Prenatal and postnatal requirements of NT-3 for sympathetic neuroblast survival and innervation of specific targets

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SUMMARY

Postnatal homozygous neurotrophin-3 mutant mice display a loss of about half the sympathetic superior cervical ganglion (SCG) neurons (Ernfors, P., Lee, K.-F., Kucera, J. and Jaenisch, R. (1994a) Cell 77, 503-512; Farinas, I., Jones, K. R., Backus, C., Wang, X. Y. and Reichardt, L. F. (1994) Nature 369, 658-661). We found that this loss is caused by excessive apoptosis of sympathetic neuroblasts leading to a failure to generate a normal number of neurons during neurogenesis. NT-3 was also found to be required postnatally. In Nt-3−/− mice, sympathetic fibers failed to invade pineal gland and external ear postnatally; whereas other targets of the external and internal carotid nerves, including the submandibular gland and the iris, displayed a normal complement of sympathetic innervation. Sympathetic fibers of mice carrying one functional copy of the Nt-3 gene (Nt-3+/− mice) invaded the pineal gland, but failed to branch and form a ground plexus. Cultured neonatal sympathetic neurons responded to NT-3 by neurite outgrowth and mRNA upregulation of the NT-3 receptor, trkC. Exogenously administered NT-3 promoted sympathetic growth and rescued the sympathetic target deficit of the mutant mice. We conclude that NT-3 is required for the survival of sympathetic neuroblasts during neurogenesis and for sympathetic innervation and branching in specific targets after birth.

Key words: neurotrophin-3, sympathetic neurons, innervation, neurogenesis, mouse

INTRODUCTION

Neurotrophins include four structurally and functionally similar polypeptides which are crucial in the development and survival of vertebrate neurons. The neurotrophins include nerve growth factor (NGF) (Levi-Montalcini, 1987; Levi-Montalcini and Angeletti, 1968), brain-derived neurotrophic factor (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3) (Ernfors et al., 1990; Hohn et al., 1990; Jones and Reichardt, 1990; Kaisho et al., 1990; Maisonpierre et al., 1990b; Rosenthal et al., 1990) and neurotrophin-4 (Hallböök et al., 1991; Berkemeyer et al., 1991; Ip et al., 1992). The temporally and spatially restricted expression of the neurotrophins in the brain and periphery suggests that their regulation is important to ensure a correct development of the nervous system (Ernfors et al., 1990a,b, 1992; Friedman et al., 1991a,b,c; Maisonpierre et al., 1990a; Phillips et al., 1990; Schecterson and bothwell, 1992).

