Regulation of Nerve Growth Factor Receptor Gene Expression in Sympathetic Neurons during Development

Sean Wyatt and Alun M. Davies

School of Biological and Medical Sciences, University of St. Andrews, St. Andrews, Fife KY16 9TS, Scotland

Abstract. We used quantitative reverse transcription (RT)/PCR to study the regulation of p75 mRNA and *trk*A mRNA expression in the developing sympathetic neurons of the mouse superior cervical sympathetic ganglion (SCG) in vivo and in vitro. At E13, the SCG contains proliferating cells that express many features of differentiated neurons. These immature neurons survived in culture without NGF, and NGF did not induce c-*fos* expression. Low levels of p75 and *trk*A mRNAs were expressed at this stage in vivo. There was no significant increase in the level of either *trk*A mRNA or p75 mRNA in E13 control cultures up to 72 h in vitro, and neither NGF nor depolarizing levels of K^+ ions (40 mM KCl) affected the expression of *trk*A mRNA. In E14 cultures, NGF induced c-*fos* expression in 10–15% of the neurons and enhanced the survival of a similar percentage of neurons. The proportion of neurons responding to NGF increased with age, reaching 90% in E18 cultures. The in vivo level of *trk*A mRNA increased markedly from E14 onward, but in contrast to sensory neurons (in which p75 and *trk*A mRNA levels increase in parallel), the level of *trk*A mRNA initially increased far more rapidly than that of p75 mRNA. After E17, the level of p75 mRNA increased rapidly and approached that of *trk*A mRNA postnatally, but at no stage did this exceed the level of *trk*A mRNA. In E14 cultures, the level of *trk*A mRNA increased in the absence of neurotrophins or 40 mM KCl. The level of p75 mRNA in E14 cultures was enhanced by NGF but was unaffected by 40 mM KCl. Our findings show that NGF receptor expression during the earliest stages of sympathetic neuron development is not affected by depolarization but indicate that by an early developmental stage (between E13 and E14 in vivo), sympathetic neurons become specified to upregulate *trk*A mRNA in culture independently of added factors. In addition, our findings reveal several distinctive features of p75 mRNA and *trk*A mRNA expression in sympathetic neurons compared with sensory neurons and provide a plausible explanation for previously observed differences in the effects of a p75 null mutation on the response of sensory and sympathetic neurons during embryonic and postnatal development.

NGF is the founder member of a family of homodimeric proteins termed neurotrophins that promote and regulate the survival of many kinds of neurons in the developing vertebrate nervous system (Davies, 1994). Experimental manipulation of NGF levels during development (Levi-Montalcini, 1987) and studies of mice with null mutations in the NGF gene (Crowley et al., 1994) and NGF receptor tyrosine kinase gene (Smeyne et al., 1994) have clearly shown that NGF is required for the survival of developing sympathetic neurons and a subset of sensory neurons that includes nociceptive and thermoceptive neurons.

NGF exerts its effects on responsive neurons by binding to two transmembrane glycoproteins: TrkA and p75. TrkA has a cytoplasmic tyrosine kinase domain that undergoes rapid transphosphorylation after NGF binding (Kaplan et al., 1991; Klein et al., 1991). Expression studies in cell lines (Cordon-Cardo et al., 1991) and neurons (Allsopp et al., 1993) indicate that TrkA is essential for NGF responsiveness. Although p75 is probably not a functional NGF receptor alone (Hempstead et al., 1989), in vitro studies of the action of NGF on neurons obtained from $p75^{-/-}$ mice indicate that p75 modulates the response of neurons to NGF. Compared with wild-type embryonic sensory neurons, p75-deficient embryonic sensory neurons are fourfold less sensitive to NGF in the mid-concentration range (Davies et al., 1993*b*; Lee et al., 1994). Intriguingly, the NGF dose response of embryonic p75-deficient sympathetic neurons is normal (Davies et al., 1993*b*) and only becomes shifted to higher NGF concentrations postnatally (Lee et al., 1994). How p75 enhances the response of embryonic sensory and postnatal sympathetic neurons

Address all correspondence to A. M. Davies, School of Biological and Medical Sciences, Bute Medical Building, University of St. Andrews, St. Andrews, Fife KY16 9TS, Scotland.

Sean Wyatt's present address is Department of Molecular Pathology, Windeyer Building, Cleveland Street, London W1P 6DB, England. Tel.: 01334 463219. Fax: 01334 463217.

to NGF is not known, and the basis of the age-related effects of the p75 mutation on the response of sympathetic neurons to NGF is not understood.

Detailed quantitative information on the timing and regulation of NGF receptor genes during development is currently available for the sensory neurons of the embryonic mouse trigeminal ganglion. These neurons initially survive independently of neurotrophins when their axons are growing to their peripheral targets and are briefly supported by brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), or NT4/5 when their axons reach these targets. Shortly afterward, the neurons become dependent on NGF and lose dependence on BDNF, NT3, or NT4/5 (Davies and Lumsden, 1984; Buchman and Davies, 1993; Davies et al., 1993). Although trigeminal neurons express low levels of p75 and *trk*A mRNAs from the earliest stages of their development, the acquisition of NGF dependence is correlated with marked increases in the levels of both p75 mRNA (Wyatt et al., 1990) and *trk*A mRNA (Wyatt and Davies, 1993) and the ability of the neurons to bind NGF (Davies et al., 1987). Although NGF maintains or upregulates the level of p75 mRNA in short-term cultures of E12 trigeminal neurons (the stage when many neurons acquire NGF dependence), the level of *trk*A mRNA increases in these cultures, irrespective of the presence of NGF (Wyatt and Davies, 1993), suggesting that the developmental increase in *trk*A mRNA is not induced by NGF.

The age-related effects of the p75 null mutation on the response of developing sympathetic neurons to NGF (Davies et al., 1993*b*; Lee et al., 1994) have raised the intriguing possibility that the levels of p75 and TrkA in sympathetic neurons may have different developmental patterns of expression from those in sensory neurons. Moreover, studies of the regulation of p75 and *trk*A mRNA expression in cell lines of the sympathoadrenal lineage raise the possibility that the expression of NGF receptor genes may be regulated differently in sympathetic and sensory neurons. In PC12 cells, a pheochromocytoma-derived cell line that stops dividing and differentiates into sympathetic-like neurons after NGF exposure, NGF increases the expression of both p75 mRNA (Doherty et al., 1988) and *trk*A mRNA (Holtzman et al., 1992). In MAH cells, a retrovirally immortalized sympathoadrenal progenitor cell line (Birren and Anderson, 1990), *trk*A mRNA is induced by depolarizing levels of KCl but not by NGF (Birren et al., 1992), whereas depolarizing levels of KCl do not affect *trk*A mRNA expression in developing sensory neurons (Wyatt and Davies, 1993). These observations prompted us to determine the normal developmental time-course of p75 and *trk*A mRNA expression in normal sympathetic neurons and to investigate the role of NGF and depolarization on p75 and *trk*A mRNA expression at different stages of development.

We chose to investigate p75 mRNA and *trk*A mRNA expression in the developing mouse superior cervical sympathetic ganglion (SCG).[1] This large collection of sympathetic neurons can be dissected from the embryo as early as E13, before the immature neurons acquire survival dependence on NGF (Coughlin and Collins, 1985). At this early stage, mammalian and avian sympathetic ganglia contain many dividing cells that possess a variety of neuronal characteristics, including catecholamines (Cohen, 1974), dense-core vesicles, high-affinity noradrenaline uptake (Rothman et al., 1978), tyrosine hydroxylase (Rothman et al., 1980), tetanus toxin receptor (Rohrer and Thoenen, 1987), neuron-specific antigens SCG10, B2 (Anderson and Axel, 1986) and Q211 (Rohrer and Thoenen, 1987), neurofilament protein (DiCicco-Bloom and Black, 1988), and neuritic processes (DiCicco-Bloom et al., 1990). These proliferating, neuronlike cells have been termed precursor cells (Anderson and Axel, 1986), neuroblasts (DiCicco-Bloom and Black, 1988), and immature neurons (Ernsburger et al., 1989*a*). Because these cells express so many of the properties that are used as markers for differentiated sympathetic neurons, we prefer to refer to these cells as immature sympathetic neurons (Rohrer, 1990).

Like early trigeminal neurons (Buchman and Davies, 1993; Davies et al., 1993*a*), immature sympathetic neurons appear to undergo a switch in neurotrophin dependence, in this case from NT3 to NGF. Although many immature sympathetic neurons survive for several days in culture (Ernsberger et al., 1989*a*), NT3 enhances the survival of these cells before they become dependent on NGF for survival (Birren et al., 1993; Dechant et al., 1993; DiCicco-Bloom et al., 1993). In situ hybridization and Northern blotting have shown that *trk*C mRNA expression precedes *trk*A mRNA expression in developing sympathetic ganglia, suggesting that the switch in neurotrophin responsiveness is a result of the sequential expression of NT3 and NGF receptor tyrosine kinases (Birren et al., 1993; DiCicco-Bloom et al., 1993).

In the study reported here, we show that expression of *trk*A and p75 mRNAs in immature sympathetic neurons is unaffected by depolarization or retinoic acid and that, by an early stage in their development, sympathetic neurons acquire the capacity to upregulate *trk*A mRNA expression in vitro independently of added factors. Major differences in the developmental profile of p75 and *trk*A mRNA expression in sympathetic and sensory neurons provide a plausible explanation for the age-related difference in the effects of the p75 null mutation on NGF responsiveness in developing sympathetic and sensory neurons.

