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Sympathetic Neurons, but Not Their Precursors

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Superior cervical ganglia of postnatal mice with a targeted disruption of the gene for neurotrophin-3 have 50% fewer neurons than those of wild-type mice. In culture, neurotrophin-3 increases the survival of proliferating sympathetic precursors. Both precursor death (W. ElShamy *et al.*, 1996, *Development* 122, 491–500) and, more recently, neuronal death (S. Wyatt *et al.*, 1997, *EMBO J.* 16, 3115–3123) have been described in mice lacking NT-3. Consistent with the second report, we found that, *in vivo*, neurogenesis and precursor survival were unaffected by the absence of neurotrophin-3 but neuronal survival was compromised so that only 50% of the normal number of neurons survived to birth. At the time of neuron loss, neurotrophin-3 expression, assayed with a lacZ reporter, was detected in sympathetic target tissues and blood vessels, including those along which sympathetic axons grow, suggesting it may act as a retrograde neurotrophic factor, similar to nerve growth factor. To explore this possibility, we compared neuron loss in neurotrophin-3-deficient mice with that in nerve growth factor-deficient mice and found that neuronal losses occurred at approximately the same time in both mutants, but were less severe in mice lacking neurotrophin-3. Eliminating one or both neurotrophin-3 alleles in mice that lack nerve growth factor does not further reduce sympathetic neuron number in the superior cervical ganglion at E17.5 but does alter axon outgrowth and decrease salivary gland innervation. Taken together these results suggest that neurotrophin-3 is required for survival of some sympathetic neurons that also require nerve growth factor.

Key Words: **neurotrophin; sympathetic neuron; survival; cell death.**

INTRODUCTION

According to the neurotrophic factor hypothesis, neurons are initially overproduced, and their number is subsequently refined by neuron loss through competition for trophic factors synthesized in restricted amounts in target tissues. Nerve growth factor (NGF) fits the criteria for a target-derived neurotrophic factor for sympathetic neurons. *In vitro*, sympathetic neurons isolated from late embryonic or early postnatal rodents die, but almost all can be rescued by NGF (Chun and Patterson, 1977; Greene, 1977; Levi-Montalcini and Angeletti, 1963). *In vivo*, NGF is expressed in sympathetic target tissues coincident with the arrival of sympathetic axons and at levels roughly corresponding to innervation density. Sympathetic neurons transport NGF from their target tissues (Hendry *et al.*, 1974; Korsching and Thoenen, 1983; Thoenen and Barde, 1980) and application of NGF to nerve terminals is sufficient to maintain cultured sympathetic neurons (Campenot, 1977). Increasing the amount of NGF available to sympathetic neurons during development rescues neurons that would normally die (Albers *et al.*, 1994; Hoyle *et al.*, 1993; Levi-Montalcini, 1987), while decreasing NGF with chronic injections of anti-NGF antisera during development results in destruction of the sympathetic nervous system (reviewed in Levi-Montalcini, 1987). The requirement for endogenous NGF has been confirmed in gene-targeting experiments: sympa-

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thetic ganglion volume is reduced by at least 80% at P3 in mice with a targeted disruption of the gene for NGF (Crowley *et al.,* 1994) and essentially no neurons remain at P9 in mice that lack the tyrosine kinase receptor for NGF, TrkA (Smeyne *et al.,* 1994; Fagan *et al.,* 1996). Taken together, these data support the hypothesis that NGF, synthesized in limiting quantities in target tissues and retrogradely transported from axon terminals, determines the number of sympathetic neurons that survive into adulthood.

Similar to postmitotic sympathetic neurons, at least 60% of dividing sympathetic precursors do not survive in serum-free cultures (Birren *et al.,* 1993; Coughlin and Collins, 1985; DiCicco-Bloom *et al.,* 1993). Unlike sympathetic neurons, they cannot be rescued by NGF. Precursors isolated from E14.5 rat sympathetic ganglia, however, can be maintained by low doses of neurotrophin-3 (NT-3) (3 ng/ml) (Belliveau *et al.,* 1997; Birren *et al.,* 1993; DiCicco-Bloom *et al.,* 1993) although similar treatment does not rescue NGF-dependent sympathetic neurons isolated later in development. Based on these results, it was hypothesized that NT-3 and NGF act sequentially *in vivo,* NT-3 to maintain dividing precursors and NGF to maintain neurons (Birren *et al.,* 1993; DiCicco-Bloom *et al.,* 1993). Rat sympathetic precursors cultured in the absence of serum do not express TrkA or become NGF-dependent. NT-3, at higher concentrations than required to promote survival, induces cell cycle arrest and, apparently as a consequence, TrkA expression and NGF responsiveness (Verdi and Anderson, 1994). These findings raise the possibility that NT-3 regulates the transition between dividing, NT-3-dependent precursors and postmitotic NGF-dependent neurons. Similarly, in cell culture, NT-3, BDNF, and NT-4/5 transiently support trigeminal neurons before they become NGF-dependent, and the change in neurotrophin dependence appears to require as yet unidentified environmental cues (Buchman and Davies, 1993; Buj-Bello *et al.,* 1994; Davies, 1994).

The dynamic neurotrophin responsiveness of maturing sympathetic precursors is accompanied by changes in the pattern of neurotrophin receptor expression. The patterns of expression of Trk receptors, which mediate the survival-promoting effects of neurotrophins (Barbacid, 1994; Bothwell, 1995), mirror the patterns of neurotrophin responsiveness: TrkC, the preferred Trk receptor for NT-3, is expressed in dividing precursors and decreases to nearly undetectable levels by birth. mRNA encoding TrkA, a functional receptor for NGF, can be detected as early as E13.5 in mice and increases to high levels by birth (Birren *et al.,* 1993; DiCicco-Bloom *et al.,* 1993; Fagan *et al.,* 1996; Wyatt and Davies, 1995; Wyatt *et al.,* 1997). Sympathetic neurons remain responsive to NT-3 after the change in receptor expression from TrkC to TrkA because NT-3 can elicit biological responses through TrkA in cell lines and cultured sympathetic neurons at 10- to 100-fold higher concentrations than required for TrkC activation (Belliveau *et al.,* 1997; Cordon-Cardo *et al.,* 1991; Davies *et al.,* 1995). NGF and NT-3 interactions with TrkA are modulated by the low-affinity neurotrophin receptor ($p75^{NTR}$), which increases the selectivity of TrkA for NGF when the two are coexpressed (Benedetti *et al.,* 1993; Clary and Reichardt, 1994; Lee *et al.,* 1994). $p75^{NTR}$ mRNA is expressed at very low levels in sympathetic ganglia during neurogenesis and does not increase in the ganglia until after E17 in mouse, reaching adult levels only during the first postnatal week (Verdi and Anderson, 1994; Wyatt and Davies, 1995).

The 50% decrease in neuron number observed in the superior cervical ganglion (SCG) of postnatal NT-3 null mice (Ernfors *et al.,* 1994; Farinas *et al.,* 1994) confirms that NT-3 has a significant role in sympathetic development *in vivo* but when and how this deficit arises is unclear. Based on tissue culture studies, *in vivo* either sympathetic precursors or sympathetic neurons (or both) could require NT-3. The two analyses of sympathetic development in NT-3-deficient mice published to date report conflicting data on when the deficit is first evident and therefore reach different conclusions concerning its origin. ElShamy and colleagues (1996) have presented evidence for excessive death of dividing sympathetic precursors in the SCG of NT-3-deficient mice from the earliest stages of sympathetic gangliogenesis through the period of neuronal proliferation and argue that NT-3 is required for the survival of precursors, but not neurons. In contrast, Wyatt *et al.* (1997) examined sympathetic ganglia of the same line of NT-3 mutants later in development and observed no deficiency in neuron number at E16, the earliest stage they studied morphologically. By estimating neuron number in the SCG, they detected a decrease in neuron number between E16 and E18. Based on these data, they propose that precursor survival is unaffected in the mutant mice and that the neuronal deficit at P0 reflects neuron death occurring late in embryogenesis.

To clarify the role(s) of NT-3 in determining sympathetic neuron number, we analyzed precursor and neuron numbers, mitosis, neuronal differentiation, and cell death throughout sympathetic development in the superior cervical and stellate ganglia from a strain of mice bearing an independently generated NT-3 mutation on a different genetic background (Farinas *et al.,* 1994). The results of the present study show that NT-3 is not required to support dividing precursors or to generate the full complement of sympathetic neurons but that it is required for the survival of the normal complement of postmitotic neurons. During the time when neuron loss occurs in the mutants, we have observed that NT-3 is expressed in sympathetic target tissues, including blood vessels along which many sympathetic axons grow. Because these results raised the possibility that NT-3 functions as a target-derived neurotrophic factor, similar to NGF, we compared neuron number and pyknosis in sympathetic ganglia from NT-3- and NGF-deficient mice. We found that neuron loss appears to coincide temporally in the two mutants, but is more severe in NGF-deficient than in NT-3-deficient mice. In mice lacking NGF with NT-3 reduced or absent, however, neuron numbers are not further decreased from those in mice

lacking NGF. Taken together, these results suggest that NT-3 is required together with NGF to maintain the full complement of newly generated sympathetic neurons.