The members of the neurotrophin family have been shown to support different classes of sensory neurons in culture (Davies et al., 1986, 1993; Ernfors et al., 1990; Hohn et al., 1990; Hory-Lee et al., 1993; Leibrock et al., 1989; Lindsay et al., 1985; Maisonpierre et al., 1990). The establishment and analysis of mice carrying deletions in the neurotrophin genes have revealed some of their physiological roles in vivo. In many instances neurotrophins support distinct classes of peripheral sensory neurons also in vivo (Conover et al., 1995; Crowley et al., 1994; Ernfors et al., 1994a,b, 1995; Farinas et al., 1994; Liu et al., 1995). For instance, in the vestibular inner ear, target-derived BDNF is crucial for neuronal survival; whereas, in the cochlea, NT-3 supports type 1 spiral ganglion neurons, and BDNF type 2 spiral ganglion neurons, (Ernfors et al., 1995). In the dorsal root ganglion (DRG), NGF supports the function and survival of neurons mediating pain sensation (see review by Lewin and Mendell, 1993), while NT-3 supports neurons mediating limb proprioception in culture (Hohn et al., 1990; Hory-Lee et al., 1993) and in vivo (Ernfors et al., 1994a; Farinas et al., 1994). The targets of innervation in the inner ear, the sensory epithelia, express BDNF and NT-3 mRNAs (Ernfors et al., 1992; Pirvola et al., 1992). Similarly, NGF mRNA is expressed in cutaneous tissues (Bandtlow et al., 1987; Davies et al., 1987; Ernfors et al., 1992) and NT-3 mRNA in the intrafusal fibers of the muscle spindle and in motor neurons (Coprav and Brouwer, 1994; Ernfors and Persson, 1991), suggesting that they act in a target-derived fashion. Thus, in these cases the neurotrophins may retrogradely support different functional classes of neurons destined to terminate in different targets.
One of the first characterised effects of NGF was its ability to elicit sympathetic outgrowth when injected into the embryo or neonate (Cohen et al., 1954; Levi-Montalcini and Booker, 1960; Olson, 1967). Exogenously added NGF also stimulates sympathetic regeneration in the adult after sympathectomy (Bjerre et al., 1973). Although postnatal sympathetic neurons respond markedly to NGF, sympathetic neurons of early developmental stages develop independently of NGF (Coughlin et al., 1977; Coughlin and Collins, 1985; Ernsberger et al., 1989). Recently, a role for NT-3 in the sympathetic system has also been shown in vivo. Mice carrying a deletion in the Nr-3 gene develop with excessive loss of sympathetic neurons (Ernfors et al., 1994a; Farinas et al., 1994). In culture, NT-3 and NGF have been shown to support sympathetic neurons sequentially; whereas NT-3 stimulates the survival of sympathetic neuroblasts (Dechent et al., 1993; Birren, 1993; DiCicco-Bloom, 1993), these neurons become NGF dependent only after terminal mitosis (Birren, 1993). This is consistent with the expression of NGF in sympathetic targets and the presence of NT-3 mRNA in the embryonic sympathetic ganglia (Bandtlow et al., 1987; Heumann et al., 1984; Schecterson and Bothwell, 1992; Shelton and Reichardt, 1984a,b). The expression of NT-3 mRNA also in some peripheral sympathetic targets (Ernfors et al., 1992; Ernfors et al., 1990; Maconsipierre et al., 1990a) suggests that NT-3 may in addition act as a target-derived factor for sympathetic neurons. We have investigated the in vivo role of NT-3 for sympathetic neuron survival and the trophic requirements of NT-3 for sympathetic target innervation.

MATERIALS AND METHODS

Labeling of mitotic and apoptotic cells

For detection of proliferating cells, bromodeoxyuridine was administered to the mice and the sympathetic cells that had incorporated the nucleotide analogue were detected by immunohistochemistry. For detection of proliferating cells, bromodeoxyuridine was administered to the mice and the sympathetic cells that had incorporated the nucleotide analogue were detected by immunohistochemistry. For detection of proliferating cells, bromodeoxyuridine was administered to the mice and the sympathetic cells that had incorporated the nucleotide analogue were detected by immunohistochemistry. For detection of proliferating cells, bromodeoxyuridine was administered to the mice and the sympathetic cells that had incorporated the nucleotide analogue were detected by immunohistochemistry. For detection of proliferating cells, bromodeoxyuridine was administered to the mice and the sympathetic cells that had incorporated the nucleotide analogue were detected by immunohistochemistry. For detection of proliferating cells, bromodeoxyuridine was administered to the mice and the sympathetic cells that had incorporated the nucleotide analogue were detected by immunohistochemistry. For detection of proliferating cells, bromodeoxyuridine was administered to the mice and the sympathetic cells that had incorporated the nucleotide analogue were detected by immunohistochemistry.

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Cell culture

The SCG were dissected from neonatal mice (P0) and cultured in F12:DMEM (1:1) substituted with 1 mg/ml bovine serum albumin (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma) on collagen-coated tissue culture dishes. In each experiment 12 ganglia were placed in the dish (1 cm²) and cultured with 20 ng/ml of recombinant NGF or NT-3 or without any neurotrophin. The ganglia were removed from the dishes for purification of RNA after 2 days in culture. SCG were also quickly frozen on dry ice at the time of dissection for purification of RNA.

RNA preparation and RNase protection assay

The tissue was frozen at −70°C until use. Total RNA was purified from 12 ganglia per experimental condition by the guanidine isothiocyanate/phenol-chloroform extraction method described by Schomczynski and Sacci (1987) and was quantified using a spectrophotometer.