Materials and Methods

Neuron Cultures

Mouse embryos were obtained from overnight matings of CD1 mice. Pregnant females were killed by cervical dislocation, and the precise stage of development of the embryos was determined by the criteria of Theiler (1972). Electrolytically sharpened tungsten needles were used to dissect SCGs from embryonic day 13 (E13) embryos to postnatal day 1 (PND1) neonates. Ganglia were incubated from 5 to 15 min (depending on age) at 37°C with 0.05% trypsin (Worthington Biochemical Corp., Freehold, NJ) in calcium- and magnesium-free HBSS. After removal of the trypsin solution, the ganglia were washed twice with 10 ml of Ham's F12 medium containing 10% heat-inactivated horse serum and were gently triturated with a fire-polished, siliconized Pasteur pipette to give a single cell suspension. The cells were plated in 35-mm plastic tissue culture dishes (1,200–2,000 neurons per dish; Nunc, Roskilde, Denmark) that had been precoated with polyornithine (0.5 mg/ml, over night) and laminin (20 μg/ml for 4 h). The cultures were incubated at 37°C in a humidified 4% CO_2 incubator. For studying the effects of NGF on neuronal survival and c-*fos* induction, Ham's F14 medium supplemented with 10% heat-inactivated horse serum (Gibco Laboratories, Grand Island, NY), 60 mg/ml penicillin, and 100 mg/

1. *Abbreviations used in this paper*: RT, reverse transcription; SCG, mouse superior cervical sympathetic ganglion.

ml streptomycin was used. For investigating the factors that regulate the expression of p75 mRNA and *trk*A mRNA expression, a defined medium consisting of Ham's F14 supplemented with 2 mM glutamine, 0.35% BSA (Pathocyte-4, ICN Biomedicals, Irvine, CA), 60 ng/ml progesterone, 16 μg/ml putrescine, 400 ng/ml L-thyroxin, 38 ng/ml sodium selenite, 340 ng/ml tri-iodothyronine, 60 mg/ml penicillin, and 100 mg/ml streptomycin was used. A limited number of experiments showed that the NGF survival response of developing SCG neurons was very similar to that for neurons grown in serum-supplemented medium and in defined medium.

Neuronal survival was estimated in control and NGF-supplemented cultures by counting the number of neurons in a 12-×-12-mm grid in the center of each 35-mm culture dish after 48 h of incubation. The difference in neuronal survival between control and NGF-supplemented cultures at different ages is expressed as the difference in the number of neurons in control and NGF-supplemented cultures (i.e., a percentage of the number of neurons in NGF-supplemented cultures). For studies of the regulation of p75 mRNA and *trk*A mRNA expression, the total number of neurons in each dish was estimated immediately before RNA was extracted by counting the number of neurons in the 12-×-12-mm grid and multiplying this number by the quotient of the total growth area of the dish and the grid area.

Immunocytochemical Detection of c-fos Protein

To provide an additional measure of the proportion of SCG neurons that respond to NGF at each age, immunocytochemistry was used to estimate the proportion of neurons that express the nuclear proto-oncogene c-*fos* in response to NGF. For these studies, the neurons were plated in Petri dishes containing 4-×-11-mm diameter wells (Greiner, Frickenhausen, Germany) at a density of 500 cells per well in a final volume of 120 μl of F14 medium supplemented with 10% heat-inactivated horse serum. After NGF exposure, the cultures were rinsed twice with PBS and were fixed in fresh 4% paraformaldehyde in PBS for 17 min at room temperature. After two additional rinses with PBS, the cultures were permeabilized with 0.3% Triton X-100 in PBS for 30 min and were incubated overnight at 4°C with rabbit polyclonal c-*fos* antiserum diluted in PBS containing 0.3% Triton. Two c-*fos* antisera were used in separate experiments: an antibody raised against a synthetic peptide corresponding to the NH_2-terminal sequence of c-*fos* (Hunt et al., 1987) was obtained from G. Evan (ICRF, London) and was used at a dilution of 1:4,000; and another antibody also directed against the NH_2-terminal domain of c-*fos* was obtained from Oncogene Science (c-*fos*/Ab-2; Manhasset, NY) and was used at a dilution 1:100. After incubation with primary antiserum, the cultures were rinsed with PBS and were incubated with biotinylated goat anti-rabbit antiserum diluted 1:500 in PBS for 3 h at room temperature followed by additional rinses with PBS and incubation for 30 min at room temperature with an avidin/biotinylated HRP macromolecular complex (Vector Laboratories, Burlingame, CA). After the cultures were rinsed with PBS, peroxidase activity was localized by incubation with a 0.5 mg/ml DAB solution in PBS containing 0.01% hydrogen peroxide, 0.01% nickel chloride, and 0.01% cobalt chloride. The reaction was stopped after 45–60 s by flooding the cultures with PBS. The cultures were then mounted in Aquamount (BDH Chemicals, Poole, England) beneath 10-mm diameter coverslips (Chance Propper, London, UK) and were viewed with a Zeiss Axioskop microscope after the walls of the Petri dishes were removed.

c-*fos* expression was recognized by intense nuclear staining. The proportion of neurons that expressed c-*fos* immunoreactivity was determined by examining several microscope fields selected at random. Each field was examined with phase contrast optics to count the total number of neurons and with bright-field optics to count the number of neurons with darkly stained nuclei. At least 100 neurons in each well were assessed in this manner to determine the proportion of c-*fos* positive neurons.

Measurement of trkA mRNA and p75 mRNA Levels by Quantitative RT/PCR

A quantitative RT/PCR technique (Wyatt and Davies, 1993) was used to measure the very low levels of *trk*A mRNA and p75 mRNA in SCG neuron cultures and dissected whole ganglia. The RT reaction and PCR were calibrated by the inclusion of control RNA templates in the RT reaction. The control RNA templates were transcribed in vitro from *trk*A and p75 cDNA clones that had been modified by the insertion between the PCR primer sites of 3 bp in the case of *trk*A cDNA and 4 bp in the case of p75 cDNA. Because the target mRNA and its control RNA template are present in the same RT reaction and PCR and use the same primers, the variables that affect amplification efficiency are nullified. To generate the p75 cDNA control template from which the control RNA template was transcribed, a 597-bp fragment of the mouse p75 cDNA corresponding to nucleotides 426 to 1023 of the rat p75 cDNA was isolated from mouse brain total RNA using RT/PCR and was cloned into pGEM 3Z (Promega Biotec, Madison, WI). The 601-bp p75 cDNA control template was constructed by cleaving the cloned p75 cDNA at a single AvaI site to generate DNA with 4 bp of 5′ overhang at each end. These overhangs were filled in with the Klenow fragment of DNA polymerase I in the presence of 5 mM dNTPs and ligating the resulting blunt ends. The control p75 cRNA template was synthesized by in vitro transcription of the control p75 cDNA from the Sp6 RNA polymerase of the pGEM vector. To generate the *trk*A cDNA control template, a 452-bp fragment of the mouse *trk*A cDNA corresponding to nucleotides 838 to 1290 was cloned into pGEM 3Z and was cleaved at a single internal PpUMI site to generate DNA with 3 bp of 5′ overhang at each end that were filled in and ligated as before. The resulting 455-bp cDNA was transcribed from the Sp6 RNA polymerase of the pGEM vector to produce the *trk*A RNA control template.

Total RNA (Chomczynski and Sacchi, 1987), spiked with known amounts of the appropriate control RNA, was reverse transcribed for 45 min at 37°C with GIBCO BRL Superscript enzyme (Gaithersburg, MD) in a 10 μl reaction containing the manufacturer's buffer supplemented with 0.5 mM dNTPs and 10 μM random hexanucleotides. Each RT reaction was then gently mixed with a 40-μl PCR solution comprising 1 × NBL Taq DNA polymerase buffer (with an additional 0.4 mM $MgCl_2$ in the case of p75), 1 U of NBL Taq DNA polymerase, 40 ng of 5′ end-labeled primers, and 0.1 mM dNTPs. The primers for p75 were (5′) 5′-CCGATACAGTGACCACTGTGATG-3′ and (3′) 5′-AGCAGCCAAGATGGAGCAATAGAC-3′. These hybridize 97 bp apart in the sequence of mouse p75 cDNA and 101 bp apart in the p75 control cDNA. The primers for *trk*A were (5′) 5′-CGTCATGGCTGCTTTTATGG-3′ and (3′) 5′-ACTGGCGAGAAGGAGACAG-3′. These hybridize 75 bp apart in the sequence of mouse *trk*A cDNA and 78 bp apart in *trk*A control cDNA.

p75 cDNA was amplified by eight cycles of 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s, followed by 15 cycles of 91°C for 60 s, 56°C for 45 s, and 72°C for 60 s. *trk*A cDNA was amplified by eight cycles of 94°C for 60 s, 56°C for 45 s, and 72°C for 45 s, followed by 12 cycles of 90°C for 60 s, 55°C for 60 s, and 72°C for 60 s. These conditions were optimal for RT and amplification of 1 fg of *trk*A and p75 control transcripts, respectively, such that the rate of reaction did not plateau. If reactions were allowed to reach the plateau phase, the formation of heteroduplex DNA products during this phase altered the ratio between homoduplex products, leading to inaccuracies.

The PCR products of the control and native cDNA templates were resolved on 7% nondenaturing polyacrylamide gels that were dried and autoradiographed. Reactions were set up such that the autoradiographic signals from the PCR products of the native and control cDNA templates were approximately equal. The autoradiographs were scanned with a densitometer (Personal Laser, Molecular Dynamics, Inc., Sunnyvale, CA), and the intensity of the respective signals was ascertained using ImageQuant software (Molecular Dynamics). These values enabled the levels of p75 mRNA or *trk*A mRNA in the initial total RNA sample to be calculated.