MATERIALS AND METHODS

Animals

The generation of animals in which the lacZ coding region replaces the NT-3 coding exon (Farinas *et al.*, 1994) and of those with targeted disruption of the gene for NGF (Crowley *et al.*, 1994) has been previously described. Animals with a targeted disruption of the NGF gene were obtained from Dr. Heidi Phillips (Genentech, San Francisco, CA). NT-3−/− and NGF−/− embryos and their wild-type littermates were obtained from crosses of heterozygous adult mice. Embryos with varying dosages of NGF and NT-3 were obtained from crosses of doubly heterozygous adult mice (NT-3+/−; NGF+/−). Staging of timed pregnancies was determined by plug date and confirmed according to external morphology (Theiler, 1972). Genotypes were determined by PCR analysis of tail snip DNA (primers: NT-3, 5′AATTACCACAGCACCCTGC3′, 5′GTTTTTGACCGGCCTGGCTT3′; lacZ, 5′GGGACAGATTGAACCAGAAG3′, 5′CCACACTTATGACTTGCTTGT3′; and NGF/Neo, primer 1, 5′TCACTCGGGCAGCTATTGG3′, primer 2, 5′GCATGCCCGACGGCGAGGAT3′, and primer3, 5′CATAGCGTAATGTCCATGTT3′) with reagents from Gibco (Gaithersburg, MD) or by Southern blot as described (Farinas *et al.*, 1994).

Morphological Analyses

Ganglia to be embedded in plastic were immersed at least 24 h at 4°C in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer. After being rinsed in phosphate buffer, tissue was fixed in 2% osmium tetroxide in 0.1 M phosphate buffer for 2 h, stained overnight at 4°C with 1% uranyl acetate, and embedded in plastic resin (Araldite/Embed 812). One-micrometer sections were stained with 0.5% toluidine blue in 35% ethanol. Cell counts were performed every 40 μm at 400× using the Neurolucida or Stereo-Investigator image analysis system. The same image analysis system was used to collect data on all animals that were directly compared. Neurons with a nucleolus in the plane of the section were counted.

Embryos and neonates processed for paraffin embedding and cell counts were fixed by immersion in Carnoy's solution (60% ethanol, 30% chloroform, 10% acetic acid). Either 7- (NT-3-deficient animals) or 10- (NGF-deficient animals) μm paraffin sections were stained with cresyl violet. At E13.5, most cells express neuronal markers and are either neuronal precursors or neurons that cannot be readily distinguished in paraffin sections. Therefore, at that stage we determined total ganglion cell number. From E15.5 to P0, neurons were identified by their large round or oval nuclei with one or more large nucleoli and could be readily distinguished from precursors, glial cells, and endothelial cells, which have small, irregular, or elongated nuclei. At E13.5 and E15.5, the SCG and stellate ganglia were photographed in every fourth section, and cells or neurons with a nucleus and nucleolus in focus in the photographs were counted (final magnification: 450×). The SCG and stellate ganglia from E17.5 and P0 animals were counted directly from slides using the Neurolucida Image analysis system; counts were performed as described above for plastic sections using a single plane of focus for each section. Neuron numbers for whole ganglia were calculated by summing counts from the individual sections and multiplying by 4 (because every fourth section was counted). Numbers were compared between +/+ and −/− animals at each age using the Student *t* test. Since cell counts were not corrected for cell size changes during development, they cannot be compared between ages. The percentage of the number of cells or neurons observed in mutant compared to wild-type ganglia was calculated by dividing the neuron number obtained from individual ganglia from mutant mice by the average for the wild-type animals of the same age; these percentages were then averaged.

Images for cell area determination were collected from paraffin sections through the SCG of the P0 animals used for neuron counts. Areas were determined using NIH Image to trace the cell perimeters and calculate areas. In each section, all neurons containing a nucleus with a clear nucleolus and whose borders could be clearly distinguished were measured. One to three nonadjacent sections through the center of the ganglia from three animals of each genotype were analyzed and at least 500 cells were measured per animal.

For histological detection of β-galactosidase activity, animals were immersed for 2–4 h in or perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and rinsed in phosphate buffer. Tissues for frozen sections were infiltrated with 30% sucrose in 0.1 M phosphate buffer. Whole embryos or pups, dissected sympathetic targets, or 20- to 30-μm frozen sections were incubated 12–24 h in β-galactosidase staining solution (20 mM $MgCl_2$, 0.02% NP-40, 0.01% deoxycholic acid, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1 mg/ml X-gal in PBS, pH7.3) at 30–37°C, rinsed in PBS, and mounted in PBS–glycerol. For immunohistochemistry combined with lacZ staining, sections stained for lacZ were postfixed in 4% paraformaldehyde and incubated in rabbit anti-tyrosine hydroxylase (TH; PelFreez, Rogers, AR) diluted 1:200 in dilution buffer (2% BSA, 0.3% Triton X-100, 0.1% sodium azide in PBS). Immunoreactivity was detected with diaminobenzidine (Sigma, St. Louis, MO) using the VectaStain ABC kit (Vector Laboratories, Burlingame, CA). Immunohistochemical detection of sympathetic axons using anti-tyrosine hydroxylase was performed as above except that goat anti-rabbit IgG conjugated to Oregon green (Molecular Probes, Eugene, OR) was used in place of the VectaStain ABC reagents. Slides were mounted in PBS–glycerol after rinsing in PBS.

RESULTS

Morphology of Developing Sympathetic Cells in the Presence and Absence of NT-3

As a first step in elucidating the role of NT-3 in sympathetic development, we compared the morphology of cells in the SCG of NT-3-deficient and wild-type mice at several embryonic stages in 1-μm plastic sections (Fig. 1). We chose to begin our analysis at E13.5, when sympathetic ganglia have coalesced and contain mainly mitotic precursors (Fernholm, 1971; Rubin, 1985), because of the likelihood that NT-3 would be required early in sympathetic development. At E13.5, ganglia were small and contained small, darkly stained cells with centrally located nuclei containing multiple small nucleoli or patches of densely stained heterochromatin (Fig. 1A). Most neurogenesis in the mouse SCG has occurred by E15.5 (Fagan *et al.*, 1996). At E15.5, the majority of SCG cells, although noticeably smaller than

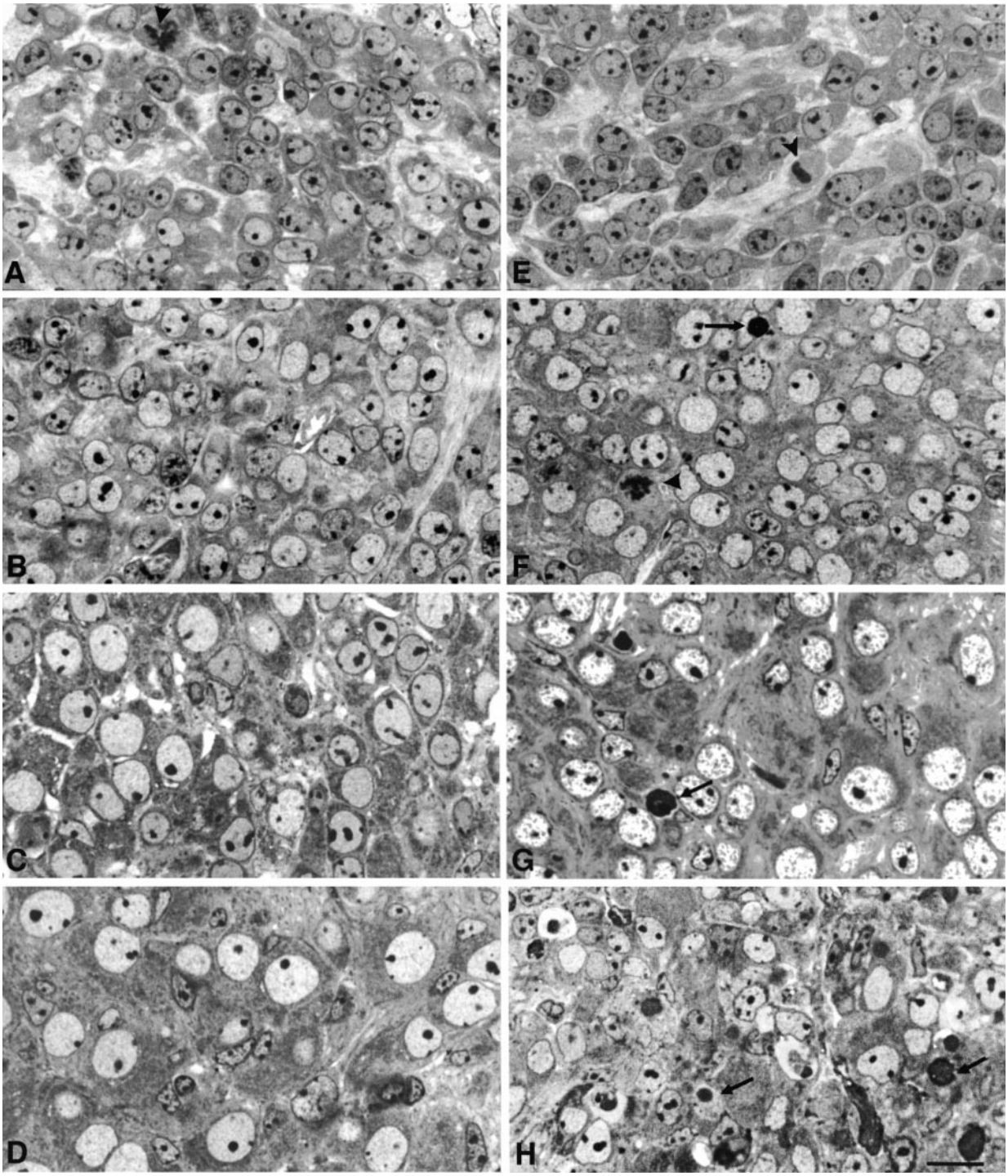


FIG. 1. (A–H) Morphology of sympathetic neurons in SCG of wild-type (A–D) and NT-3-deficient (E–H) mice in 1-μm toluidine blue-stained plastic sections. (A and E) E13.5; (B and F) E15.5; (C and G) E17.5; (D and H) P0. Arrowheads point to mitotic figures and arrows to pyknotic or degenerating cells. Scale bar, 10 μm. (I) Histogram of cross-sectional areas of neurons from SCG of P0 animals. The mean neuronal area was significantly smaller in SCG from NT-3 $-/-$ mice than in those from wild-type mice (+/+, 60 $\pm$ 0.83 μm^2; $-/-$, 54 $\pm$ 0.73 μm^2; $P < 0.0001$).