The RNase protection assay was performed with the RPAII Ribonuclease Protection Assay Kit (Ambion, Austin, TX). The 378 bp trkC cRNA probe was synthesised by in vitro transcription from a DNA fragment encompassing nucleotides 1945-2317 in the rat trkC sequence (Merlot et al., 1992) covering the protein tyrosine kinase domain of the trkC receptor. The probes were labeled with (α-32P)CTP to a specific activity of 200 Ci/mmol (1-2×10⁹ cpm/μg) by transcription with T7 RNA polymerase. The RNA protection assay was carried out as described by the manufacturer. Protected cRNA fragments were separated on 4% polyacrylamide gels under denaturing condition and the gels were exposed to X-ray films. Sequence reactions of unrelated DNA with known sequences were used as size markers. The full-length trkC receptor without insertions in the kinase domain resulted in a 378 bp protected band. The presence of any insertions between nucleotides 2184 and 2185 resulted in the digestion of the 378 bp fragment into two fragments of 138 bp and 240 bp.

Fast blue tracing

Fast blue (Sigma) was used as a 4% aqueous solution. Two Nr-3-/- mice and two control mice were injected subcutaneously (0.5 μl) in the external ear at postnatal day (P) 7 using a 40 μm diameter pulled glass pipette forged to have a sealed tip and a round opening on the side. 35 μm from the tip. The mice were anaesthetized with ether during the injections and perfused transcardially with 4% paraformaldehyde 24 hours later. The SCG were dissected, immersion fixed for 1 hour in 4% paraformaldehyde, embedded in 30% sucrose overnight and cut into 20 μm thick sections. The sections were viewed with a Nikon microscope equipped with a UV filter.

Injections of NT-3 in the external ear

Nr-3-/- mice (P7) were injected with NT-3 (1 μl of a 1.7 μg/μl solution of NT-3) or saline (0.9% NaCl) in the external ear as described for fast blue tracing. The mice were perfused with paraformaldehyde (4%) 5 days later and processed for immunohistochemistry. Three animals per experimental group were injected.
Fluorescence of sympathetic fibers

The presence of sympathetic fibers was revealed by a modification of the glyoxylic acid technique for monoamine fluorescence (Gloster and Diamond, 1992; Torre, 1980). This method took advantage of the ability of the monoamine uptake system to take up not only their normal transmitter, noradrenaline, but also the false transmitter, $\alpha$-methyl noradrenaline ($\alpha$-CH$_3$-NA). Because this monoamine fluoresces, the method led to enhanced fiber fluorescence seen with the glyoxylic acid technique. Mice were injected intraperitoneally with 0.1 ml of $\alpha$-CH$_3$-NA (1 mg/ml; Sigma). Tissue was dissected 60-90 minutes later, frozen and cryostat sections (20 $\mu$m) were collected. These were incubated with the glyoxylic acid solution for 5 seconds, dried with a stream of dry cool air, covered with mineral oil, and incubated in a 95°C oven for 3 minutes. A cover-slip was then added with more mineral oil.

Immunohistochemistry

For immunohistochemistry, mice were perfused with 4% paraformaldehyde, dissected and postfixed in paraformaldehyde for 2 hours. Sections were cut at 10 $\mu$m on a cryostat. For fluorescence immunohistochemistry the sections were preincubated in dilution buffer (0.5 M NaCl, 0.01 M phosphate buffer pH 7.3, 3% bovine serum albumin and 0.3% Triton X-100) for 1 hour followed by overnight incubation with the indicated concentration of antisera in dilution buffer. After 4 washes in PBS, sections were incubated for 2 hours with the appropriate rhodamine-conjugated secondary antiserum, washed 3 times for 10 minutes and covered with glycerol/PBS (9:1) for viewing. The rabbit anti-TH was diluted 1:200 (Peel Freeze); the rabbit anti-VIP, 1:500 (Amersham); the rabbit anti-NPY, 1:1000 (Peninsula Laboratories); the mouse anti-AChE, 1:200 (Chemicon); the rabbit anti-NF 150 kDa, 1:500 (Affinity Res. Prod.). Rhodamine-conjugated goat antimouse and goat anti-rabbit antisera were used as secondary antisera. The ABC protocol was used when peroxidase immunohistochemistry was performed (Vectastain ABC kit, Vector Laboratories, CA). The sections were postfixed in paraformaldehyde for 5 minutes, washed in PBS (3x 15 minutes), treated with 50% ethanol in PBS (3x 15 minutes) and endogenous peroxidase activity blocked in 0.3% hydrogen peroxide in PBS/50% ethanol for 15 minutes, washed in PBS and blocked for 1 hour in 10% goat serum diluted in PBS. The sections were drained and incubated with a rabbit anti-TH antibody (1:300; Peel Freeze) diluted in PBS, 0.1% Tween overnight. The immunohistochemistry was then}