To compare the relative levels of p75 and *trk*A mRNAs in different preparations of purified neurons, the level of the mRNA encoding the ubiquitous, constitutively expressed L27 ribosomal protein was also measured in RNA samples from these neurons by quantitative RT/PCR. To determine the level of L27 mRNA, 10-μl aliquots of reverse transcribed neuronal total RNA were added to 50-μl PCRs containing NBL Taq DNA polymerase in the supplied buffer plus 5 μl of the following labeled primers: (5′)5′-GAAGAACATCGATGATGGCAC-3′ and (3′) 5′-TAGCGGTCGATTCCAGCCACC-3′. These hybridize 75 bp apart in the L27 sequence and were labeled as described for the *trk*A and p75 primers. L27 cDNA was amplified by 25 cycles of 92°C for 60 s, 55°C for 60 s, and 72°C for 60 s. There was a final 72°C incubation for 10 min. These conditions are optimal for RT and amplification of 500 fg of L27 gene run-off transcripts such that the rate of reaction does not plateau. The level of L27 mRNA in the extracted RNA was quantified by coamplifying with standard run-off transcripts from the L27 cDNA, which have an additional 4-bp insert between the primer annealing sites.

Purification of SCG Neurons

For measurement of p75 mRNA and *trk*A mRNA levels in purified SCG neurons, these neurons were separated from satellite cells in E16 and older SCGs by low-temperature, differential sedimentation (Davies,

1986). Briefly, SCGs were dissected in ice-cold HBSS, incubated with 0.05% trypsin (Worthington Biochemical Corp.) in phosphate-buffered saline for 10 min at 37°C, washed in HBSS containing 10% heat-inactivated horse serum (HIHS), and dissociated in serum-free HBSS by gentle trituration with a fire-polished Pasteur pipette. The resulting cell suspension was layered on a precooled column of HBSS containing 10% HIHS in a dropping funnel at 2°C. After 60 min, aliquots were removed and the fractions containing neurons were identified by phase-contrast microscopy. The neurons were pelleted in siliconized Eppendorf tubes, frozen, and stored at −80°C until required.

Results

To determine when SCG neurons start responding to NGF during development, two variables of NGF responsiveness were studied—neuronal survival and c-*fos* induction.

Developmental Changes in the Survival Response of SCG Neurons to NGF

Dissociated, low-density cultures of E13 to E18 SCG neurons were grown in control cultures and cultures containing 10 ng/ml NGF. In E13 cultures, the majority of the immature neurons survived for 48 h of incubation in control cultures, there was no difference in the number of neurons in control and NGF-supplemented cultures. In E14 cultures, 15–20% more neurons were surviving in NGF-supplemented cultures after 48 h of incubation compared with control cultures (Fig. 1 *A*). The magnitude of the NGF survival response increased with age, and by E18 the great majority of neurons were surviving with NGF (Fig. 1 *A*). Thus, the first SCG neurons become dependent on NGF for survival at or shortly after E14, and the majority of the remaining neurons become NGF dependent over the next 5 days of development.

The dose response of E18 SCG neurons to NGF is shown in Fig. 1 *B*. Interpolation of the data revealed a half-maximally effective NGF concentration of 134 pg/ml. Because saturation was reached at a concentration of 10 ng/ml, this level of NGF was used in subsequent experiments.

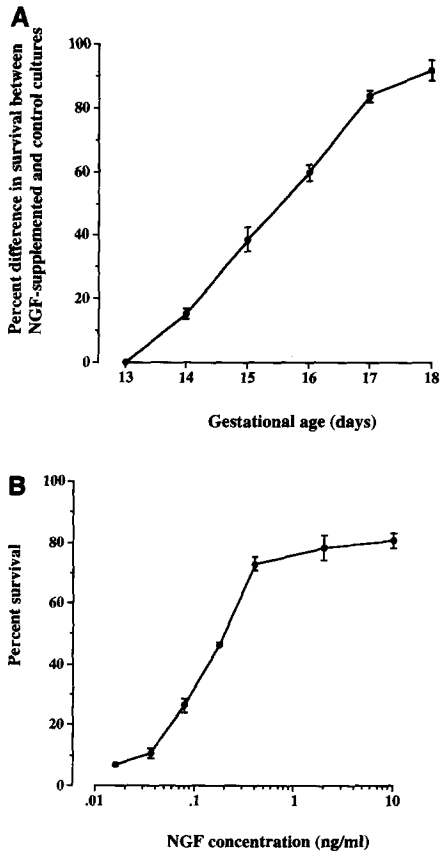

Figure 1. Survival response of developing SCG neurons to NGF. (*A*) Cultures of E13 to E18 SCG neurons were grown under control conditions (no NGF) and with NGF (10 ng/ml) for 48 h of incubation, when the number of surviving neurons were counted. The difference between survival in NGF-supplemented cultures and control cultures is expressed as a percentage of the survival in NGF-supplemented cultures. The means and the standard errors of three separate experiments are plotted. (*B*) E18 SCG neurons were grown with different concentrations of NGF, and the percentage of survival was estimated after 48 h of incubation. The means and the standard errors of three separate experiments are plotted.

Developmental Changes in c-fos Induction by NGF

Initial experiments showed that 2 h of NGF exposure induced c-*fos* expression in about 80% of E18 SCG neurons, whereas <1% of the neurons were labeled in control cultures (no NGF). The same results were obtained with both c-*fos* antisera, but because the c-*fos* antiserum obtained from G. Evan gave the most intense nuclear staining, this was used in subsequent experiments. No nuclear staining was observed when the c-*fos* antiserum was omitted.

To determine the time-course of c-*fos* induction, NGF was added to E18 SCG neurons 2 h after plating. After incubation periods with NGF ranging from 15 min to 6 h, the cultures were processed for c-*fos* immunoreactivity. Expression of c-*fos* was first discernible in a small number of neurons after 30 min of NGF exposure, increased to maximum after 3 h of NGF exposure and declined thereafter (Fig. 2 *A*). The intensity of nuclear staining was also maximal after 3 h. For these reasons, neurons were exposed to NGF for 3 h in subsequent studies of c-*fos* expression. The c-*fos* induction dose response of E18 SCG neurons to NGF is shown in Fig. 2 *B*. Interpolation of the data revealed a half-maximally effective NGF concentration of 118 pg/ml, which is very similar to the half-maximally effective NGF concentration for promoting neuronal survival.

To ascertain the developmental changes in c-*fos* induction by NGF, cultures of SCG neurons were set up at daily intervals from E13 to E18. The neurons were grown either in the absence of NGF (control cultures) or with 10 ng/ml NGF. After 3 h of incubation, the cultures were fixed and processed for c-*fos* immunoreactivity. In E13 cultures, NGF did not induce c-*fos* expression in any neurons. In E14 cultures, NGF induced c-*fos* expression in ~10% of the neurons. The percentage of c-*fos* induction by NGF increased with age to reach >80% in E17 and E18 cultures (Fig. 3). Thus, both the survival response of SCG neurons to NGF and the induction of c-*fos* by NGF follow the same developmental time course, indicating that the earliest neurons respond to NGF at or shortly after E14.

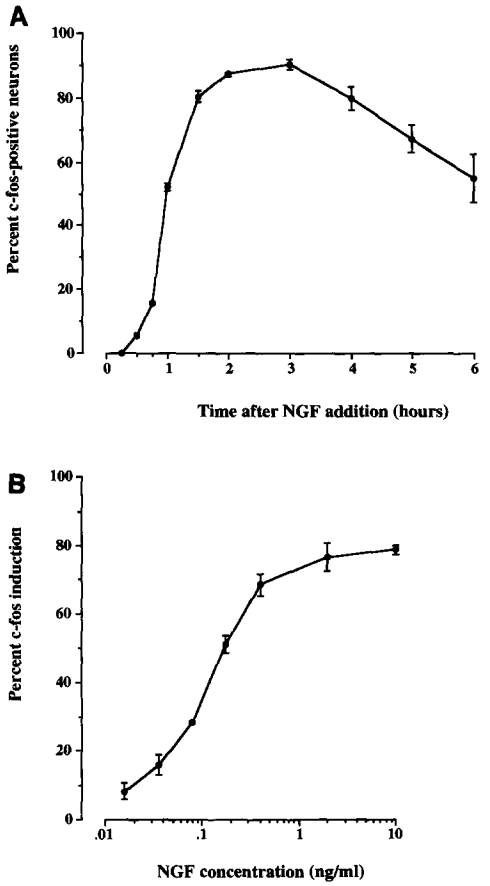

Figure 2. Induction of c-*fos* in cultured SCG neurons by NGF. (*A*) Time-course of c-*fos* induction by NGF in E18 SCG neurons. The percentage of c-*fos*–positive neurons is plotted against time of NGF exposure. The means and the SEMs of three separate estimates of c-*fos* induction are plotted. (*B*) c-*fos* induction dose–response of E18 SCG neurons to NGF. E18 SCG neurons were exposed to different concentrations of NGF for 3 h before fixation and estimation of the percentage of c-*fos*–positive neurons. The means and the standard errors of three separate experiments are plotted.

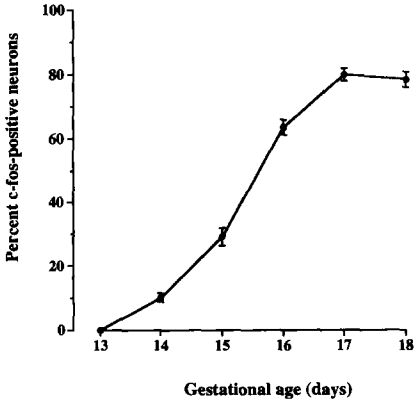

Figure 3. Age-related changes in c-*fos* induction in developing SCG neurons by NGF. Cultures of E13 to E18 SCG neurons were exposed to NGF for 3 h. The percentage of c-*fos*–positive neurons is plotted at each age. The means and the standard errors of three separate estimates of c-*fos* induction are plotted.