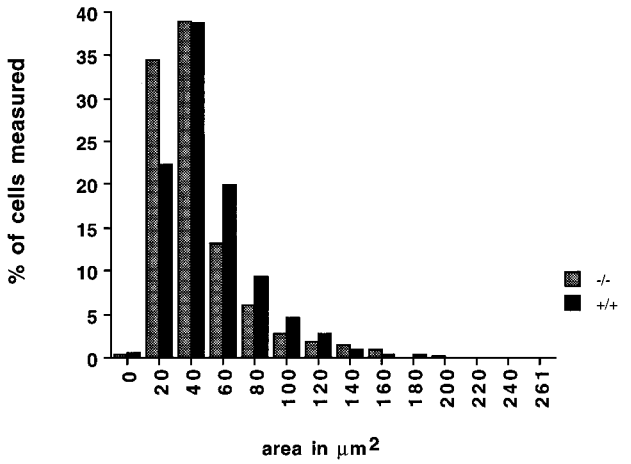


FIG. 1—*Continued*

mature sympathetic neurons, possessed a characteristic neuronal morphology, with a larger, less densely stained nucleus than at E13.5 and a small number of nucleoli (Fig. 1B). At E17.5, the neurons had larger nuclei and increased cytoplasmic volume. Glial cells or their precursors were identifiable throughout the ganglia at E17.5, often in clusters as previously reported for rat (Fig. 1C; Hall and Landis, 1992). Mitotic figures were rare at this age, consistent with the fact that neurogenesis is largely complete, and the major period of gliogenesis has not yet begun (Fagan *et al.*, 1996). By E17.5, tyrosine hydroxylase-immunoreactive sympathetic axons were present in many target tissues, including the submandibular gland, heart, and intrascapular brown adipose tissue (data not shown; see also Rubin, 1985). At P0, the neurons were large and possessed eccentric nuclei; some were associated with satellite glial cells (Fig. 1D). Finally, although the major period of naturally occurring cell death, at least in rat, is thought to occur postnatally (Wright, 1983), pyknotic profiles, indicative of dying cells, were evident throughout the ganglia from as early as E15.5.

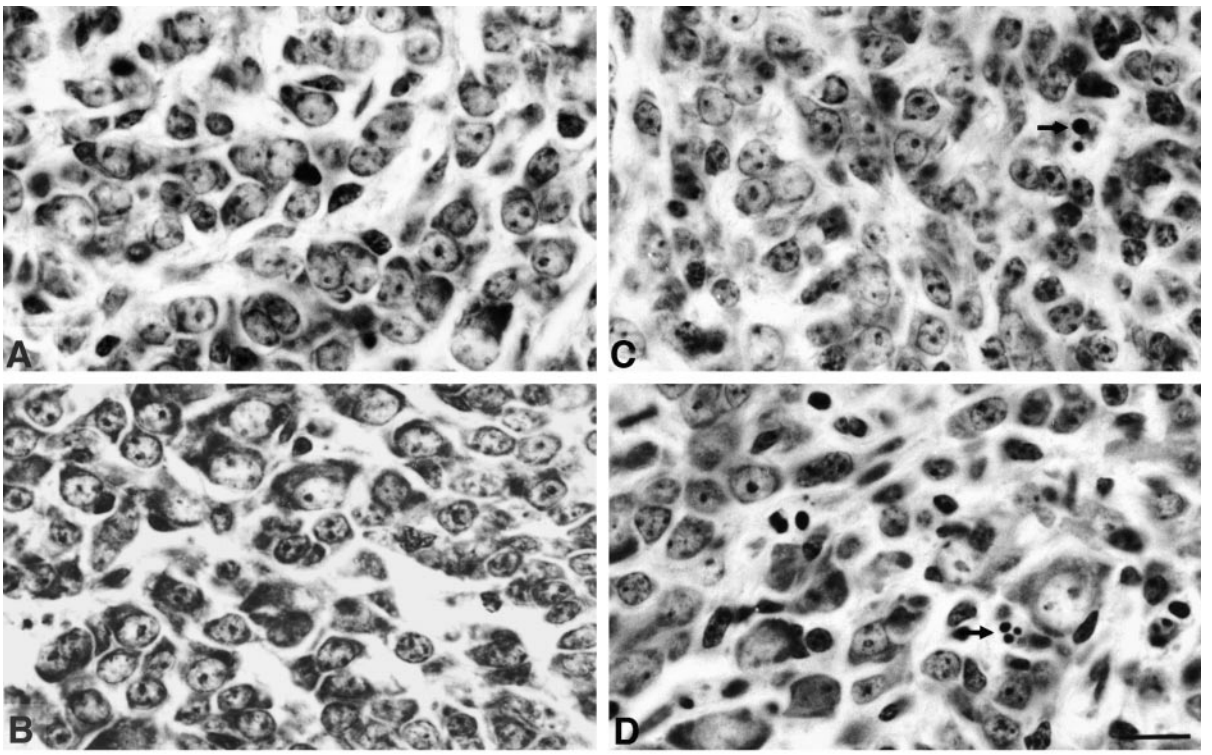
No differences in the morphologies of sympathetic precursors or newly generated postmitotic neurons were observed between the SCG of wild-type mice and those lacking NT-3 at E13.5 or E15.5 (Figs. 1E and 1F). This suggests that sympathetic neurogenesis and the early stages of neuronal differentiation were not significantly disrupted in the absence of NT-3. There was no evidence for cell death in ganglia from wild-type or NT-3-deficient animals until E15.5, at which time scattered pyknotic profiles were present. Morphological differences between neurons in the SCG of mice lacking NT-3 and those from wild-type mice were apparent at E17.5 (Figs. 1G and 1H; see also Fig. 2). At this age, the SCG from control animals contained many differentiating neurons while those from mice lacking NT-3 appeared to contain a greater proportion of neurons that were smaller and at an earlier stage of differentiation. At P0, differences between ganglia from mice of the two genotypes were more pronounced (Figs. 1D and 1H). When the cross-sectional areas of neurons were measured in paraffin sections of the SCG of P0 animals from both genotypes, the ganglia of mice lacking NT-3 contained both large and small neurons, similar to those from wild-type mice, but the distribution of areas was shifted toward smaller sizes (Fig. 1I) and the mean neuron area was significantly less in the mutant ganglia. Many pyknotic cells were present in ganglia from NT-3 null mice (Table 1; Figs. 1G and 1H), as well as darkly stained, shrunken neurons apparently in early stages of degeneration.

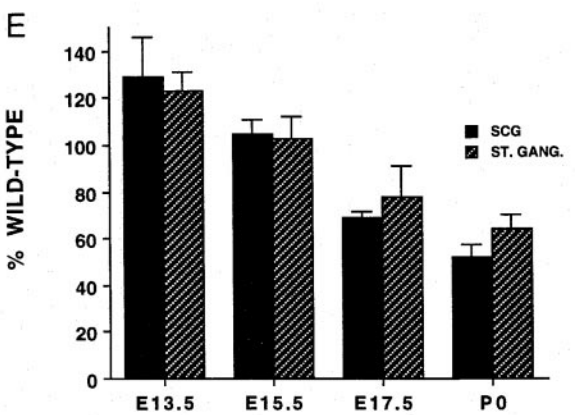
NT-3 Is Not Required for Survival of Sympathetic Precursors, but Is Required for Sympathetic Neuron Survival

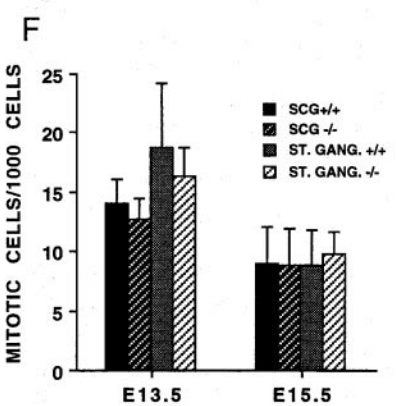
To determine when sympathetic ganglia from NT-3-deficient mice first contain fewer sympathetic neurons or precursors than control ganglia, total cells were counted in the SCG from wild-type and NT-3-null mice at E13.5 and neurons at E15.5, E17.5, and P0. Initially, counts at E13.5 and E15.5 were performed on paraffin sections and no statistically significant differences were detected in the number of cells (E13.5) or neurons (E15.5) (Table 1A; Fig. 2E) in ganglia from wild-type compared to those from NT-3-deficient mice.

