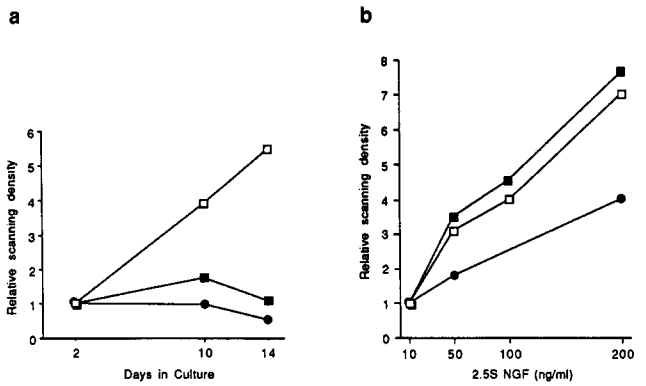

Figure 4. Quantitation of the representative Northern blots using scanning laser densitometry. Results are expressed relative to the scanning density at day 2 (*a*) or at 10 ng/ml 2.5S NGF (*b*). (*a*) Timecourse of expression of LNGF receptor (□), Tα1 α-tubulin (●), and tyrosine hydroxylase (■) mRNAs 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 LNGF receptor, Tα1 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*.

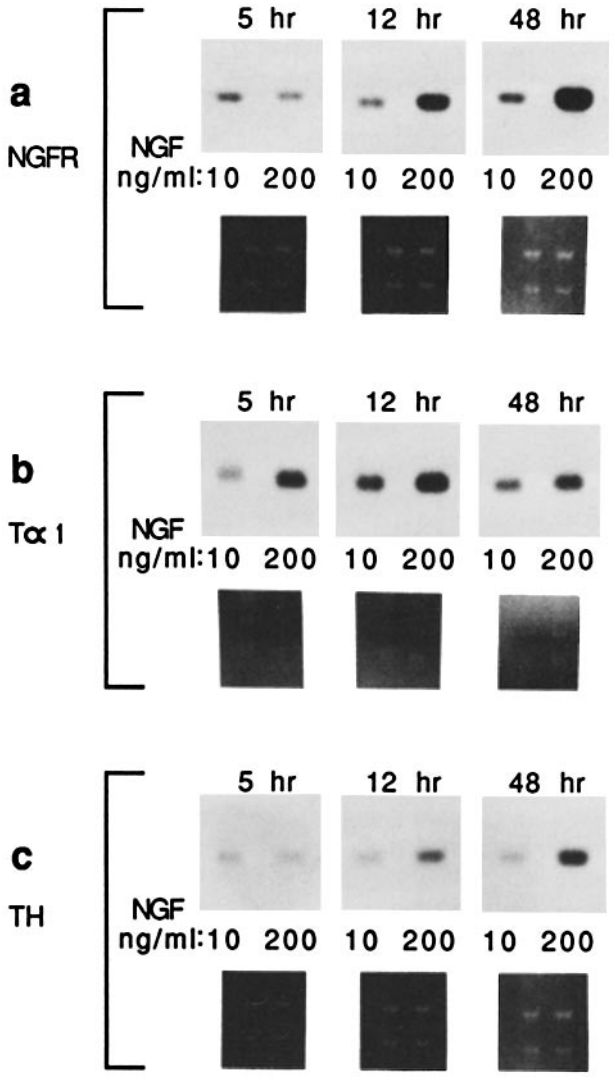

Figure 5. Expression of LNGF receptor (*a*), Tα1 α-tubulin (*b*), and tyrosine hydroxylase (*c*) mRNAs in neonatal sympathetic neurons cultured in the presence of 10 ng/ml 2.5S NGF for 5 d, followed by the addition of 200 ng/ml 2.5S NGF for 5, 12, or 48 h. The upper panels are photographs of autoradiographs 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.

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, LNGF receptor, Tα1 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 T26 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, 1977*b*; 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-

gans by unilateral ganglionectomy (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 Tα1 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 increased levels of Tα1 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 timecourses 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., 1991*b*). However, Hempstead et al. (1991) have hypothesized that the high-affinity NGF receptor is a complex composed of p75 LNGF receptor and the trk protooncogene, both of which bind NGF with lower affinity than the complex (Kaplan et al., 1991*a*; Hempstead 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.