Developmental Time Course of p75 mRNA and trkA mRNA Expression in SCGs

Quantitative RT/PCR was carried out to determine levels of p75 mRNA and *trk*A mRNA in SCGs at daily intervals from E13 to PND1. The sensitivity, specificity, and accuracy of the competitive RT/PCR technique used for quantifying the absolute levels of these mRNAs have been documented previously (Wyatt and Davies, 1993). Low levels of p75 mRNA and *trk*A mRNA were present in the SCGs at E13 and E14 (Fig. 4). Although the levels of p75 mRNA and *trk*A mRNA were similar at E14, between E14 and E15, the level of *trk*A mRNA increased more than sixfold. In contrast, the level of p75 mRNA showed only a small increase over the same period (Fig. 4). The level of *trk*A mRNA continued to increase with age, reaching almost 100 pg per ganglion just before birth. In contrast with *trk*A mRNA, the level of p75 mRNA increased only very gradually until E17, when its level was only 15% of the *trk*A mRNA level. After E17, the level of p75 mRNA increased more rapidly in the ganglion, reaching 38% of the *trk*A mRNA level by birth (Fig. 4). A limited number of assays at later ages indicated that the relative level of p75 mRNA reached 60% of that of *trk*A mRNA by PND3 (data not shown).

Although the majority of cells in the early sympathetic chain are proliferating immature sympathetic neurons (Rohrer and Thoenen, 1987; Ernsberger et al., 1989*a*, 1989*b*; Rohrer, 1990), at later ages an increasing proportion of satellite cells is present in the ganglion. To determine if p75 mRNA and *trk*A mRNA are expressed predominantly in neurons, differential sedimentation was used to prepare purified neurons (greater than 95% neurons) from E17 and newborn SCGs. RT/PCR assays using RNA extracted from these neurons and from whole ganglia at the same age showed that the relative levels of *trk*A mRNA to p75 mRNA were very similar in purified neurons and whole ganglia (Fig. 5). These findings indicate that the relative levels of *trk*A mRNA to p75 mRNA observed in whole ganglia during development (Fig. 4) accu-

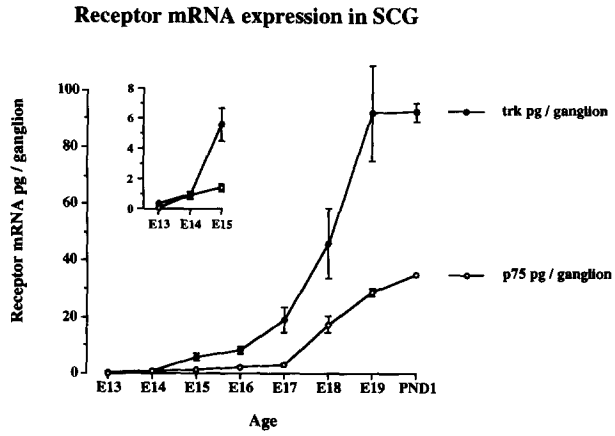

Figure 4. Graph of the levels of p75 mRNA and *trk*A mRNA per SCG from E13 to PND1. The inset shows in detail the changes occurring in p75 mRNA and *trk*A mRNA expression occurring between E13 and E15. The means ± SEM of at least four separate measurements at each age are shown.

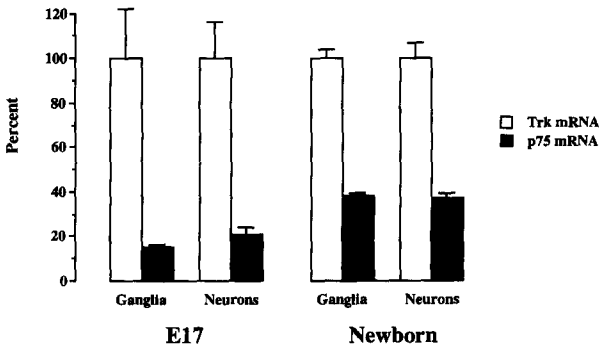

Figure 5. Bar chart of the relative levels of p75 mRNA and *trk*A mRNA in whole SCG and purified SCG neurons from E17 embryos and postnatal day 1 neonates. In each of three separate preparations of neurons, there was <5% contamination by satellite cells. The relative levels of p75 and *trk*A mRNAs in different neuronal preparations were determined by standardizing the levels of p75 and *trk*A mRNAs to the level of L27 mRNA in these preparations. To compare the relative levels of mean levels of p75 mRNA and *trk*A mRNA in whole ganglia and purified neurons, the mean levels of *trk*A mRNA were normalized to 100%. The means ± SEM for three separate estimations of p75 mRNA and *trk*A mRNA in ganglia and purified neurons are plotted.

rately reflect relative levels of these mRNAs in the sympathetic neurons.

Regulation of p75 mRNA and trkA mRNA Expression

To investigate the extent to which the developmental increases in *trk*A mRNA and p75 mRNA expression are influenced by NGF or depolarization, low density cultures of the immature neurons in E13 and E14 SCG were studied at intervals in culture. An advantage of studying neurotrophin receptor expression in these early neuronal cultures is that the majority of the immature neurons survive for several days in the absence of NGF (more than 3 d in E13 cultures and more than 2 d in E14 cultures). Also, because at this stage the SCGs consist predominantly of immature neurons, there is negligible contamination by nonneuronal cells in these cultures. Positive identification of immature neurons by neurofilament immunocytochemistry (Buchman and Davies, 1993) showed that the great majority of cells in early SCG cultures were neurons. In E14 cultures, nonneuronal cells constituted only 2.8 ± 0.4% (mean ± SEM) of the cells after 24 h in defined medium and 3.0 ± 0.6% of the cells after 48 h.

In each experiment, approximately the same number of cells were plated in 35-mm culture dishes (<5% variation between dishes in each experiment). Typically, the number of cells plated ranged between 1,200 and 2,000 cells per dish in different experiments. There was no significant difference ($P > 0.05$, Student's t test) in the number of immature neurons grown under different experimental conditions in E13 cultures, and in E14 cultures ~20% more neurons died in control cultures between 24 and 48 h compared with cultures supplemented with NGF at concentrations >0.4 ng/ml. Because the number of neurons in each dish was counted before RNA extraction, differences in neuron number between dishes were taken into account in calculating the mean level of p75 mRNA and *trk*A mRNA per neuron under different experimental conditions. The levels of p75 mRNA and *trk*A mRNA were measured in all dishes.

trkA mRNA

The low mean neuronal level of *trk*A mRNA expression in E13 control cultures (no added factors) was unchanged between 24 and 72 h in vitro (Fig. 6 *A*). In these control cultures <1% of the neurons died between 24 and 48 h and 40.3 ± 3.2% (mean ± SEM) of the neurons were lost between 48 and 72 h. In contrast, in E14 control cultures the mean neuronal level of *trk*A mRNA increased about threefold between 24 and 48 h in vitro (Fig. 7 *A*). In these experimental cultures, 27 ± 6.7% of the neurons died between 24 and 48 h.

Because neurons increase in size with time in culture and the total amount of RNA per neuron increases concomitantly, it is possible that the increase in *trk*A mRNA levels that we observed between 24 and 48 h in E14 cul-

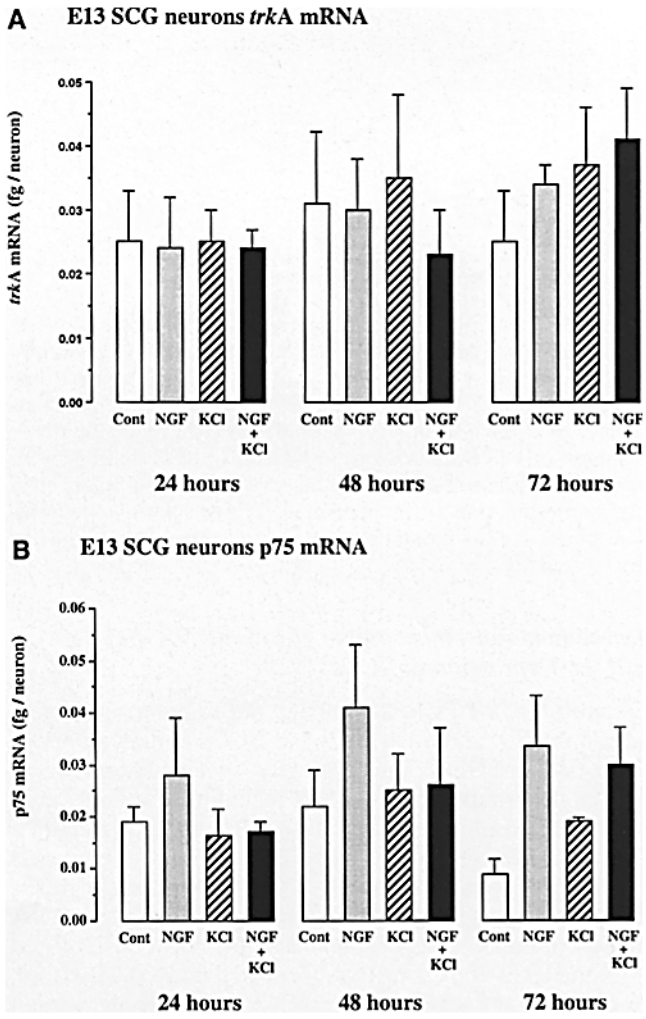

Figure 6. Bar charts of the levels of (*A*) *trk*A mRNA and (*B*) p75 mRNA per neuron in cultures of E13 SCG neurons grown for 24, 48, and 72 h in unsupplemented culture medium (*Cont*), with 10 ng/ml NGF, 40 mM KCl, or NGF plus KCl. The bar charts show the mean and standard error of results from three separate experiments (each with triplicate culture dishes for each condition at each time point).