If NT-3 were important to support the survival of dividing sympathetic precursors, one would predict that NT-3-deficient animals would have fewer neurons at E15.5 when most neurons have been born. Thus, this age is crucial in distinguishing an early effect on precursors from a later effect on neurons. Because we encountered difficulty in counting immature sympathetic neurons in paraffin sections at E15.5 when the cells are small and tightly packed, we wished to confirm that there were no differences between wild-type and NT-3-deficient mice at this age using optimally prepared material. Therefore, a second set of SCG from NT-3-null and wild-type E15.5 embryos was embedded in plastic and the number of neurons were counted in 1-μm sections (Table 1B). Consistent with the counts obtained from paraffin sections and the absence of morphological differences in the plastic sections, no statistically significant difference in neuron number was found between the two genotypes. In fact, one of the NT-3-deficient animals analyzed had many more neurons than any of the wild-type animals. The other animals had numbers similar to those of wild type, as was seen in the analysis of paraffin sections. When mitotic spindles and pyknotic profiles were counted in both paraffin and plastic sections, we found that neither the proportion of the population proliferating nor of that undergoing cell death was altered by the absence of NT-3 (Tables 1A and 1B; Figs. 2F and 2G). Thus, our analysis of early sympathetic development provides evidence that neither precursor survival and proliferation nor neurogenesis requires NT-3.

Although the SCG is the most intensively studied sym-







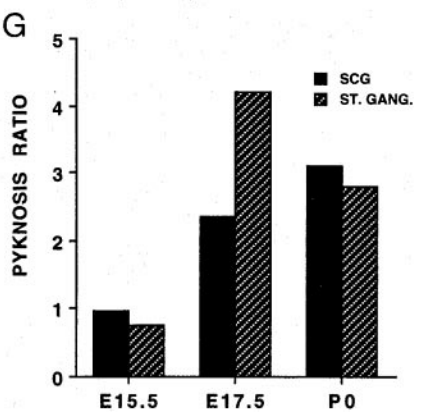


TABLE 1
Analysis of Sympathetic Ganglia in NT-3−/− Mice

A. Superior cervical ganglion–paraffin sections

Age		No. cells/neurons ±sem (*n*)	*P*	Pyknotic cells ±sem	Mitotic cells ±sem
E13.5	+/+	5863 ± 3460 (3)	0.10	0	79 ± 5
	−/−	7561 ± 1005 (3)		0	100 ± 28
E15.5	+/+	6034 ± 395 (4)	0.28	218 ± 39	57 ± 19
	−/−	6356 ± 338 (4)		236 ± 51	55 ± 22
E17.5	+/+	18,739 ± 3154 (4)	0.07	364 ± 44	53 ± 16
	−/−	12,056 ± 1124 (3)		584 ± 102	57 ± 24
P0	+/+	16,536 ± 1046 (3)	0.003	332 ± 101	16 ± 7
	−/−	8427 ± 1018 (3)		556 ± 185	16 ± 6

B. Superior cervical ganglion–plastic sections

Age		No. neurons ±sem (*n*)	*P*	Pyknotic cells ±sem	Mitotic cells ±sem
E15.5	+/+	14,643 ± 823 (3)	0.13	188 ± 27	87 ± 10
	−/−	21,261 ± 5041 (3)		386 ± 176	67 ± 13

C. Stellate ganglion–paraffin sections

Age		No. cells/neurons ±sem (*n*)	*P*	Pyknotic cells ±sem	Mitotic cells ±sem
E13.5	+/+	4859 ± 1542 (3)	0.20	0	73 ± 11
	−/−	5991 ± 391 (4)		0	97 ± 8
E15.5	+/+	3399 ± 483 (4)	0.43	181 ± 34	34 ± 17
	−/−	3500 ± 291 (6)		141 ± 28	33 ± 5
E17.5	+/+	16,588 ± 2686 (3)	0.17	117 ± 38	24 ± 2
	−/−	12,977 ± 2085 (3)		315 ± 78	23 ± 4
P0	+/+	18,236 ± 2438 (3)	0.07	180 ± 76	40 ± 14
	−/−	11,616 ± 1064 (2)		298 ± 118	50 ± 30

Note. P values represent comparison of neuron or cell numbers between ganglia from +/+ and −/− animals at each age using the Student *t* test. Numbers were not compared between ages because of changes in neuron size (see Materials and Methods). Both SCG were counted in 5 of the 6 animals used for plastic sections (B) and the two numbers were averaged for each animal; *n* represents the numbers of animals.

pathetic ganglion, recent data indicate that it differs from the rest of the paravertebral sympathetic chain in several respects. It is derived from cranial, rather than thoracic or lumbar, neural crest (Durbec *et al.,* 1996b) and its precursors require trophic support from members of the glial-derived neurotrophic factor (GDNF) family (Durbec *et al.,* 1996a; Moore *et al.,* 1996). Therefore, we examined a second large ganglion, the stellate ganglion. Cells at E13.5, and neurons at older ages, were counted, and as in the case of the SCG, cell numbers at E13.5 and E15.5 in mutant were similar to those of wild-type stellate ganglia (Table 1C, Fig. 2E).

As predicted by the morphological analysis, the first difference in neuron number between the SCG of wild-type and NT-3-deficient animals was detected at E17.5. Neuron counts were performed in paraffin sections at E17.5 and P0, when neurons can be readily distinguished from other ganglionic cells (Fig. 2). Although the morphological changes in neurons are not as apparent in paraffin sections as in plastic sections, glial cells and pyknotic profiles are

FIG. 2. Analysis of sympathetic ganglia from NT-3−/− mice. (A–D) Cresyl violet-stained paraffin sections through SCG of wild-type (A and C) and NT-3-−/− (B and D) mice at E17.5 (A and B) and P0 (C and D). Arrowheads point to pyknotic cells. Scale bar, 10 μm. (E) Bars indicate % wild type number of neurons, except at E13.5 when total cells were counted (see Materials and Methods) and are the average of counts from at least 3 animals ± SEM. (F) Mitotic spindles were counted in the same sections used to determine cell (E13.5) and neuron (E15.5 and later) number. (G) Pyknotic cells were counted in the same sections used to determine neuron number. The pyknosis ratio was calculated as the number of pyknotic cells per 1000 neurons in ganglia of NT-3−/− mice compared to the number in ganglia of wild-type mice.

more obvious (compare Figs. 1G and 1H to 2C and 2D). Mice lacking NT-3 had only 64% as many SCG neurons as wild-type mice at E17.5 (Fig. 2E) and had a corresponding increase in pyknotic profiles (Fig. 2G). Increased cell death and fewer neurons were also found in the stellate ganglion of NT-3-deficient mice (Table 1C; Figs. 2E and 2F). At P0, as previously reported (Farinas *et al.*, 1994), NT-3-deficient animals have a 50% decrease in SCG neuron number. A more than threefold increase in pyknotic profiles was detected in NT-3-deficient mice compared to wild-type controls at P0 (Table 1). The number of mitotic spindles, which at E17.5 and P0 are likely to correspond mainly to dividing glial precursors (Hall and Landis, 1992; Hendry, 1977), was similar for the two genotypes (Table 1). Comparison of developing sympathetic ganglia from wild-type and NT-3-deficient mice thus indicates that the deficit present at P0 is due to excessive neuronal death late in embryogenesis. Because expression of TrkC is low and TrkA expression is high at E17.5 and later (Birren *et al.*, 1993; DiCicco-Bloom *et al.*, 1993; Fagan *et al.*, 1996; Wyatt *et al.*, 1997), NT-3 likely signals through TrkA to promote survival. This notion is consistent with the finding that mice lacking TrkC do not have sympathetic deficits (Fagan *et al.*, 1996; Tessarollo *et al.*, 1998).

NT-3 Is Expressed in Peripheral Tissues but Not Sympathetic Ganglia Late in Embryogenesis

Having ascertained that the sympathetic deficit resulting from a lack of NT-3 is not detectable until E17.5, it was important to determine the potential source(s) of NT-3 for sympathetic neurons at this stage. The lacZ gene that was inserted in place of the NT-3 gene in mutant mice allows detection of sites of NT-3 mRNA expression via β-galactosidase histochemistry in mice carrying one or two copies of the disrupted NT-3 gene. As previously described, NT-3 is synthesized around sympathetic ganglia before E15 (data not shown; Verdi *et al.*, 1996); however, NT-3 expression in or around sympathetic ganglia was not detected at E15.5 or later with the exception of rare lacZ-positive cells in the SCG. As indicated in Table 2, NT-3 is expressed in some but not all targets of the SCG and stellate ganglion at E17.5 and P0, including blood vessels (Scarisbrick *et al.*, 1993), submandibular gland, pineal gland, and ear (see also Ernfors *et al.*, 1990). In NT-3-expressing glands, expression was limited to cells in secretory tubules and ducts (Figs. 3C and 3E). Expression in the ear was intense and widespread, although the sympathetic innervation in the ear is restricted to the array of small blood vessels. In the eye, the most intense NT-3 expression was detected in the iris, although positive cells were also present in the retina and cornea. The robust expression of NT-3 in smooth muscle cells of blood vessels (Fig. 3) (Scarisbrick *et al.*, 1993) is of particular interest; the sympathetic chain forms along the dorsal aorta and the SCG forms in the bifurcation of the carotid artery, many sympathetic axons in peripheral nerve trunks follow the vasculature into and through tissues, and arteries are an important sympathetic target. As shown in Fig. 3A, sympathetic axons exit the ganglion in close-proximity to large NT-3-expressing blood vessels and course along them to target areas. NT-3 from the dorsal aorta and carotid artery could diffuse into sympathetic ganglia at early embryonic stages. Because of the increasing distance between the major vessels and the ganglia as well as the ensheathing connective tissue present at later embryonic ages, however, it is more likely that neurons retrogradely transport NT-3 derived from blood vessels and target tissues.