We thank Grace Dotto and Dwight Draker for excellent technical assistance, and Richard Murphy for the gift of 2.5S NGF and purified "A" chain NGF. We also thank Ann Acheson, Richard Murphy, Chako Mathew, Jean Toma, and Phil Barker for frequent discussions and advice.

This work was supported by grants from the Medical Research Council of Canada and the Canadian Network of Centers of Excellence for Neural Regeneration. Freda Miller and Robert Campenot are Scholars of the Alberta Heritage Foundation for Medical Research, and Yanling Ma is supported by a fellowship from the Alberta Paraplegic Association.

Received for publication 29 July 1991 and in revised form 12 December 1991.

References

Acheson, A., P. A. Barker, R. F. Alderson, F. D. Miller, and R. A. Murphy. 1991. Detection of brain-derived neurotropic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. *Neuron.* 7: 265–275.

Aloe, L., C. Cozzari, P. Calissano, and R. Levi-Montalcini. 1981. Somatic and behavioural postnatal effects of fetal injections of nerve growth factor antibodies in the rat. *Nature (Lond.).* 291:413–415.

Angeletti, P. U., R. Levi-Montalcini, and F. Caramia. 1971. Analysis of the effects of the antiserum to the nerve growth factor in adult mice. *Brain Res.* 92:343–355.

Barde, Y.-A., D. Edgar, and H. Thoenen. 1982. Purification of a new neurotrophic factor from mammalian brain. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:549–553.

Bernd, P., and L. A. Greene. 1984. Association of 125I-nerve growth factor with PC12 pheochromocytoma cells. Evidence for internalization via high-affinity receptor only and for long-term regulation by nerve growth factor of high- and low-affinity receptor. *J. Biol. Chem.* 259:15509–15516.

Bocchini, V., and P. U. Angeletti. 1969. The nerve growth factor: purification as a 30,000-molecular-weight protein. *Proc. Natl. Acad. Sci. USA.* 64:787–794.

Buck, C. R., H. J. Martinez, I. B. Black, and M. V. Chao. 1987. Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and brain. *Proc. Natl. Acad. Sci. USA.* 84:3060–3063.

Campenot, R. B. 1982. Development of sympathetic neurons in compartmentalized cultures. I. Local control of neurite growth by nerve growth factor. *Dev. Biol.* 93:1–12.