Fig. 1. Embryonic proliferation and apoptosis of sympathetic neurons in the $Nt-3^{-/-}$ mice. (A) The number of SCG neurons of $Nt-3^{-/-}$ mice as a percentage of age-matched control mice at embryonic and postnatal stages. Note the reduction in neuronal numbers in the mutant between E11 and E17. (B) Number of apoptotic cells in the mutant SCG as a percentage of the control, detected with the TUNEL method at several embryonic stages. Note the significant increase in apoptotic SCG cells at E11, E12 and E14, and almost normal levels at E17. (C) Number of proliferating cells in the mutant SCG as a percentage of the control detected with BrdU immunohistochemistry at several embryonic stages. Note the progressive decline in the number of proliferating cells in the SCG from the mutant mice. (D,E) Photomicrographs of E14.5 control (D) and mutant (E) SCG stained for the detection of TH. Note the reduced size of the mutant SCG. (F) Control and (G) mutant E14.5 SCG stained by the TUNEL method reveals numerous apoptotic cells in the mutant ganglion (arrowheads). (H,I) BrdU immunohistochemistry of E14.5 control (H) and mutant (I) SCG. Note reduced number of stained cells in the mutant SCG. Scale bars in D and H are 50 $\mu$m and E and I are at the same magnification. Scale bar in F is 25 $\mu$m and G is at the same magnification. Student’s t-test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
performed according to the manufacturers instructions.

RESULTS

Excessive apoptosis of early SCG neurons in Nt-3−/− mice

To determine the developmental period of sympathetic neuron loss in mice completely lacking a functional Nt-3 gene (Nt-3−/− mice), heads of E11, E12, E14, E17 and P7 Nt-3−/− mice and control mice were sectioned and the number of the SCG neurons was counted. Furthermore, 50-100 neurons were sampled to measure the neuron size at each stage and genotype. No significant size difference was detected between control and Nt-3−/− mice of the same embryonic age. However, differences in neuronal size were seen between embryos of different stages. Thus, the actual number of neurons can only be compared between control and Nt-3−/− mice of the same embryonic stage. At the earliest time point that the sympathetic chain could be identified (E11), there was only a slight reduction in the number of neurons, compared to age matched controls. However, at all stages examined between E11 and E17 the SCG of Nt-3−/− mice displayed a significant reduction in the number of neurons relative to control, and at E17 it was reduced to almost half the normal complement (Fig. 1A,D,E; Table 1). Because no significant further decrease in the number of remaining neurons was evident between E17 and P7 (Fig. 1A), these results show that the neuronal deficiency caused by the lack of NT-3 starts at the time of ganglion formation and continues to E17.

To examine the cause of the loss of SCG neurons in Nt-3−/− mice, sections were processed with the TUNEL

Fig. 2. Lack of innervation in specific sympathetic targets. Postnatal day 15 mice, (A,C,E,G) controls, (B,D,F,H) NT-3 homozygous mutants. (A,B,E,F) Monoamine fluorescence, (C,D,G,H) TH immunohistochemistry. A similar pattern of fluorescent fibers was seen in the iris (A and B) and submandibular gland (E,F) of the mutant mice (B,F), as compared to control mice (A,E), whereas the Nt-3−/− pineal gland (D) and external ear (H) lacked sympathetic innervation. Arrowheads in G indicate TH stained nerve fibers and the star and asterisk indicate autofluorescing hair shafts. Scale bar in A (for A-D,G,H) is 25 μm and (E,F) 50 μm.
method for detection of apoptotic cells. The TUNEL method allows visualization of cells containing fragmented DNA, a hallmark of apoptosis. Consistent with a role for NT-3 in the survival of neurons between E11 and E17, the number of apoptotic cells in the Nr-3−/− mice were significantly increased at E11, E12 and E14 (143%, 156% and 185%, respectively; Table 1A, Fig. 1B,F,G), as compared to age matched controls. No significant excessive apoptosis was seen at E17 (Table 1A, Fig. 1B).