TABLE 2

NT-3 Expression in the Sympathetic Nervous System and Peripheral Tissues

Tissue	E17.5	P0
Sympathetic ganglia	–	–
Superior cervical ganglion targets		
Submandibular gland	+	+
Iris	++	n.d.
Extraorbital lacrimal gland	++	++
Harderian gland	+	+
Ear	++	++
Pineal gland	++	++
Thyroid gland	–	–
Thymus	–	–
Blood vessels	+	+
Stellate ganglion targets		
Thymus	–	–
Blood vessels	+	+
Intrascapular brown adipose tissue	–	–
Atrium	–	–
Ventricle	–	–
Aorta	++	++

Note. NT-3 expression was inferred from β-galactosidase histochemistry, which localizes expression of the lacZ gene that replaces the NT-3 gene. Both heterozygous and homozygous mutants were analyzed, with similar results except that the staining was more intense in homozygotes. Tissue from at least three animals was examined at each age. n.d., not determined.

NT-3 and NGF Are Required at Similar Developmental Stages

The findings that NT-3 is required late in embryogenesis and is strongly expressed in peripheral tissues suggest that it may function in a manner similar to NGF and use the same receptor, since TrkA is the major neurotrophin receptor expressed in sympathetic neurons at this stage (Birren *et al.*, 1993; DiCicco-Bloom *et al.*, 1993; Fagan *et al.*, 1996; Wyatt *et al.*, 1997). In NGF-deficient animals, increased pyknosis was not detected until at least E16.5 (Hong Zhang and S.C.L., unpublished observation) and neuron counts indicate that neuronal losses due to the absence of NGF are not evident at either E15.5 or E16.5 (C.B. and S.C.L.,

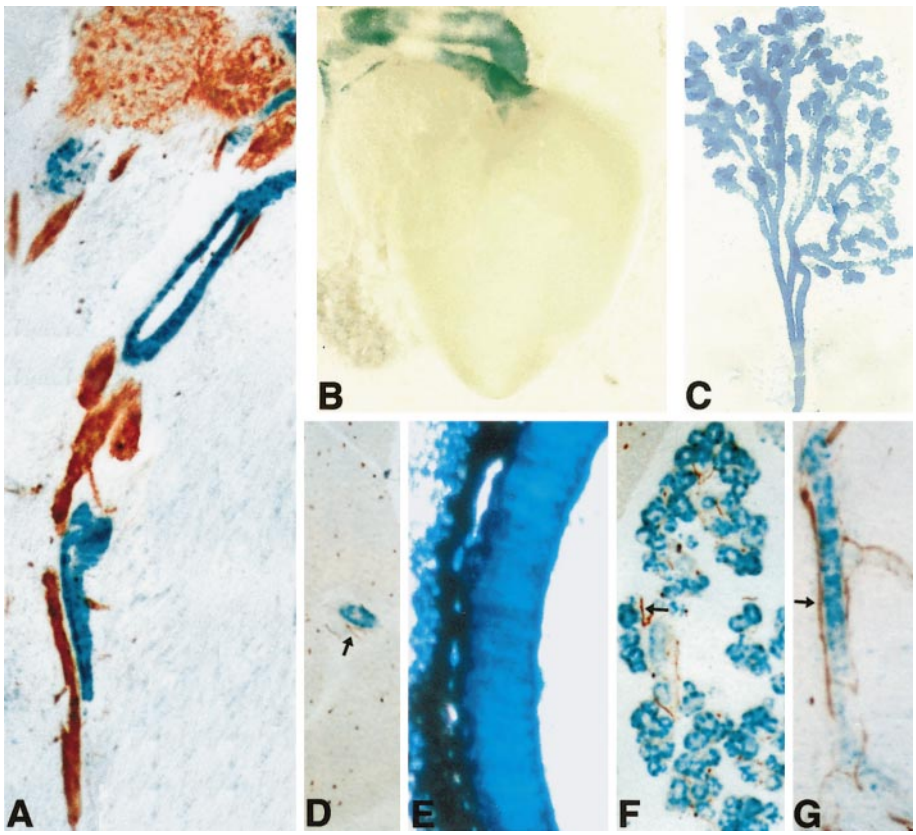


FIG. 3. β-Galactosidase (β-gal) histochemistry to visualize sites of NT-3 expression. (A) A section through SCG stained for β-gal and TH. Note large, TH-positive axon fascicles exiting the ganglion and traveling along the NT-3-positive carotid artery. Magnification 18×. (B and C) Whole-mount β-gal histochemistry of the heart (B) and extraorbital lacrimal gland (C). Magnification 13× (B) and 35× (C). (D) TH-immunostained axons (arrow) associated with an NT-3-positive blood vessel coursing through intrascapular brown adipose tissue, a sympathetic target tissue that does not express NT-3. The brown spots on the tissue are blood cells that express endogenous peroxidase activity. Magnification 100×. (E) A sagittal section through the iris. Magnification 500×. (F and G) TH-positive sympathetic fibers (arrows) in association with NT-3-positive submandibular gland cells (F) and blood vessels coursing through peripheral tissues (G). Magnification 90× (F) and 180× (G). (A, D, E, and F) E17.5 NT-3+/− (B and D) P0 NT-3−/−.

unpublished results). This suggests that excessive neuronal loss in NGF-deficient mice begins between E16.5 and E17.5, similar to that for NT-3-deficient mice. To explore the possibility that NT-3 and NGF act at the same stage of sympathetic development, neuronal numbers in the SCG and stellate ganglia were compared at E17.5 and P0 in mice lacking NGF. At E17.5, although differentiated neurons were present in ganglia of NGF-deficient mice, the ganglia contained only 39% as many sympathetic neurons as their wild-type littermates and pyknotic profiles were increased fourfold (Table 3, Fig. 4F). There was a similar but less severe reduction of 35% in neuron number in stellate ganglia (Table 3, Figs. 4E and 4F). By P0, the SCG of the mutant animals contained mainly glial cells (Figs. 4B and 4D). Consistent with the reduction in SCG volume previously reported (Crowley *et al.*, 1994), mice lacking NGF had more than a 90% decrease in neuron number in the SCG at P0 (Table 1; Fig. 4E) and increased pyknosis (Fig. 4F). A slightly larger proportion of neurons (30%) remained in the stellate ganglion at P0. Neuron loss in mice lacking NGF thus coincides in time with, but is more severe than, that in mice lacking NT-3. Therefore, because almost all SCG neurons are lost by birth in the absence of NGF, and half of them in the absence of NT-3, the vast majority of neurons that require NT-3 must also require NGF at some stage prior to P0.

Sympathetic Loss in NGF-Deficient Mice Is Not Exacerbated by Decreases in NT-3

The requirement for NGF for the survival of almost all sympathetic neurons and NT-3 for the survival of half could be explained in at least three ways. First, NT-3 could transiently support some sympathetic neurons independent of NGF. Second, the same neurons which require NT-3 could also require NGF. Finally, NT-3 may have an essen-

tial role in the development of some NGF-dependent sympathetic neurons that does not involve a direct effect on neuron survival. To determine whether NT-3 can support sympathetic neuron survival *in vivo* independent of NGF, SCG from E17.5 mice lacking NGF were compared to those from mice lacking NGF and one or both NT-3 alleles. If NT-3 normally maintains some sympathetic neurons during embryogenesis independent of NGF, reducing NT-3 levels should exacerbate neuronal losses in mice lacking NGF. In contrast, if the same neurons that require NT-3 also require NGF prior to E17.5, or if the role of NT-3 in neuronal survival is indirect, decreasing or eliminating NT-3 might not affect neuronal survival in the absence of NGF.

No differences were detected in the average number of neurons in the SCG among E17.5 NGF-deficient mice that had varying gene doses of NT-3 (Fig. 5; Table 4) when 1-μm plastic sections were analyzed. Consistent with E17.5 being near the peak of sympathetic neuron loss in both NGF- and NT-3-deficient mice, neuron number in NGF-deficient ganglia was quite variable, even among mice with the same NT-3 genotype. Although we pooled neuron counts from ganglia obtained from five litters of animals to obtain the averages shown in Fig. 5, we were concerned that the variability, particularly related to slight differences in timing, might obscure small differences in neuron number between genotypes. Even when neuron numbers in the SCG of NGF-deficient animals with varying doses of NT-3 were compared within individual litters, however, we did not detect significant differences. Therefore, we conclude that decreasing or eliminating NT-3 in the absence of NGF does not further exacerbate neuronal losses in the SCG at E17.5 (Table 4). Cell death, assessed by the number of pyknotic figures, was also not increased in NGF−/− animals lacking one or both NT-3 alleles (Table 4) compared to those lacking NGF alone. It should be noted, however, that of the 9 NGF−/−;NT-3+/− animals we analyzed, 2 had at least a 50% reduction in neuron number compared with NGF−/−;NT-3+/+ animals, and the neurons had abnormal morphologies, although none of the NGF−/−;NT-3−/− had this phenotype. Taken together, these results do not provide evidence for an NGF-independent survival-promoting role for NT-3. They do not, however, rule out the possibility that NT-3 has an NGF-independent role slightly later in development, although the dependence of most of the neurons on NGF at this time (Table 3) makes this less likely.

TABLE 3
Analysis of Sympathetic Ganglia in NGF−/− Mice

Age		No. neurons ±sem (*n*)	*P*	Pyk. cells ±sem
A. Superior cervical ganglion				
E17.5	+/+	9948 ± 455 (3)	≤0.001	135 ± 24
	−/−	3863 ± 312 (3)		563 ± 26
P0	+/+	8273 ± 1497 (4)	≤0.001	511 ± 99
	−/−	719 ± 111 (4)		332 ± 143
B. Stellate ganglion				
E17.5	+/+	10,373 ± 1099 (4)	0.011	63 ± 1
	−/−	6660 ± 531 (4)		371 ± 53
P0	+/+	10,409 ± 1681 (3)	0.007	291 ± 42
	−/−	3048 ± 560 (3)		221 ± 46

Note. *P* values represent comparison of neuron numbers between ganglia from +/+ and −/− animals at each age using the Student *t* test.