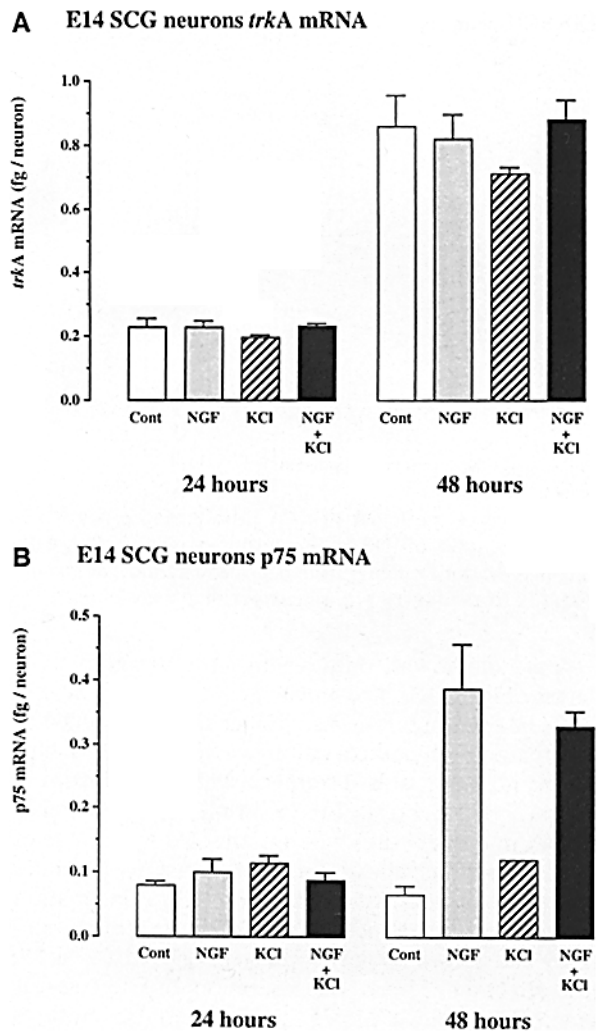

Figure 7. Bar charts of the levels of (*A*) *trk*A mRNA and (*B*) p75 mRNA per neuron in cultures of E14 SCG neurons grown for 24 and 48 h in unsupplemented culture medium (*Cont*), with 10 ng/ml NGF, 40 mM KCl, or NGF plus KCl. The bar charts show the mean and standard error of results from three separate experiments (each with triplicate culture dishes for each condition at each time point).

tures was nonspecific and reflected a growth-related increase in total mRNA content per neuron. To address this issue, we used RT/PCR to measure the levels of L27 mRNA (which codes for a ubiquitous, constitutively expressed ribosomal protein) in E14 cultures after 24 and 48 h of incubation. In contrast with the threefold increase in *trk*A mRNA levels observed between 24 and 48 h, there was only a 32% increase in the level of L27 mRNA (from 112.4 ± 12.4 fg to 148.3 ± 24.4 fg per neuron). Thus, the increase in *trk*A mRNA observed in E14 cultures is not a consequence of a nonspecific growth-related increase in total mRNA content.

The presence of NGF did not affect the expression of *trk*A mRNA in either E13 or E14 cultures. There was no significant difference between the mean neuronal level of *trk*A mRNA in control cultures and cultures containing NGF at matching time points in E13 cultures and at matching time points in E14 cultures ($P > 0.05$, *t* test). The increase in *trk*A mRNA expression that occurs in E14 neurons between 24 and 48 h was of the same magnitude in the presence and absence of NGF. Thus, the developmental increase in *trk*A mRNA expression is independent of NGF. NGF was used at concentrations ranging from 0.016 ng/ml to 250 ng/ml (0.016, 0.08, 0.4, 10, 50, and 250 ng/ml). Because there was no significant difference in the levels of *trk*A mRNA in different concentrations of NGF used (data not shown), only results with 10 ng/ml NGF are shown (Figs. 6 *A* and 7 *A*). At later ages, when the neurons become increasingly dependent on NGF for survival, exposing neurons to a wide range of NGF concentrations above the minimum required for the maximal neuronal survival did not significantly affect the expression of *trk*A mRNA (Fig. 9).

Depolarizing levels of KCl (40 mM) did not increase *trk*A mRNA expression in either E13 or E14 cultures (Fig. 6 *A* and 7 *A*). There was no significant difference between the mean neuronal level of *trk*A mRNA in control cultures and cultures containing 40 mM KCl at the same time points in E13 cultures ($P > 0.05$, *t* test). The level of *trk*A mRNA was slightly lower in E14 cultures containing 40 mM KCl compared with control cultures at this age. Furthermore, exposure of E13 or E14 cultures to 40 mM KCl plus NGF did not significantly increase the expression of *trk*A mRNA compared with cultures containing NGF alone ($P > 0.05$, *t* test). In E18 cultures, when the great majority of neurons depend on NGF for survival, the mean neuronal level of *trk*A mRNA was not significantly different between cultures containing NGF alone and cultures containing NGF plus 40 mM KCl ($P > 0.05$, *t* test) after both 24 and 48 h of incubation (Fig. 8 *A*).

Because retinoic acid has been shown to induce NGF survival dependence and high-affinity NGF receptors in immature sympathetic neurons from chicken embryos (Rodriguez-Tebar and Rohrer, 1991), we investigated whether retinoic acid induces *trk*A mRNA expression in immature sympathetic neurons from mouse embryos. E13 SCG cultures were grown with a range of retinoic acid concentrations with and without NGF in the culture medium. For all experiments, a fresh batch of retinoic acid was dissolved in the dark in a small volume of ethanol immediately before being diluted in culture medium (control cultures received the same volume of ethanol). Because of the light sensitivity of retinoic acid, the cultures were kept in the dark until RNA extraction. After 48 h of incubation, the level of *trk*A mRNA in control cultures (0.067 ± 0.010 fg/neuron) was very similar to that in cultures containing retinoic acid alone at concentrations of 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M (0.055 ± 0.004, 0.052 ± 0.009, 0.053 ± 0.015, and 0.037 ± 0.008, respectively; mean ± SEM) and cultures containing the same concentrations of retinoic acid plus 10 ng/ml NGF (0.068 ± 0.002, 0.057 ± 0.009, 0.060 ± 0.004, and 0.040 ± 0.008, respectively; $n = 3$ per experimental condition). Thus, retinoic acid does not induce *trk*A mRNA expression in immature sympathetic neurons.

Because NT3 promotes the survival of immature sympathetic neurons (Birren et al., 1993; Dechant et al., 1993; DiCicco-Bloom et al., 1993), we also investigated the effect of NT3 on *trk*A mRNA expression. E13 SCG cultures were grown without added neurotrophins and with 2 ng/ml

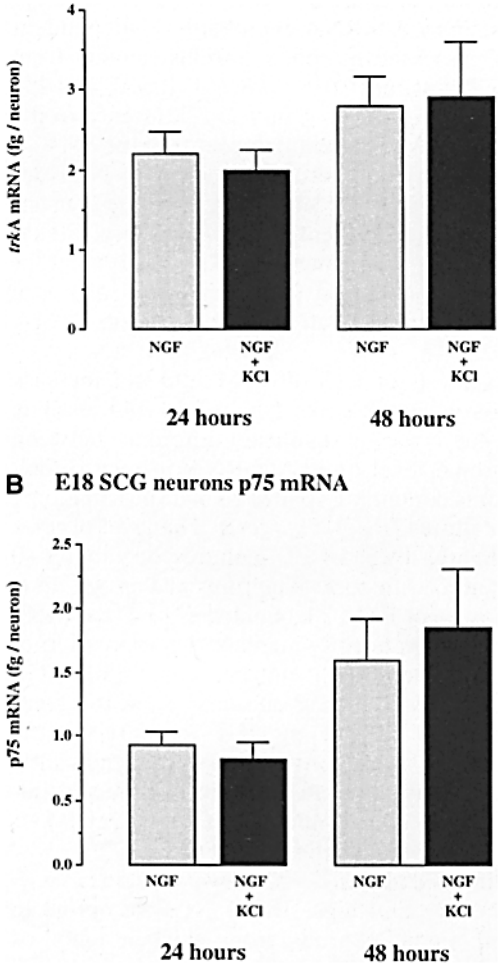

Figure 8. Bar charts of the levels of (*A*) *trk*A mRNA and (*B*) p75 mRNA per neuron in cultures of E18 SCG neurons grown for 24 and 48 h with 10 ng/ml NGF or NGF plus 40 mM KCl. The bar charts show the mean and standard error of results from three separate experiments (each with triplicate culture dishes for each condition at each time point).

NT3. In these experiments, the level of *trk*A mRNA in control cultures after 48 h was 0.032 ± 0.013 fg/neuron compared with 0.035 ± 0.017 fg/neuron in cultures containing NT3, indicating that NT3 at this concentration does not induce *trk*A mRNA expression.

p75 mRNA

In contrast to the lack of effect of NGF on *trk*A mRNA expression in early sympathetic neurons, NGF affected the expression of p75 mRNA in E14 cultures. Although there was little difference in the mean neuronal level of p75 mRNA in control and NGF-supplemented cultures after 24 h of incubation in E14 cultures, the mean neuronal level of p75 mRNA in NGF-supplemented cultures had increased to almost sixfold of its level in control cultures (Fig. 7 *B*). NGF was used at concentrations ranging from 0.016 ng/ml to 250 ng/ml (0.016, 0.08, 0.4, 10, 50, and 250 ng/ml). Because the effect of NGF on p75 mRNA induction is maximal at concentrations of NGF in excess of 0.4

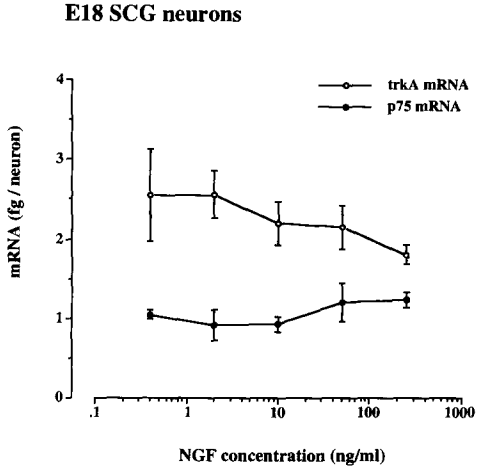

Figure 9. Graphs of the levels of *trk*A mRNA and p75 mRNA per neuron in cultures of E18 SCG neurons grown for 24 h with NGF at concentrations ranging from 0.4 to 250 ng/ml. The means and standard errors of three separate experiments are plotted.

ng/ml (data not shown), only results with 10 ng/ml NGF are shown (Fig. 7 *B*). The mean neuronal level of p75 mRNA in E13 cultures was also higher in the presence of NGF compared with control cultures after 48 and 72 h of incubation; however, this difference was not statistically significant ($P > 0.05$, *t* test). Like *trk*A mRNA, the level of p75 mRNA in E18 cultures was not affected by a range of NGF concentrations above those required to promote maximal neuronal survival after either 24 h of incubation (Fig. 9), or 48 or 72 h of incubation (data not shown).