The pregnant females were injected with BrdU to detect proliferating cells in the SCG immunohistochemically. The excessive loss of cells in the SCG coincided with the period of abundant proliferation (Table 1). Abundant proliferation was detected at E11, E12 and E14 in the control mice, with a peak in proliferation at E12 (Table 1B). Few proliferating cells were detected at E17 in the control mice. The excessive apoptosis in the mutant SCG caused a progressive decline during development in the number of proliferating cells (Table 1B, Fig. 1C, H and I), but did not lead to a change in the proportion of proliferating cells to total number of cells, compared to age matched controls (Table 1B). Thus, together these results suggest that NT-3 does not affect proliferation, but it supports the survival of sympathetic neuroblasts.

NT-3 appeared not to influence expression of tyrosine hydroxylase (TH), because TH-immunoreactivity was detected at E11 in the SCG of Nr-3−/− mice, similar to control mice (data not shown).

**Postnatal sympathetic innervation of peripheral targets in Nr-3−/− mice**

In order to establish whether the sympathetic neuron loss led to a deficit in target innervation, tissues from several different sympathetic targets (spleen, duodenum, sweat gland, heart, kidney, vas deference, thymus and hairy skin) of P15 Nr-3−/− mice and controls were dissected, sectioned and stained for TH-immunoreactivity to detect sympathetic nerve fibers. Qualitatively normal sympathetic innervation was found in all these targets. Sympathetic neurons innervating sweat glands switch phenotype postnatally from possessing noradrenaline to containing choline acetyltransferase, acetylcholinesterase and vasoactive intestinal peptide (VIP) (Landis et al., 1988; Leblanc and Landis, 1986). The lack of NT-3 did not affect the phenotypic switch of sympathetic neurons innervation of the sweat gland, as revealed by acetylcholinesterase and VIP immunohistochemistry.

In addition to the above targets of sympathetic innervation, four tissues innervated by the SCG were analysed in more detail. Two targets supplied by the external carotid nerve, the pineal gland and the iris, and two targets supplied by the internal carotid nerve, the submandibular gland and the external ear. Sympathetic nerve fibers of the iris and submandibular gland were detected by glyoxylic acid fluorescence and TH immunohistochemistry. The ground plexus of fluorescent nerve fibers in the Nr-3−/− iris and submandibular gland (Fig. 2B and F, respectively) appeared similar to control (Fig. 2A and E, respectively). In contrast to these targets, the pineal gland and the external ear of Nr-3−/− mice displayed a complete absence of sympathetic innervation as revealed by glyoxylic acid fluorescence and TH immunoreactivity (Fig. 2D and H, Nr-3−/−; C and G, controls).

**Developmental failure of sympathetic target innervation in Nr-3−/− mice**

The developmental sequence of pineal gland and external ear innervation in the mutant mice and control mice were analysed to establish whether nerve fibers initially reached these targets and were subsequently lost, or if the nerve fibers failed to reach the targets in the mutant mice. A few unbranched TH-immunoreactive nerve fibers were detected

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**Table 1. Number of apoptotic and proliferating neurons in the SCG of control and Nr-3−/− mice at several stages of development**

<table>
<thead>
<tr>
<th>Age</th>
<th>Control</th>
<th>Nr-3−/−</th>
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<tbody>
<tr>
<td></td>
<td>Total number of neurons</td>
<td>Number of apoptotic neurons</td>
</tr>
<tr>
<td></td>
<td>Total number BrdU* neurons</td>
<td>BrdU*/total</td>
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<tr>
<td>A. Number of apoptotic and proliferating neurons in the SCG</td>
<td></td>
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<tr>
<td>E11.5</td>
<td>3084±193</td>
<td>935±63</td>
</tr>
<tr>
<td>E12.5</td>
<td>4540±172</td>
<td>376±56</td>
</tr>
<tr>
<td>E11.5</td>
<td>4540±172</td>
<td>376±56</td>
</tr>
<tr>
<td>E17.5</td>
<td>10235±1114</td>
<td>420±44</td>
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Apoptotic cells were detected by the TUNEL method and proliferation by BrdU immunohistochemistry. The number of positive neurons in the SCG was counted in every other section. Differences in neuronal size were seen between embryos of different stages. Thus, the actual number of neurons can only be compared between control and Nr-3−/− mice of the same embryonic stage.