NT-3 Affects Axon Outgrowth and Target Innervation

NT-3 can stimulate sympathetic neurite outgrowth both in culture and *in vivo* (Belliveau *et al.*, 1997; ElShamy *et al.*, 1996), and we find that NT-3 is highly expressed in blood vessels that are adjacent to sympathetic ganglia and that some sympathetic axons follow to their target tissues or innervate (Figs. 3A and 3G). Therefore one way NT-3 could promote NGF-dependent survival is by stimulating or supporting axon outgrowth to NGF-expressing target tissues. To examine this possibility, we compared outgrowth of sympathetic axons from the SCG to a nearby target, the salivary gland, by tracing sympathetic axon trajectories in serial sections from E17.5 embryos that were NGF−/−;NT-3+/+ or NGF−/−;NT-3+/−. Similar to what was found for neuron numbers, we found a variable innervation phenotype. Figure 6 shows several examples from each genotype which represent the range of phenotypes observed. In three of five NGF−/−;NT-3+/+ (Figs. 6A and 6B) animals we observed relatively robust growth of TH-immunoreactive axons to the salivary gland and axons within the gland itself. In the other two animals analyzed, a small number of axons were detected near the salivary gland and none within it. In NGF−/−;NT-3+/− animals, we also observed a variable phenotype; of the eight animals we analyzed, however, none approached the level of innervation present in NGF−/−;NT-3+/+ animals (Figs. 6C–6E). In one case, axons could be seen reaching and entering the gland (Fig. 6E), but in most cases, there were very few or no axons along the pathway and no gland innervation. In NGF−/−;NT-3+/+ animals, axons were typically found in thick fascicles, even when innervation was sparse, while in NGF−/−;NT-3+/− animals, TH-immunoreactive axon fascicles were thinner. These results suggest that reducing NT-3 levels in the absence of NGF disrupts axonal growth and target innervation and are consistent with the possibility that the presence of NT-3 is important to help axons reach NGF-expressing targets.

DISCUSSION

We have presented several lines of evidence in support of the hypothesis that NT-3 is important for the survival of

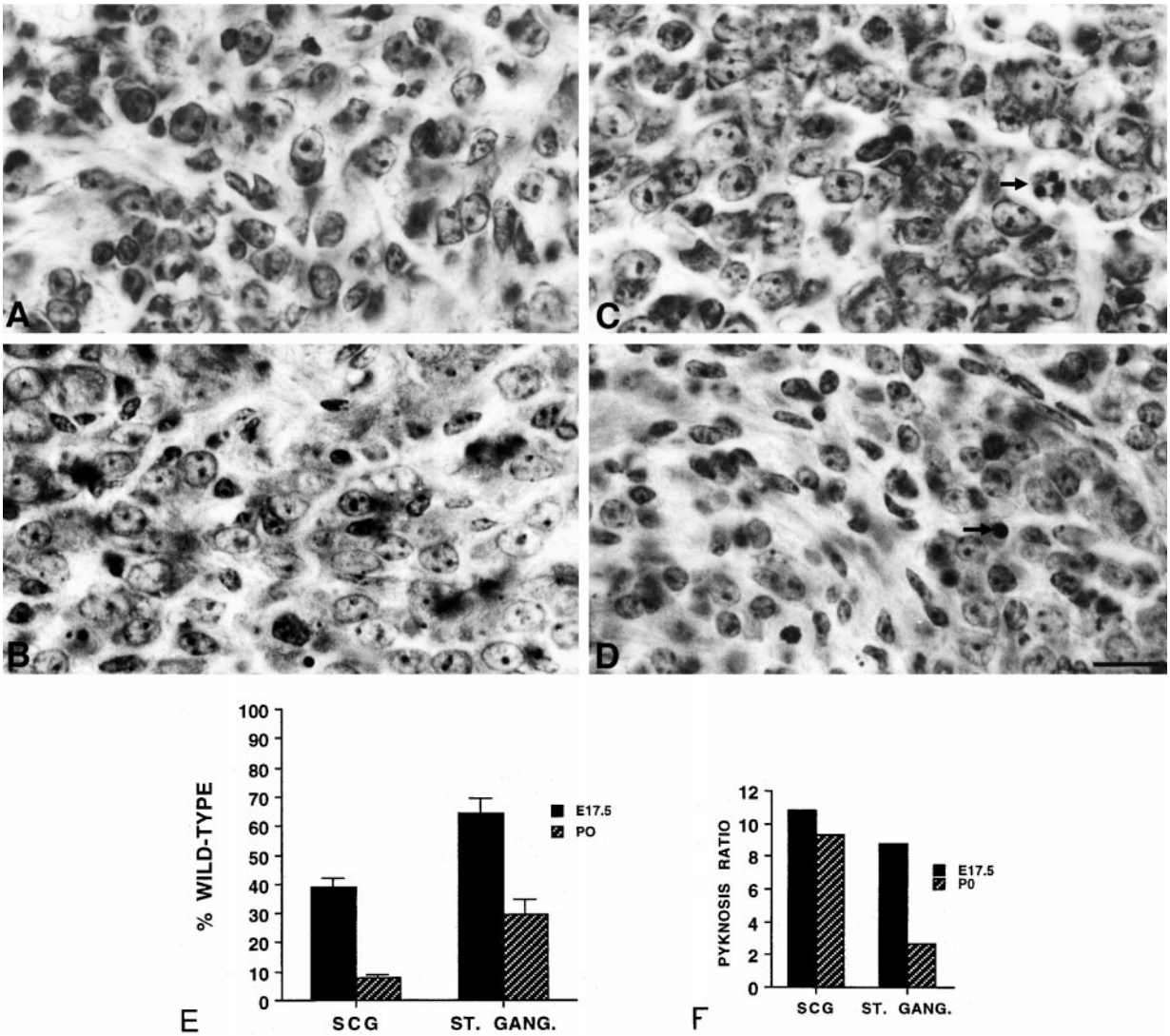


FIG. 4. Analysis of sympathetic ganglia of NGF−/− mice. (A–D) Cresyl violet-stained paraffin sections through SCG of wild-type (A and C) and NGF−/− (B and D) mice at E17.5 (A and B) and P0 (C and D). Arrowheads point to pyknotic cells and scale bar is 10 μm. (E) Bars indicate % wild-type number of neurons and are the average of counts from at least three animals ± SEM. (F) Pyknotic profiles were counted in the same sections used for neuron counts and the pyknosis ratio was calculated as for Fig. 2G.

immature sympathetic neurons rather than of sympathetic precursors. Morphological analysis and neuron counts of the relevant developmental stages indicate that sympathetic precursor survival, proliferation, and neuronal differentiation proceed normally in mice lacking NT-3, but that the lack of NT-3 leads to excessive neuronal death late in embryogenesis, when axons are growing to or arriving at their targets. While the initial study of sympathetic ganglion development in a different line of NT-3-deficient mice described fewer SCG cells and excessive death during neurogenesis (E11–E14) (ElShamy *et al.*, 1996), a subsequent analysis of mice derived from the same line revealed a reduction in neuron number only at a later stage, between E16 and E18 (Wyatt *et al.*, 1997). We have compared neuron number in NT-3-deficient and wild-type mice from E13.5 to P0 and find that differences between NT-3-null and wild-type mice are first evident at E17.5, consistent with the more limited analysis of Wyatt *et al.* (1997). The absence of statistically significant differences at E13.5 and E15.5 eliminates the possibility that early deficits also exist in NT-3 mutants. During the time that neurons are lost in the NT-3 mutants, NT-3 is available to the terminals of growing axons because it is expressed in blood vessels that some sympathetic axons have grown along as well as in a number of target tissues that are innervated by sympathetic neurons. NGF, also synthesized in target tissues, is required for