At E13 and E14, depolarizing levels of KCl (40 mM) had little effect on p75 mRNA expression. In E13 cultures the mean neuronal level of p75 mRNA in control cultures was not significantly greater than in cultures containing 40 mM KCl after 24 and 48 h ($P > 0.05$, *t* test) (Fig. 6 *B*). Although the level of p75 mRNA was slightly higher in E14 cultures containing 40 mM KCl compared with control cultures, this was negligible compared with the marked effect of NGF on the expression of p75 mRNA after 48 h of incubation (Fig. 7 *B*). In E18 cultures, the mean neuronal level of p75 mRNA was not significantly different between cultures containing NGF alone and cultures containing NGF plus 40 mM KCl ($P > 0.05$, *t* test; Fig. 8 *B*).

We also investigated whether retinoic acid induces p75 mRNA expression in immature sympathetic neurons. E13 SCG cultures were grown with a range of retinoic acid concentrations with and without NGF in the culture medium. After 48 h of incubation, the level of p75 mRNA in control cultures (0.046 ± 0.017 fg/neuron) was greater than in cultures containing retinoic acid alone at concentrations of 10^{-9} M, 10^{-8} M, 10^{-7} M, and 10^{-6} M (0.038 ± 0.006, 0.027, 0.014 ± 0.0005, and 0.017 ± 0.009, respectively; mean ± SEM). Likewise, the level of p75 mRNA in cultures containing 10 ng/ml NGF (0.072 ± 0.003 fg/neuron) was greater than in cultures containing NGF plus the same range of retinoic acid concentrations (0.067 ± 0.011, 0.048 ± 0.014, 0.037 ± 0.007, and 0.025 ± 0.006, respectively; $n = 3$ per experimental condition). Thus, retinoic acid does not induce the expression of p75 mRNA in immature sympathetic neurons.

We also investigated whether NT3 induces p75 mRNA expression in immature sympathetic neurons by culturing E13 SCG neurons with and without NT3. In these experiments, the level of p75 mRNA in control cultures after 48 h was 0.023 ± 0.006 fg/neuron compared with 0.029 ± 0.011 fg/neuron in cultures containing 2 ng/ml NT3, indicating that NT3 at this concentration does not significantly increase the expression of p75 mRNA in immature sympathetic neurons.

Discussion

Acquisition of NGF Responsiveness

In agreement with previous studies of developing sympathetic neurons in rodents (Coughlin and Collins, 1985; DiCicco-Bloom and Black, 1988) and birds (Rohrer and Thoenen, 1987; Ernsberger et al., 1989*a*; Rodriguez-Tebar and Rohrer, 1991), we have shown that the survival of immature sympathetic neurons is initially independent of NGF. In E13 mouse SCG cultures, the majority of immature neurons survive independently of NGF for at least 3 d and show no enhanced survival with NGF. NGF begins to enhance the survival of a small percentage of neurons in E14 cultures. The proportion of SCG neurons that depend on NGF for survival increases in cultures established at later embryonic ages, reaching 90% in E18 cultures. Acquisition of the NGF survival response correlates with c-*fos* induction by NGF. With both developmental age and NGF concentration, the proportion of neurons that survive with NGF and express c-*fos* in response to NGF is very similar. Thus, by two criteria, survival and c-*fos* induction, the first SCG neurons start responding to NGF at or shortly after E14.

Developmental Expression of trkA mRNA and p75 mRNA

Using quantitative RT/PCR, we determined the levels of *trk*A mRNA and p75 mRNA in the developing SCGs. At E13, the levels of these mRNAs are very low, which is in accord with the unresponsiveness of cultured SCG neurons to NGF at this early stage of development. Between E14 and E15, there is a sixfold increase in the level of *trk*A mRNA, which is closely related to the onset of NGF responsiveness in a proportion of the neurons in vitro at this stage. In marked contrast to the NGF-dependent sensory neurons of the developing mouse trigeminal ganglion, in which the levels of *trk*A mRNA and p75 mRNA are very similar and increase in parallel with the acquisition of the NGF survival response (Wyatt and Davies, 1993), the increases in *trk*A mRNA and p75 mRNA are out of step with one another in the developing SCG. The level of *trk*A mRNA initially increases much more rapidly than that of p75 mRNA, so that by E17 the level of *trk*A mRNA is almost sevenfold higher than that of p75 mRNA. However, from E17 onward the level of p75 mRNA increases more rapidly. The difference between *trk*A mRNA and p75 mRNA is only threefold by birth (Fig. 4) and less than twofold by the fourth postnatal day (data not shown). The relative levels of *trk*A mRNA and p75 mRNA in whole ganglia are likely to reflect the relative levels of these mRNAs in individual SCG neurons because a similar ratio is observed between *trk*A mRNA and p75 mRNA in purified neuronal preparations and whole ganglia at the same stage of development. Although it was not possible to use differential sedimentation to obtain purified neurons from SCGs before E16, several studies have shown that early sympathetic ganglia are composed mainly of cells expressing neuronal markers (Rohrer and Thoenen, 1987; DiCicco-Bloom and Black, 1988; Ernsberger et al., 1989*b*). Thus, at stages before purified neurons can be obtained, the relative levels of *trk*A mRNA and p75 mRNA in whole ganglia are also likely to reflect the relative levels of these mRNAs in early sympathetic neurons.

The different developmental profiles of *trk*A mRNA and p75 mRNA expression in sensory and sympathetic neurons suggest an explanation for the age-related differences in the effect of a null mutation in the p75 gene on the NGF survival response of sensory and sympathetic neurons. Trigeminal sensory neurons of $p75^{-/-}$ embryos are threefold less sensitive to NGF in the mid-concentration range than are trigeminal neurons of wild-type embryos, whereas the NGF dose response of embryonic SCG neurons is unaffected by this mutation (Davies et al., 1993*b*). However, by the postnatal period, the dose response of p75-deficient SCG neurons has shifted to higher NGF concentrations compared with wild-type SCG neurons (Lee et al., 1994). Likewise, mutant NGF molecules that have negligible binding to p75 but bind TrkA normally are less active on embryonic trigeminal and postnatal sympathetic neurons than are wild-type NGF, whereas mutant and wild-type NGF molecules are equally active on embryonic sympathetic neurons (A. Horton, J. Winslow, and A. M. Davies, unpublished data). Although we do not know the relationship between mRNA and protein levels for TrkA and p75 in developing sensory and sympathetic neurons, our present findings suggest that embryonic sympathetic neurons are unaffected by the p75 null mutation because these neurons normally express low levels of p75 relative to TrkA. Embryonic trigeminal and postnatal sympathetic neurons are affected by the p75 mutation because they normally express relatively higher levels of p75 than embryonic sympathetic neurons.

In situ hybridization (Birren et al., 1993) and Northern blotting (DiCicco-Bloom et al., 1993) have shown that the expression of *trk*A mRNA increases markedly during embryonic development as sympathetic neurons acquire a survival response to NGF. More recently, Verdi and Anderson (1994) used RT/PCR to compare the levels of p75 and *trk*A mRNAs in immature sympathetic neurons (neuroblasts) and sympathetic ganglia with the levels of these mRNAs in PC12 cells. Although these authors failed to detect p75 and *trk*A mRNAs in neuroblasts from early sympathetic ganglia when, as we have shown, the levels of these mRNAs are similar, they found that *trk*A mRNA expression precedes p75 mRNA expression during development. The stages at which *trk*A mRNA and p75 mRNA where first detected in sympathetic neuroblasts/neurons of the rat embryo roughly correspond with the developmental stages at which we demonstrate marked increases in the levels of expression of these mRNAs in the mouse SCG. However, in contrast to our direct measurements of p75 and *trk*A mRNA levels, which show that the level of p75 mRNA approaches but does not exceed that of *trk*A

mRNA postnatally, Verdi and Anderson claim that the level of p75 mRNA is tenfold greater than that of *trk*A mRNA by the first postnatal day. The reason for this discrepancy is most likely a result of differences in the methods used to measure mRNA levels. To control for variations in PCR reaction conditions, Verdi and Anderson used a synthetic internal control DNA template in each PCR reaction that was amplified alongside the target cDNA templates (p75, *trk*A, and actin cDNAs). However, this control DNA was unrelated to the target cDNAs and was amplified by different primers to those used to amplify the target cDNAs. Because amplification efficiency is greatly influenced by the lengths and sequences of the templates and PCR primers, it is best to use an internal control template that is as similar as possible to the target cDNA template and to amplify both control and target templates with the same set of primers. For these reasons we synthesized separate specific control DNAs for p75 and *trk*A that were identical to the target p75 and *trk*A cDNAs, except they were 3 or 4 bp longer (sufficient to resolve target and control products on a PAGE gel). Moreover, to control for differences in RT efficiency, these specific control templates were added as cRNA to the RT reactions. To normalize RNA levels between neuroblasts and PC12 cells, Verdi and Anderson used parallel RT/PCRs for actin mRNA, which could introduce additional problems, not only because these reactions were standardized with an unrelated control template, but because any developmental changes in actin mRNA expression would affect the comparison of p75 and *trk*A mRNA levels between neuroblasts and PC12 cells. Because we measured the absolute levels of p75 and *trk*A mRNAs using known amounts of specific cRNA control templates in each reaction, we did not need to resort to using another mRNA species to standardize our results to p75 and *trk*A mRNAs in some cell line. Moreover, the accuracy of our quantitative RT/PCR assay has been verified by directly comparing the same samples assayed by this assay and by quantitative Northern blotting using specific RNA calibration and recovery standards (Wyatt and Davies, 1993).