Campenot, R. B., and D. D. Draker. 1989. Growth of sympathetic nerve fibers

in culture does not require extracellular calcium. *Neuron.* 3:733–743.
Chun, L. L. Y., and P. H. Patterson. 1977*a*. Role of nerve growth factor in the development of rat sympathetic neurons *in vitro*. I. Survival, growth, and differentiation of catecholamine production. *J. Cell Biol.* 75:694–704.
Chun, L. L. Y., and P. H. Patterson. 1977*b*. Role of nerve growth factor in the development of rat sympathetic neurons in vitro. II. Developmental studies. *J. Cell Biol.* 75:705–711.
Crutcher, K. A., and J. P. Chandler. 1985. Evidence for sprouting specificity following medial septal lesions in the rat. *J. Comp. Neurol.* 237:116–125.
Diamond, J., M. Coughlin, L. MacIntyre, M. Holmes, and B. Visheau. 1987. Evidence that endogenous β nerve growth factor is responsible for the collateral sprouting, but not the regeneration, of nociceptive axons in the adult rats. *Proc. Natl. Acad. Sci. USA.* 84:6596–6600.
Dornay, M., V. H. Gilad, and G. M. Gilad. 1985. Compensatory changes in contralateral sympathetic neurons of the superior cervical ganglion and in their terminals in the pineal gland following unilateral ganglionectomy. *J. Neurosci.* 5:1522–1526.
Edwards, R. M., W. J. Rutter, and D. Hanahan. 1989. Directed expression of NGF to pancreatic β cells in transgenic mice leads to selective hyperinnervation of the islets. *Cell.* 58:161–170.
Feinstein, D. L., and D. Larhammar. 1990. Identification of a conserved protein motif in a group of growth factor receptors. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 272:7–11.
Gizang-Ginsberg, E., and E. B. Ziff. 1990. Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-Fos. *Genes and Dev.* 4:477–491.
Gorin, P. D., and E. M. Johnson, Jr. 1990. Effects of long-term nerve growth factor deprivation on the nervous system of the adult rat: an experimental autoimmune approach. *Brain Res.* 198:27–42.
Green, S. H., R. E. Rydel, J. L. Connolly, and L. A. Greene. 1986. PC12 mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. *J. Cell Biol.* 102: 830–843.
Hawrot, E., and P. H. Patterson. 1979. Long-term cultures of dissociated sympathetic neurons. *Methods Enzymol.* 28:574–584.
Hefti, F., H. Gnahn, M. E. Schwab, and H. Thoenen. 1982. Induction of tyrosine hydroxylase by nerve growth factor and by elevated K+ concentrations in cultures of dissociated sympathetic neurons. *J. Neurosci.* 2:1554–1566.
Hempstead, B. L., L. S. Schleifer, and M. V. Chao. 1989. Expression of functional nerve growth factor receptor after gene transfer. *Science (Wash. DC).* 243:373–375.
Hempstead, B. L., D. Martin-Zanca, D. R. Kaplan, L. F. Parada, and M. V. Chao. 1991. High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature (Lond.).* 350:678–682.
Hendry, I. A., and J. Campbell. 1976. Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. *J. Neurocytol.* 5:351-360.
Hohn, A., J. Leibrock, K. Bailey, and Y.-A. Barde. 1990. Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature (Lond.).* 334:339–341.
Hosang, M., and E. M. Shooter. 1985. Molecular characteristics of nerve growth factor receptor on PC12 cells. *J. Biol. Chem.* 260:655–662.
Johnson, D., A. Lanahan, C. R. Buck, A. Sehgal, C. Morgan, E. Mercer, M. Bothwell, and M. Chao. 1986. Expression and structure of the human NGF receptor. *Cell.* 47:545–554.
Johnson, Jr., E. M., P. D. Brandeis, and J. Pearson. 1980. Dorsal root ganglion neurons destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science (Wash. DC).* 210:916–918.
Kaplan, D. R., B. L. Hempstead, D. Martin-Zanca, M. V. Chao, and L. F. Parada. 1991*a*. The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science (Wash. DC).* 252:554–558.
Kaplan, D. R., D. Martin-Zanca, and L. F. Parada. 1991*b*. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature (Lond.).* 350:158–160.
Kessler, J. A., and I. B. Black. 1980. Nerve growth factor stimulates the development of substance P in sensory ganglia. *Proc. Natl. Acad. Sci. USA.* 77:649–652.
Klein, R., S. Jing, V. Nanduri, E. O'Rourke, and M. Barbacid. 1991. The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell.* 65:189–197.
Leibrock, J., F. Lottspeich, A. Hohn, M. Hofer, B. Hengerer, P. Masiakowski, H. Thoenen, and Y.-A. Barde. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature (Lond.).* 341:149–152.
Levi-Montalcini, R., and B. Booker. 1960*a*. Excessive growth of the sympathetic ganglia evoked by a protein isolated from mouse salivary glands. *Proc. Natl. Acad. Sci. USA.* 42:373–384.
Levi-Montalcini, R., and B. Booker. 1960*b*. Destruction of the sympathetic ganglia in mammals by an antiserum to the nerve-growth promoting factor. *Proc. Natl. Acad. Sci. USA.* 42:384–391.
Lewis, E. J., A. W. Tank, N. Weiner, and D. M. Chikaraishi. 1983. Regulation of tyrosine hydroxylase mRNA by glucocorticoid and cyclic AMP in a rat pheochromocytoma cell line. *J. Biol. Chem.* 258:14632–14637.