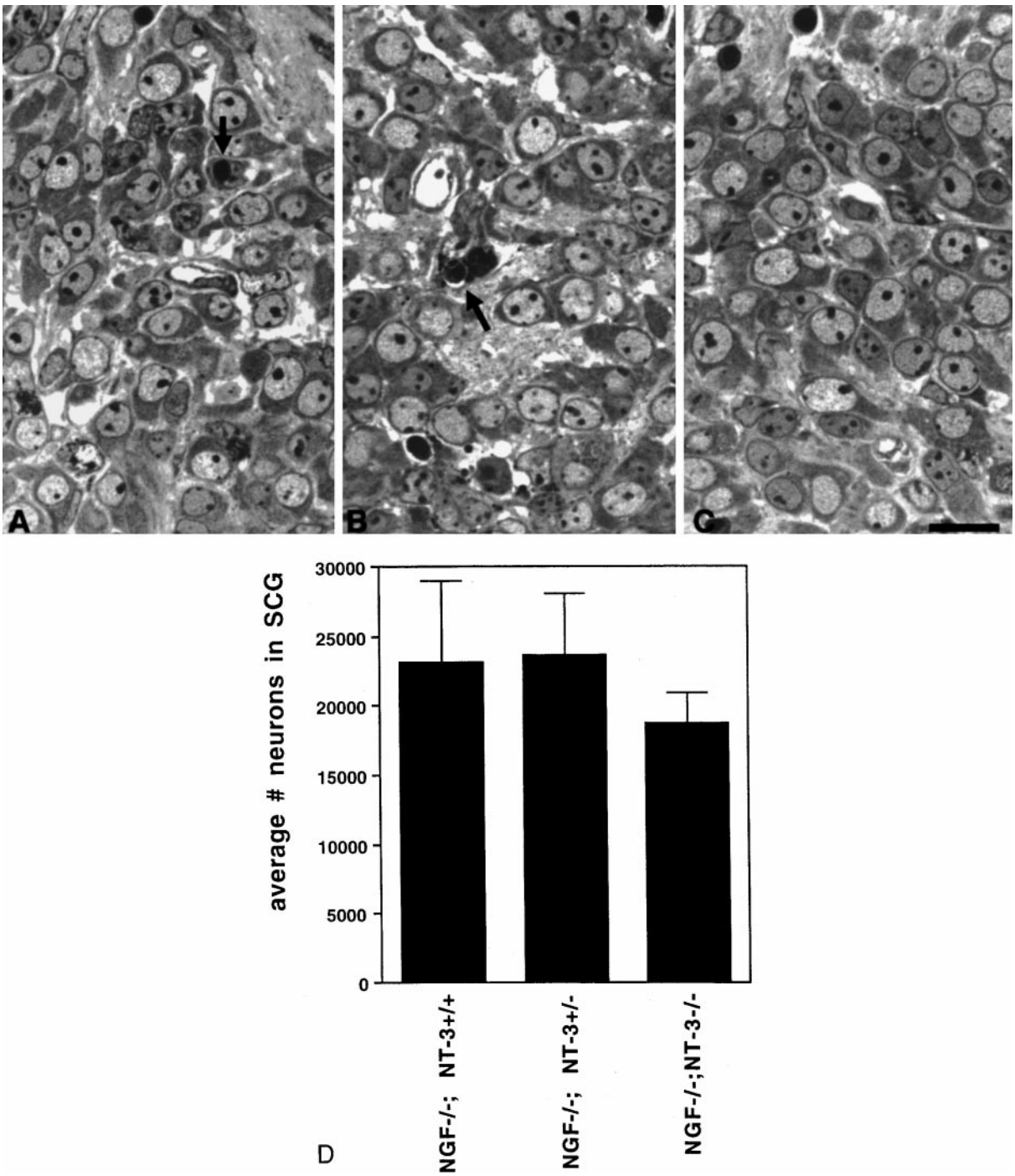


FIG. 5. Neuron counts in SCG from NGF−/−;NT-3+/+, NGF−/−;NT-3+/−, and NGF−/−;NT-3−/− mice. (A, B, and C) 1-μm toluidine blue-stained plastic sections through the SCG of an E17.5 NGF−/−;NT-3+/+ mouse (A), an E17.5 NGF−/−;NT-3+/− mouse (B), or an E17.5 NGF−/−;NT-3−/− mouse (C). Scale bar is 10 μm. (D) Bars indicate the average number of neurons in the SCG of mutant mice ± SEM.

survival of sympathetic neurons during the same developmental period. Our results, and those of Wyatt *et al.* (1997), provide compelling evidence that endogenous NGF is not sufficient for survival of the full complement of sympathetic neurons developing *in vivo* because loss of NT-3 causes excessive neuronal death at the same time that NGF

TABLE 4
Analysis of SCG in NGF/NT-3 Compound Mutants

A. SCG neuron numbers

Genotype	No. neurons ±sem (*n*)	*P*	Pyknotic cells ±sem (*n*)
NGF−/−; NT-3+/+	23,171 ± 5741 (8)	n.a.	934 ± 147
NGF−/−; NT-3+/−	23,665 ± 4373 (9)	0.66	1556 ± 229
NGF−/−; NT-3−/−	18,792 ± 2106 (3)	0.95	967 ± 214

B. SCG, % of littermates

Genotype	Average % of lm ±sem (*n*)	Pyknotic cells/ 1000 neurons
NGF−/−; NT-3+/+	n.a.	50 ± 9 (8)
NGF−/−; NT-3+/−	90 ± 18 (5)	70 ± 6 (9)
NGF−/−; NT-3−/−	106 ± 10 (3)	51 ± 8 (3)

Note. Average % of lm, the % of littermates. The *n* value is reduced for NGF−/−; NT-3+/− in B compared to A because not all animals have matching littermates. NGF−/−; NT-3+/− animals were compared to NGF−/−; NT-3+/+ littermates. NGF−/−; NT-3−/− animals were compared to either NGF−/−; NT-3+/+ or NGF−/−; NT-3+/− littermates.

is required for sympathetic neuron survival. Analysis of NGF/NT-3 compound mutants suggests that although NT-3 and NGF both make essential contributions to sympathetic neuron survival, the same neurons which require NT-3 also require NGF before E17.5. This dual dependency could reflect the fact that the effect of NT-3 on sympathetic neuron survival is dependent on NGF or that there is a sequential requirement for NT-3 and NGF.

Earlier studies have suggested that NT-3 is important for survival and/or maturation of dividing sympathetic precursors *in vitro* (Birren *et al.*, 1993; DiCicco-Bloom *et al.*, 1993; Verdi and Anderson, 1994) and *in vivo* (ElShamy *et al.*, 1996). Our results, however, indicate that sympathetic precursors are present in normal numbers and that their survival, proliferation, and differentiation are not perturbed in the absence of NT-3. Because, in some cases, we found an increase in neuronal numbers in sympathetic ganglia from mice lacking NT-3 at E13.5 and E15.5, we cannot exclude the possibility that NT-3 contributes to early determination of sympathetic neuron number. It is also possible that NT-3 supports survival of proliferating precursors, but at later developmental stages (E17.5) than predicted. We think this is unlikely because most sympathetic neurons have been born by this age (Hendry, 1977). Second, the similar numbers of mitotic figures between NT-3-deficient and wild-type mice do not support the idea that dividing precursors are dying. Rather, the increase in pyknotic figures in NT-3-deficient mice at E17.5 correlates well with the decrease in neuron number (Table 1).

The results of our analysis of early sympathetic development in NT-3-deficient mice do not match those of ElShamy *et al.* (1996), who found massive death of dividing precursors early in sympathetic development, but are consistent with those of Wyatt *et al.* (1997) who suggested that "the early loss previously reported was either erroneous or due to a different genetic background." The finding that NT-3 is sufficient to support sympathetic precursors in culture, but not necessary to support them *in vivo*, could reflect redundancy with other growth factors. For example, receptors for members of the GDNF family and for hepatocyte growth factor have been implicated in sympathetic precursor development and survival (Durbec *et al.*, 1996b; Maina *et al.*, 1998; Moore *et al.*, 1996). Because culture studies implicating NT-3 in sympathetic precursor survival used rat cells, and *in vivo* studies were carried out in mice, it is also possible that sympathetic precursors from the two species differ in their growth factor requirements.

The finding that some sympathetic neurons, but not their precursors, are dependent on NT-3 for survival raises the question of how NT-3 acts to support survival. *In vitro* studies of the actions of NT-3 on sympathetic neurons suggest it can promote sympathetic neuron survival directly, but only at much greater concentrations than required to activate its high-affinity receptor TrkC. This is consistent with the low level of TrkC expression detected in sympathetic neurons at late embryonic and early postnatal stages (Birren *et al.*, 1993; DiCicco-Bloom *et al.*, 1993; Fagan *et al.*, 1996; Wyatt and Davies, 1995; Wyatt *et al.*, 1997) and suggests that these survival-promoting effects of NT-3 are mediated by an alternative receptor. SCG neuronal numbers are not reduced in mice lacking the kinase domain of TrkC (Fagan *et al.*, 1996) or the complete TrkC receptor (Tessarollo *et al.*, 1997), further suggesting that NT-3-mediated survival via TrkC activation is not essential for sympathetic precursor survival. Instead, TrkA, rather than TrkC, is likely to mediate the essential NT-3 signals. At high concentrations, NT-3 can activate TrkA, and NT-3 maintains TrkC-null sympathetic neurons as efficiently as wild-type sympathetic neurons in culture (Belliveau *et al.*, 1997; Birren *et al.*, 1993; Davies *et al.*, 1995; Lee *et al.*, 1994; Verdi and Anderson, 1994). In a careful comparison with NGF, however, it was found that the effects of NT-3 are qualitatively different from those of NGF even though both require TrkA signaling. Thus, NT-3 induced similar levels of TrkA phosphorylation as saturating doses of NGF, but did not support similar levels of survival at any of a range of concentrations tested (Belliveau *et al.*, 1997). In contrast to its weak survival-promoting activity, in these same assays, NT-3 promoted neurite outgrowth and upregulated expression of certain genes as effectively as NGF and did so through activation of TrkA.