Regulation of trkA mRNA and p75 mRNA Expression

In E13 SCG cultures, before any of the immature neurons have become NGF dependent, the low level of *trk*A mRNA expression is stably maintained for at least 3 d. Over the same period in vivo, the level of *trk*A mRNA in the ganglion increases almost 30-fold as many neurons acquire NGF dependence. Thus, when removed from their normal environment at a very early stage of their development, immature sympathetic neurons do not display the normal developmental increase in *trk*A mRNA expression. This low level of *trk*A mRNA expression in immature sympathetic neurons is also not affected by exposure to NGF. These findings are in accord with the observation that immature sympathetic neurons do not acquire NGF dependence in vitro even if cultured in the presence of NGF (Leah and Kidson, 1983; Rodriguez-Tebar and Rohrer, 1991).

In E14 control cultures, the mean neuronal level of *trk*A mRNA is already ~10-fold higher after 24 h than the level in cultured E13 neurons and increases a further threefold between 24 and 48 h of incubation. This suggests that by E14, at least some immature neurons have become specified to upregulate *trk*A mRNA expression in defined medium in the absence of added factors. Because the level of *trk*A mRNA does not increase in E13 cultures, this suggests that these immature neurons become specified to upregulate *trk*A mRNA expression between E13 and E14 in vivo. The increase in *trk*A mRNA in E14 cultures does not depend on physical contact with other cells, because it is observed in very low density cultures. It is also independent of NGF, occurring to the same extent whether or not NGF is present in the culture medium. A similar phenomenon is observed in developing trigeminal ganglion neurons in culture; at E12, when many neurons are acquiring NGF dependence, the level of *trk*A mRNA increases independently of the presence of NGF in the culture medium (Wyatt and Davies, 1993).

In contrast to our demonstration that at least a proportion of immature neurons become specified by E14 to upregulate *trk*A mRNA in defined medium in the absence of added factors, Verdi and Anderson (1994) did not observe increases in the level of *trk*A mRNA in cultures of E14.5 or E15.5 rat immature sympathetic neurons (neuroblasts). Because *trk*A mRNA expression in these cultures was increased by NT3, it was concluded that NT3 may normally induce *trk*A mRNA expression in these cells. However, the level of NT3 required to induce *trk*A mRNA expression was two orders of magnitude higher than the level of NT3 that enhances the survival of these cells (Verdi and Anderson, 1994) and is four orders of magnitude higher than the level of NT3 that promotes the maturation of early sensory neurons (Wright et al., 1992). Whether such high concentrations of NT3 are available to immature sympathetic neurons under normal physiologic conditions is not known.

Although we have shown that NGF does not affect *trk*A mRNA expression in developing sympathetic and sensory neurons (Wyatt and Davies, 1993), some studies suggest that NGF increases *trk*A mRNA expression in vivo. However, the reported effects are small and difficult to interpret because the data were obtained from grain counting in autoradiograms. Intraventricular infusion of NGF over a 2-wk period caused a 1.7-fold increase in grain density over forebrain cholinergic neurons in sections hybridized with a *trk*A probe (Holtzman et al., 1992). NGF infusion partially restored the level of *trk*A mRNA in adult DRG neurons after peripheral nerve section (Verge et al., 1992). However, the effect was small (grain counts were 48% of normal after nerve section alone and were elevated to only 61% of normal after nerve section plus NGF infusion), and NGF infusion did not affect the level of *trk*A mRNA expression in unlesioned DRG neurons. Likewise, injection of NGF into the anterior chamber of the eye of adult rats increased p75 mRNA but not *trk*A mRNA grain density in the innervating SCG neurons (Miller et al., 1994).

Although we have shown that the developmental increase in *trk*A mRNA is unaffected by NGF exposure, p75 mRNA expression was increased by NGF from the earliest stages studied. Even in E13 cultures, when NGF neither induces c-*fos* induction nor affects neuronal survival, the level of p75 mRNA was threefold higher in the presence of NGF than in control cultures after 3 d of incuba-

tion. In E14 cultures, the effect of NGF on p75 mRNA expression was more marked; after 48 h of incubation, the level of p75 mRNA was eightfold higher in the presence of NGF. These findings are consistent with other in vitro and in vivo studies showing that NGF increases the level of p75 mRNA expression in embryonic trigeminal ganglion neurons (Wyatt and Davies, 1993), postnatal and adult sympathetic neurons (Miller et al., 1991, 1994), adult basal forebrain cholinergic neurons (Cavicchioli et al., 1989; Higgins et al., 1989), and adult DRG neurons (Lindsay et al., 1990; Verge et al., 1992).

Mature sympathetic neurons and adrenal chromaffin cells are derived from cells that express markers characteristic of both lineages (Anderson, 1993). These cells are present in early sympathetic ganglia and the early adrenal medulla, and because they can give rise to either mature adrenal chromaffin cells or mature sympathetic neurons under appropriate culture conditions, they have been termed sympathoadrenal progenitor cells (Anderson, 1993). As development proceeds, chromaffin-specific markers are lost from cells in sympathetic ganglia, whereas neuron-specific markers are lost from cells in the adrenal medulla (Anderson et al., 1991; Carnahan and Patterson, 1991*a*, 1991*b*). To provide a convenient source of large numbers of sympathoadrenal progenitor cells for experimental studies, Birren and Anderson (1990) infected sympathoadrenal progenitor cells isolated from E14.5 rat adrenal glands with a *v-myc*–containing retrovirus to generate several cell lines. These MAH cells exhibit high-affinity norepinephrine uptake, express tyrosine hydroxylase and neurofilament protein, and possess short processes (Birren and Anderson, 1990), but neither respond to NGF or express *trk*A mRNA by Northern blotting (Birren et al., 1992), although very low levels of *trk*A mRNA are expressed by RT/PCR (Verdi et al., 1994). However, in the presence of depolarizing levels of KCl (40 mM), *trk*A mRNA became detectable by Northern blotting within 24 h and reached maximal levels within 3 d (Birren et al., 1992). In contrast, we have clearly shown that depolarizing levels of KCl do not affect *trk*A mRNA expression in immature sympathetic neurons before they become dependent on NGF for survival or as they acquire NGF dependence. In E13 SCG cultures, which are mainly composed of proliferating, NGF-independent, immature sympathetic neurons that are analogous to the sympathoadrenal progenitor cells described in rat SCG at an equivalent stage of development, the level of *trk*A mRNA is unaffected by 40 mM KCl up to 3 d in culture. The increase in *trk*A mRNA that takes place in E14 SCG cultures, as some cells acquire NGF dependence, occurs irrespective of the presence of 40 mM KCl in the culture medium. There may be several reasons for the apparent discrepancy between these two studies. First, the process of immortalizing sympathoadrenal progenitor cells from the early adrenal medulla may have significantly changed the behavior of these cells so that MAH cells are not fully representative of their normal counterparts. Second, MAH cells are derived from sympathoadrenal progenitor cells present in the early adrenal medulla. Although cells expressing neuronal and chromaffin markers in the early adrenal medulla and early sympathetic ganglia are generally thought of as being equivalent (Anderson, 1993), perhaps those in the early adrenal medulla (which normally only give rise to chromaffin cells) respond differently to depolarization than their counterparts in early sympathetic ganglia.

In contrast to the induction by retinoic acid of high-affinity NGF receptors and an NGF survival response in immature sympathetic neurons of chicken embryos (Rodriguez-Tebar and Rohrer, 1991), we have shown that retinoic acid over a broad range of concentrations (from 10^{-9} to 10^{-6} M), either alone or in combination with NGF, does not induce the expression of either *trk*A mRNA or p75 mRNA in immature sympathetic neurons of mouse embryos. It is possible that this is a result of species differences in the effect of retinoic acid on neuronal development. Retinoic acid does not, for example, induce NGF dependence in early trigeminal sensory neurons of mouse embryos (Paul and Davies, 1995).

We are grateful to Eric Shooter, Phillip Barker, Susan Meakin, Monte Radeke, and Luis Parada for the p75 and *trk*A cDNA clones used in this study. Our thanks to John Winslow and Gene Burton of Genentech for the purified recombinant NGF, Gerard Evan for the c-fos antiserum, and to Aviva Tolkovsky for advice on c-*fos* immunocytochemistry.

This work was supported by a grant from the Wellcome Trust, Medical Research Council and Cancer Research Campaign. The findings of this study were reported in their entirety at the Third International Conference on Nerve Growth Factor and Related Molecules at Lake Louise, Alberta in April, 1994.

Received for publication 16 December 1994 and in revised form 27 May 1995.

References

Allsopp, T., M. Robinson, S. Wyatt, and A. M. Davies. 1993. Ectopic trkA expression mediates a NGF survival response in NGF-independent sensory neurons but not in parasympathetic neurons. *J. Cell Biol.* 123:1555–1566.

Anderson, D. J. 1993. Molecular control of cell fate in the neural crest: the sympathoadrenal lineage. *Ann. Rev. Neurosci.* 16:129–158.

Anderson, D. J., and R. Axel. 1986. A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoids. *Cell.* 47: 1079–1090.

Anderson, D. J., J. F. Carnahan, A. Michelsohn, and P. H. Patterson. 1991. Antibodies markers identify a common progenitor to sympathetic neurons and chromaffin cells *in vivo* and reveal the timing of commitment to neuronal differentiation in the sympathoadrenal lineage. *J. Neurosci.* 11:3507–3519.

Birren, S. J., and D. J. Anderson. 1990. A *v-myc*-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. *Neuron.* 4:189–201.