Student’s t-test; *P< 0.05, **P< 0.01, ***P< 0.001.
in the pineal gland of control mice at birth (Fig. 3A). At P5 and P15 a marked increase was seen in the density of fibers (Fig 3B; P5). In contrast to the control mice, the pineal gland of \( Nt-3^{-/-} \) mice lacked TH-positive sympathetic nerve fibers at all stages examined (Fig. 3E and F; P0 and P5, respectively). The analysis of eight mutant pineal glands from P7, or older mutant mice, revealed only a few unbranched immunoreactive fibers. Similar results were obtained using antisera directed against neuropeptide Y or neurofilament 150 kDa.

Interestingly, mice that carry one functional copy of the \( Nt-3 \) gene (\( Nt-3^{+/} \) mice) displayed a clear deficit in fiber branching and the formation of a ground plexus in the pineal gland. Whereas the initial sympathetic fibers appeared at a similar density and developmental stage to that of the control mice (Fig. 3C; P0), the P5 and P15 \( Nt-3^{+/} \) mice displayed a markedly reduced density of immunoreactive fibers (Fig. 3D; P5), as compared to age matched controls (Fig. 3B). These results indicate that NT-3 is present in limiting concentrations and is required for sympathetic terminal branching in the pineal gland.

In the external ear, TH-positive nerve fibers of control mice were detected in close proximity to blood vessels and hair follicles, the latter presumably sympathetic pilomotor fibers. No immunoreactive material was detected at P0, a few nerve fibers at P5 (data not shown) and a marked increase in the density of TH-positive nerve fibers was seen at P15 in control mice (Fig. 2G). \( Nt-3^{-/-} \) mice displayed a complete absence of TH-immunoreactive nerve fibers at all stages examined (Fig. 2H; P15). An absence of monoaminergic terminals in the external ear was confirmed by glyoxylic acid fluorescence after administration of the synthetic catecholamine analogue \( \alpha-CH_3-NA \). Furthermore, subcutaneous injection of the fluorescent tracer fast blue in the external ear led to retrogradely labelled sympathetic neurons in the SCG of control mice, whereas no fluorescent neurons were detected in the \( Nt-3^{-/-} \) mice. However, injection of fast blue at the base of the external ear led to retrogradely labelled sympathetic neurons in the SCG of the \( Nt-3^{-/-} \) mice (data not shown).

Thus, an absence of NT-3 leads to a failure of sympathetic fibers to invade specific targets.

**Induction of trkC mRNA expression and fiber outgrowth of postnatal sympathetic neurons by NT-3**

Because the excessive loss of sympathetic neurons occurs prior to innervation of the pineal gland and the external ear, and the failure of fibers to reach these targets occur postnatally, it appeared possible that the deficit of innervation in the \( Nt-3^{-/-} \) mice was not caused by the prenatal neuronal loss, but a requirement for NT-3 in sympathetic fiber growth postnatally. To test this hypothesis in vitro, explanted sympathetic ganglia were cultured with NGF, NT-3 or without trophic factor for 2 days and scored for neurite outgrowth. NGF elicited a profound outgrowth from explanted sympathetic ganglia (Fig. 4A), as

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**Fig. 3.** Developmental failure of pineal gland sympathetic innervation. Immunohistochemical detection of TH in postnatal day 0 (A,C,E) and 5 (B,D,F) control mice (A, B), \( Nt-3^{+/} \) mice (C,D) and \( Nt-3^{-/-} \) mice (E and F). Note the lack of fluorescent fibers in the \( Nt-3^{-/-} \) mice at both P0 (E) and P5 (F), as compared to age matched controls (A and B, respectively). Also note the reduced density of fibers in the P5 \( Nt-3^{+/} \) (D), as compared to the age matched control (B). Scale bar in A (for all panels) is 25 μm.
compared to cultures without any neurotrophin (Fig. 4C). NT-3 was also found to induce sympathetic fiber outgrowth (Fig. 4B), although fewer fibers were seen compared to cultures with NGF. RNase protection assay was performed on the cultured ganglia to determine mRNA expression of the trkC gene. The anti-sense cRNA probe that was used in the protection detects only full-length trkC receptor mRNAs and distinguishes between trkC full-length receptors without inserts (trkC FL−), and trkC full-length receptors that contain inserts in the tyrosine kinase domain (trkC FL+). The levels of trkC mRNA in freshly dissected sympathetic ganglia, or those cultured with NGF, or without neurotrophins were below the detection limit (Fig. 4D). However, abundant levels of trkC mRNA were detected in sympathetic ganglia cultured with NT-3 (Fig. 4D), indicating that NT-3 induces trkC mRNA expression in postnatal sympathetic neurons. In contrast to the expression of similar amounts of trkC receptors with and without inserts in the brain (Fig. 4D), NT-3 predominantly induced expression of receptors containing inserts in sympathetic neurons (Fig. 4D).