Although both NT-3 and NGF are required between E15.5 and E17.5 for survival of the full complement of neurons (the present study; C. Brennan, unpublished observation), reduction or elimination of NT-3 in mice that lack NGF does not exacerbate neuronal losses during this period. This indicates that the neurons whose survival depends on NT-3 prior to E17.5 also require NGF. There are several possible mechanisms that could account for this dual

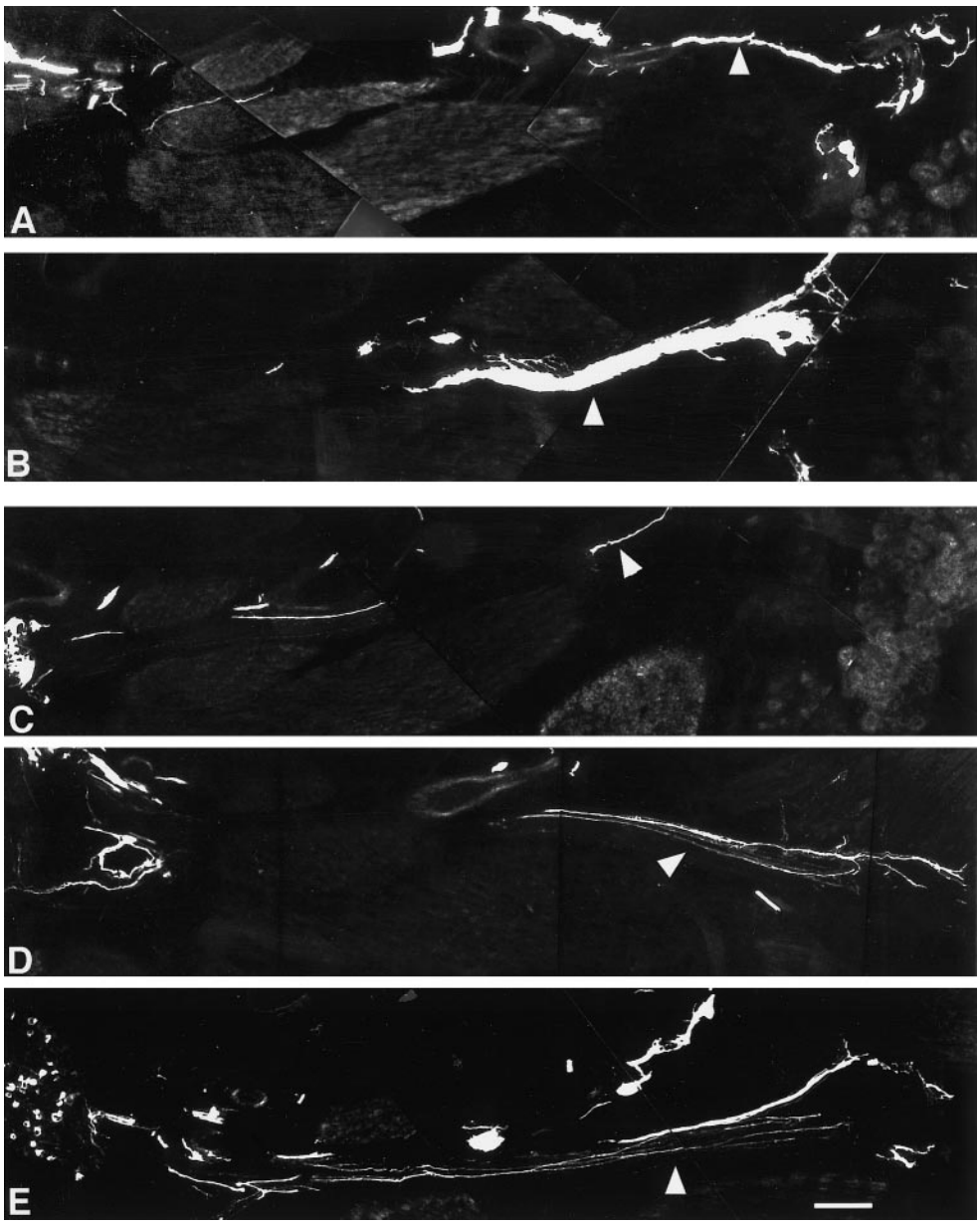


FIG. 6. Innervation of salivary gland of NGF−/−;NT-3+/+ and NGF−/−;NT-3+/− mice. 30-μm sections were immunostained with antibodies to tyrosine hydroxylase to identify sympathetic axons. The pathway between the SCG and the salivary gland was photographed in the section containing the maximal number of axons after comparing serial sections. Note that this pathway is the same as that in Fig. 3A. In each case, the ganglion is on the left (not shown), while the gland is on the right. (A and B) Examples of NGF−/−;NT-3+/+ and (C–E) NGF−/−;NT-3+/−. A and C represent the least amount of innervation seen for these genotypes, while B and E represent the most innervation seen for each genotype. Arrowheads point out thick axon fascicles in NGF−/−;NT-3+/+ (A and B) animals compared to thin axon bundles in NGF−/−;NT-3+/− animals (C–E). Scale bar, 120 μm.

dependency. One is that the weak survival-promoting activity of NT-3 on sympathetic neurons is relevant *in vivo* and that the NT-3-dependent subpopulation of sympathetic neurons is dependent on NGF and NT-3, simultaneously or sequentially. Another possible role of NT-3 that is supported by studies *in vitro* is induction of NGF responsiveness. Although we cannot formally rule out the possibility that in the absence of NT-3 *in vivo*, sympathetic neurons fail to acquire appropriate NGF responsiveness, we believe this is unlikely. NGF dependence seems to correlate very

well with NGF responsiveness and expression of TrkA. Both RT-PCR (Wyatt *et al.,* 1997) and immunohistochemical analysis of NT-3-deficient mice (our unpublished results) suggest that TrkA is expressed at appropriate levels and at the appropriate time in the absence of NT-3.

An alternative mechanism by which NT-3 could act to influence sympathetic neuron survival is suggested by the finding that NT-3 promotes neurite outgrowth and upregulation of expression of growth-related genes through TrkA in cultured sympathetic neurons and does so much more effectively than it promotes survival (Belliveau *et al.,* 1997). NT-3 could thus act in conjunction with NGF to promote neurite outgrowth, either to ensure that axons reach their targets or to permit growth into and branching within targets so that neurons access the maximal amount of NGF. Consistent with this possibility, ElShamy *et al.* (1996) found that sympathetic axons do not innervate blood vessels in the ears of NT-3-deficient mice, but that injection of NT-3 into the ear caused axons to invade this target. Our finding that innervation of the salivary gland is disrupted in mice with reduced levels of NT-3 and lacking NGF compared to animals lacking NGF alone provides further support for this mechanism. One model for how both the survival and the neurite outgrowth-promoting activities of the TrkA ligands NGF and NT-3 could be required for the development and maintenance of the appropriate number of sympathetic neurons is as follows. Signaling through TrkA may be critical first to stimulate neurite outgrowth to NGF-expressing targets and ultimately for neuronal survival. Although NGF could fulfill both requirements, its restricted distribution may impose an additional requirement for NT-3 *in vivo*. Initially, NT-3 is expressed at high levels adjacent to sympathetic ganglia and later in tissues such as blood vessels along which sympathetic axons grow. Therefore NT-3 is potentially available to stimulate neurite outgrowth allowing more sympathetic axons to reach NGF-expressing targets. This model is consistent with all the reported findings in NGF-, NT-3-, and TrkA-deficient mice. First, in mice that lack TrkA, and thus both NT-3- and NGF-mediated signal transduction, both neurite outgrowth and survival are severely compromised: axons do not reach the salivary gland, and most neurons do not survive (Fagan *et al.,* 1996). In mice that lack NGF, most neurons do not survive, although some axons do reach the salivary gland, likely due to stimulation of neurite outgrowth by NT-3. In the absence of NT-3, we suggest that some neurons may not survive because their axons fail to reach NGF-expressing targets. This model suggests that effects of neurotrophins on survival and neurite outgrowth, which can be separated by their concentration dependence *in vitro,* are less readily distinguished *in vivo* due to the restricted distribution of neurotrophins that makes survival dependent on neurite outgrowth. Although the available data are consistent with a neurite-outgrowth-promoting role for NT-3 in sympathetic development, this possibility cannot at present be distinguished from the alternatives discussed above, involving dual or sequential dependence on both NT-3 and NGF for survival.

The emerging picture for the role of endogenous neurotrophic factors in sculpting the sympathetic nervous system is more complex than predicted by early experiments identifying NGF as the sole neurotrophin required for survival and maturation of these neurons. It is also different from the model derived from more recent studies suggesting that NT-3 and NGF are sequentially required for survival at discrete developmental stages, NT-3 for precursor survival and/or differentiation and NGF for survival of postmitotic neurons. Our findings provide evidence for a dual requirement for NT-3 and NGF to support sympathetic neuron survival, which appears to differ from the requirement of some sensory neurons for different neurotrophins sequentially to support survival. Thus, trigeminal sensory neurons can be supported by NT-3, BDNF, or NT-4/5 before they require target-derived NGF so that mice lacking either BDNF or NT-3 have reduced numbers of trigeminal neurons (reviewed in Davies, 1994, 1997). In contrast, NGF and NT-3 are required simultaneously for survival of the normal complement of sympathetic neurons and may function by distinct, rather than interchangeable, mechanisms. A second difference between sensory and sympathetic neurotrophin requirements is that the Trk receptors mediating survival responses change in trigeminal sensory neurons, while both NGF and NT-3 appear to act through TrkA in sympathetic neurons.

Of the neurotrophins, NT-3 seems to have the most diverse and complex roles, functioning through multiple receptors and multiple cellular mechanisms in different types of neurons. NT-3 contributes to the determination of dorsal root ganglion sensory neuron number first by influencing the timing of precursor differentiation and second by promoting the survival of postmitotic neurons (Farinas *et al.,* 1996). Furthermore, NT-3 acts through both TrkC and TrkB to promote survival in developing DRG (Farinas *et al.,* 1998). In developing sympathetic neurons, NT-3 seems to act through TrkA to promote survival. While our studies provide evidence for the importance of NT-3 in supporting neurite outgrowth to target tissues, which may account for its effect on survival, NT-3 may also have other roles in postnatal sympathetic development. Analyses of NT-3-deficient mice suggest that sympathetic neurons depend on NT-3 for survival only transiently because neuron loss does not continue postnatally (Ernfors *et al.,* 1994). The finding that treatment of postnatal rats with NT-3 antibodies causes sympathetic neuron loss raises the possibility that sympathetic neurons may continue to require NT-3 during the first several postnatal weeks (Zhou and Rush, 1995).

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