Birren, S. J., J. M. Verdi, and D. J. Anderson. 1992. Membrane depolarization induces $p140^{trk}$ and NGF responsiveness, but not $p75^{LNGFR}$, in MAH cells. *Science (Wash. DC).* 257:395–397.

Birren, S. J., L. Lo, and D. J. Anderson. 1993. Sympathetic neuroblasts undergo a developmental switch in trophic dependence. *Development.* 119:597–610.

Buchman, V. L., and, A. M. Davies. 1993. Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. *Development.* 118:989–1001.

Carnahan, J. F., and P. H. Patterson. 1991*a*. The generation of monoclonal antibodies that bind preferentially to adrenal chromaffin cells of embryonic sympathetic ganglia. *J. Neurosci.* 11:3493–3506.

Carnahan, J. F., and P. H. Patterson. 1991*b*. Isolation of the progenitor cells of the sympathoadrenal lineage from embryonic sympathetic ganglia with the SA monoclonal antibody. *J. Neurosci.* 11:3520–3530.

Cavicchioli, L., T. P. Flanigan, G. Vantini, M. Fusco, P. Polato, G. Toffano, F. S. Walsh, and A. Leon. 1989. NGF amplifies expression of NGF receptor messenger RNA in forebrain cholinergic neurons of rats. *Eur. J. Neurosci.* 1:258–262.

Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.

Cohen, A. M. 1974. DNA synthesis and cell division in differentiating avian adrenergic neuroblasts. *In* Wenner-Gren Center International Symposium Series: Dynamics of Degeneration and Growth in Neurons. K. Fuxe, L. Olson, and Y. Zotterman, editors. Pergamon Press, Oxford, UK.

Cordon-Cardo, C. C., P. Tapley, S. Q. Jing, V. Nanduri, E. O'Rourke, F. Lamballe, K. Kovary, R. Klein, K. R. Jones, L. F. Reichardt, and M. Barbacid. 1991. The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell.* 66:173–183.

Coughlin, M. D., and M. B. Collins. 1985. Nerve growth factor-independent development of embryonic mouse sympathetic neurons in dissociated cell culture. *Dev. Biol.* 110:392–401.

Crowley, C., S. D. Spencer, M. C. Nishimura, K. S. Chen, S. Pitts-Meek, M. P. Armanini, L. H. Ling, S. B. McMahon, D. L. Shelton, A. D. Levinson, et al. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell.* 76:1001–1011.

Davies, A., and A. Lumsden. 1984. Relation of target encounter and neuronal death to nerve growth factor responsiveness in the developing mouse trigeminal ganglion. *J. Comp. Neurol.* 223:124–137.

Davies, A. M. 1986. The survival and growth of embryonic proprioceptive neurons is promoted by a factor present in skeletal muscle. *Dev Biol.* 115:56–67.

Davies, A. M. 1994. The role of neurotrophins during development. *J. Neurobiol.* 25:1334–1348.

Davies, A. M., C. Bandtlow, R. Heumann, S. Korsching, H. Rohrer, and H. Thoenen. 1987. Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature (Lond.).* 326:353–358.

Davies, A. M., A. Horton, L. E. Burton, C. Schmelzer, R. Vandlen, and A. Rosenthal. 1993*a*. Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons. *J. Neurosci.* 13:4961–4967.

Davies, A. M., K. F. Lee, and R. Jaenisch. 1993*b*. p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron.* 11:565–574.

Dechant, G., T. A. Rodriguez, R. Kolbeck, and Y. A. Barde. 1993. Specific high-affinity receptors for neurotrophin-3 on sympathetic neurons. *J. Neurosci.* 13:2610–2616.

DiCicco-Bloom, E., and I. B. Black. 1988. Insulin growth factors regulate the mitotic cycle in cultured rat sympathetic neuroblasts. *Proc. Natl. Acad. Sci. USA.* 85:4066–4070.

DiCicco-Bloom, E., E. Townes-Anderson, and I. B. Black. 1990. Neuroblast mitosis in dissociated culture: regulation and relationship to differentiation. *J. Cell Biol.* 110:2073–2086.

DiCicco-Bloom, E., W. J. Friedman, and I. B. Black. 1993. NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. *Neuron.* 11:1101–1111.

Doherty, P., P. Seaton, T. P. Flanigan, and F. S. Walsh. 1988. Factors controlling the expression of the NGF receptor in PC12 cells. *Neurosci. Lett.* 92:222–227.

Ernsberger, U., D. Edgar, and H. Rohrer. 1989*a*. The survival of early chick sympathetic neurons in vitro is dependent on a suitable substrate but independent of NGF. *Dev. Biol.* 135:250–262.

Ernsberger, U., M. Sendtner, and H. Rohrer. 1989*b*. Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. *Neuron.* 2:1275–1284.

Hempstead, B. L., L. S. Schleifer, and M. V. Chao. 1989. Expression of functional nerve growth factor receptors after gene transfer. *Science (Wash. DC).* 243:373–375.

Higgins, G. A., S. Koh, K. S. Chen, and F. H. Gage. 1989. NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. *Neuron.* 3:247–256.

Holtzman, D. M., L. Yiwen, F. F. Parada, S. Kinsman, C. Chen, J. S. Valletta, J. Zhou, J. B. Long, and W. C. Mobley. 1992. $p140^{trk}$ mRNA marks NGF-responsive forebrain neurons: evidence that *trk* gene expression is induced by NGF. *Neuron.* 9:465–478.

Hunt, S. P., A. Pini, and G. Evan. 1987. Induction of c-fos-like protein in spinal neurons following sensory stimulation. *Nature (Lond.).* 328:632–634.

Kaplan, D. R., Z. D. Martin, and L. F. Parada. 1991. Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature (Lond.).* 350:158–160.

Klein, R., S. Q. 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.

Leah, J., and C. Kidson. 1983. Survival of chick embryo sympathetic neurons in cell culture. *Int. J. Dev. Neurosci.* 1:403–409.

Lee, K. F., E. Li, L. J. Huber, S. C. Landis, A. H. Sharpe, M. V. Chao, and R. Jaenisch. 1992. Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell.* 69:737–749.

Lee, K. F., A. M. Davies, and R. Jaenisch. 1994. p75-deficient embryonic dorsal root sensory and neonatal sympathetic neurons display a decreased sensitivity to NGF. *Development.* 120:1027–1033.

Levi-Montalcini, R. 1987. The nerve growth factor 35 years later. *Science (Wash. DC).* 237:1154–1162.

Lindsay, R. M., E. M. Shooter, M. J. Radeke, T. P. Misko, G. Dechant, H. Thoenen, and D. Lindholm. 1990. Nerve growth factor regulates expression of the nerve growth factor receptor gene in adult sensory neurons. *Eur. J. Neurosci.* 2:389–396.

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.

Miller, F. D., A. Speelman, T. C. Mathew, J. Fabian, E. Chang, C. Pozniak, and J. G. Toma. 1994. Nerve growth factor derived from terminals selectively increases the ratio of p75 to trkA NGF receptors on mature sympathetic neurons. *Dev. Biol.* 161:206–217.

Paul, G., and A. M. Davies. 1995. Sensory neurons require extrinsic signals to switch neurotrophin dependence during the early stages of target field innervation. *Dev. Biol.* In press.

Rodriguez-Tebar, A., and H. Rohrer. 1991. Retinoic acid induces NGF-dependent survival response and high-affinity NGF receptors in immature chick sympathetic neurons. *Development.* 112:813–820.

Rohrer, H. 1990. The role of growth factors in the control of neurogenesis. *Eur. J. Neurosci.* 2:1005–1015.

Rohrer, H., and H. Thoenen. 1987. Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. *J. Neurosci.* 7:3739–3748.

Rothman, T. P., M. D. Gershon, and H. Holtzer. 1978. The relationship of cell division to the acquistion of adrenergic characteristics by developing sympathetic ganglion cell precursors. *Dev. Biol.* 65:322–341.

Rothman, T. P., L. A. Speccht, M. D. Gershon, T. H. John, G. Teitelman, V. M. Pickel, and D. J. Reis. 1980. Catecholamine biosynthetic enzymes are expressed in replicating cells of the peripheral but not the central nervous system. *Proc. Natl. Acad. Sci. USA.* 77:6221–6225.

Smeyne, R. J., R. Klein, A. Schnapp, L. K. Long, S. Bryant, A. Lewin, S. A. Lira, and M. Barbacid. 1994. Severe sensory and sympathetic neuropathies in mice carrying a disrupted TRK/NGF receptor gene. *Nature (Lond.).* 368: 246–249.

Theiler, K. 1972. The House Mouse (Development and Normal Stages from Fertilization to 4 Weeks). Springer-Verlag, Berlin.

Verdi, J. M., and D. J. Anderson. 1994. Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. *Neuron.* 13:1359–1372.

Verdi, J. M., S. J. Birren, C. F. Ibanez, H. Persson, D. R. Kaplan, M. Benedett, M. V. Chao, and D. J. Anderson. 1994. $p75^{LNGFR}$ regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron.* 12:733–745.

Verge, V. M. K., J. Merlio, J. Grondin, P. Ernfors, H. Persson, R. J. Riopelle, T. Hokfelt, and P. M. Richardson. 1992. Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. *J. Neurosci.* 12:4011–4022.

Wright, E., K. S. Vogel, and A. M. Davies. 1992. Neurotrophic factors promote the maturation of developing sensory neurons before they become dependent on these factors for survival. *Neuron.* 9:139–150.

Wyatt, S., and A. M. Davies. 1993. Regulation of expression of mRNAs encoding the nerve growth factor receptors p75 and *trk*A mRNA in developing sensory neurons. *Development.* 119:635–647.

Wyatt, S., E. M. Shooter, and A. M. Davies. 1990. Expression of the NGF receptor gene in sensory neurons and their cutaneous targets prior to and during innervation. *Neuron.* 4:421–427.