Lingappa, J. R., and R. E. Zigmond. 1987. A histochemical study of the adrenergic innervation of the rat pineal gland: evidence for overlap of the innervation from the two superior cervical ganglia and for sprouting following unilateral decentralization. *Neurosci.* 21:893–902.
Maisonpierre, P. C., L. Belluscio, S. Squinto, N. Y. Ip, M. E. Furth, R. M. Lindsay, and G. D. Yancopoulos. 1990. Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science (Wash. DC).* 247:1446–1451.
Martin-Zanca, D., R. Oskam, G. Mitra, T. Copeland, and M. Barbacid. 1989. Molecular and biochemical characterization of the human trk proto-oncogene. *Mol. Cell Biol.* 9:24–33.
Mathew, T. C., and F. D. Miller. 1990. Increased expression of Tα1 α-tubulin mRNA during collateral and NGF-induced sprouting of sympathetic neurons. *Dev. Biol.* 141:84–92.
Meakin, S. O., and E. M. Shooter. 1991. Molecular investigations on the high-affinity nerve growth factor receptor. *Neuron.* 6:153–163.
Miller, F. D., C. C. G. Naus, M. Durand, F. E. Bloom, and R. J. Milner. 1987. Isotypes of α-tubulin are differentially regulated during neuronal maturation. *J. Cell Biol.* 105:3065–3073.
Miller, F. D., T. C. Mathew, and J. G. Toma. 1991. Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. *J. Cell Biol.* 112:303–312.
Mobley, W. C., A. Schenker, and E. M. Shooter. 1976. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry.* 15:5543–5552.
Monroy, A. F. 1988. Staining immobilized RNA ladder. *Focus.* 10:1.
Murphy, R. A., V. Chlumecky, L. B. Smillie, M. Carpenter, M. Natriss, J. K. Anderson, J. A. Rhodes, P. A. Barker, K. Siminoski, R. B. Campenot, and J. Haskins. 1989. Isolation and characterization of a glycosylated form of beta nerve growth factor in mouse submandibular glands. *J. Biol. Chem.* 264:12502–12509.
Otten, U., M. Goedert, N. Mayer, and F. Lembeck. 1980. Requirement of nerve growth factor for development of substance P-containing sensory neurons. *Nature (Lond.).* 287:158–159.
Pleasure, S. J., U. R. Reddy, G. V. Venkatakrishnan, A. K. Roy, J. Chen, A. H. Ross, J. Q. Trojanowski, D. E. Pleasure, and V. M. Y. Lee. 1990. Introduction of NGF receptor into a medulloblastoma cell line results in expression of high- and low-affinity receptors but not NGF-mediated differentiation. *Proc. Natl. Acad. Sci. USA.* 87:8496–8500.
Purves, D., W. D. Snider, and J. T. Voyvodic. 1988. Trophic regulation of nerve cell morphology and innervation in the autonomic nervous system. *Nature (Lond.).* 336:123–128.
Radeke, M. J., T. P. Misko, C. Hsu, L. A. Herzenberg, and E. M. Shooter. 1987. Gene transfer and molecular cloning of the rat nerve growth factor receptor. *Nature (Lond.).* 325:593–597.
Rave, N., R. Crkvenjakov, and H. Boedtker. 1979. Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. *Nucleic Acids Res.* 6:3559–3567.
Raynaud, B., N. Faucon-Biguet, J. Mallet, and M. J. Weber. 1988. Regulation of neurotransmitter metabolic enzymes and tyrosine hydroxylase mRNA levels by nerve growth factor in cultured sympathetic neurons. *Development.* 102:361–368.
Rodriguez-Tebar, A., G. Dechant, and Y.-A. Barde. 1990. Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron.* 4:487–492.
Rohrer, H., U. Otten,and H. Thoenen. 1978. On the role of RNA synthesis in the selective induction of tyrosine hydroxylase by nerve growth factor. *Brain Res.* 159:436–439.
Rosenthal, A., D. V. Goeddel, T. Nguyen, M. Lewis, A. Shih, G. R. Laramee, K. Nikolics, and J. W. Winslow. 1990. Primary structure and biological activity of a novel human neurotrophic factor. *Neuron.* 4:767–773.
Ruit, K. G., P. A. Osborne, R. E. Schmidt, E. M. Johnson, Jr., and W. D. Snider. 1990. Nerve growth factor regulates sympathetic ganglion cell morphology and survival in the adult mouse. *J. Neurosci.* 10:2412–2419.
Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Isolation of cytoplasmic RNA from mammalian cells. *In* Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 7.12–7.15.
Smith, P. G., H. Reddy, and P. Venkataraman. 1990. Sympathetic sprouting into neonatally denervated contralateral target: superior cervical ganglion neuronal numbers and sizes. *Dev. Brain Res.* 53:208–214.
Snider, W. D. 1988. Nerve growth factor enhances dendritic arborization of sympathetic ganglion cells in developing mammals. *J. Neurosci.* 8:2628–2634.
Thoenen, H., P. V. Angeletti, R. Levi-Montalcini, and R. Kettler. 1971. Selective induction by nerve growth factor of tyrosine hydroxylase and dopamine-β-hydroxylase in the rat superior cervical ganglia. *Proc. Natl. Acad. Sci. USA.* 68:1598–1602.
Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragment transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201–5215.
Wright, L. L., T. J. Cunninghan, and A. J. Smolen. 1983. Developmental neuron death in the rat superior cervical sympathetic ganglion: cell counts and ultrastructure. *J. Neurocytol.* 12:727–738.