**Remainder sympathetic neurons of the Nt-3−/− mice respond to exogenously administered NT-3 by target innervation**

To test whether NT-3 can induce growth in vivo, the NT-3 protein was injected into the external ear of Nt-3−/− mice at P7 and the ear was processed 5 days later for TH-immunoreactivity. Whereas TH immunoreactive material was completely absent in Nt-3−/− mice receiving control injections of saline (data not shown), mice injected with NT-3 protein displayed numerous TH-immunoreactive nerve fibers and bundles in the external ear (Fig. 5A,B). These results show that the deficit of innervation in the mutant mice is likely not to be caused by the prenatal excessive loss of sympathetic neurons. Furthermore, it shows that NT-3 can induce sympathetic growth in the postnatal mouse in vivo.

**DISCUSSION**

Earlier work on the role of neurotrophins in the sympathetic system has shown that late embryonic and postnatal sympathetic neurons require NGF retrogradely for their survival (Cohen et al., 1954; Heumann et al., 1984; Levi-Montalcini and Booker, 1960; Olson, 1967; Shelton and Reichardt, 1984b). Recent studies have shown that late embryonic and postnatal sympathetic neurons grown in dissociated cultures are dependent on NGF for survival, whereas greater survival of embryonic sympathetic neurons is elicited by NT-3 (Birren et al., 1993; DiCicco-Bloom et al., 1993). NT-3 induces terminal mitosis of cultured sympathetic neuroblasts and might be involved in a change of neurotrophin receptor expression (Verdi and Anderson, 1994). Early neurons express the NT-3 receptor, trkC, which decreases during development, but is replaced by abundant expression of the NGF receptor, trkA (Birren et al., 1993; DiCicco-Bloom et al., 1993; Verdi and Anderson, 1994). We find that NT-3 is physiologically required in vivo for the survival of sympathetic neurons during prenatal stages. Thus, excessive cell death of sympathetic neurons caused by the absence of NT-3 does not occur during the period of programmed cell death (Wright et al., 1983), but coincides with proliferation and differentiation of sympathetic precursor cells and abundant levels of trkC mRNA in the ganglion. If NT-3 supports the survival of proliferating neuroblasts, a loss of these should lead to a reduced number of proliferating neuroblasts in the SCG of Nt-3−/− mice. We detected a significant decline in the number of proliferating sympathetic neuroblasts between E11 and E17 in the Nt-3−/− mice. The decrease in the number of proliferating neuroblasts coincided with an increase in the number of apoptotic cells and a decreased number of neurons remaining in the sympathetic ganglion. Our results therefore indicate that NT-3 is required for the survival of proliferating sympathetic neuroblasts in vivo. This conclusion is reasonable since we found that the lack of NT-3 did not affect proliferation, as shown by the unchanged proportion of proliferating
cells in the Nt-3−/− mice compared to control mice. Thus, the deficit of almost half of the sympathetic neurons in the Nt-3−/− mice is caused by apoptosis of sympathetic neuroblasts leading to a reduced generation of postmitotic neurons.

Sympathetic neurons continue to divide after differentiation (Rothman et al., 1978, 1980; Rohrer and Thoenen, 1987). The catecholamine synthesizing enzymes TH and dopamine β-hydroxylase therefore appear early in development, and are already present when the migrating neuroblasts condense into a ganglion (Cochard et al., 1978, 1979). Since NT-3 supports the survival of sympathetic neuroblasts during this period, it seemed plausible that NT-3 also influenced catecholamine synthesizing enzyme expression. However, the presence of abundant TH-immunoreactivity already at E11 in the Nt-3−/− mice suggests that NT-3 influences the survival of neuroblasts at this stage, but not differentiation into the catecholaminergic phenotype.

The differentiation of sympathetic neurons involves more than the expression of particular neurotransmitters and neuropeptides. For instance, sympathetic iris neurons innervating a denervated pineal gland cannot restore pineal function (Lingappa and Zigmond, 1987). The finding that Nt-3−/− mice show a deficit of approximately 50% of the SCG neurons indicate that NT-3 supports only a subpopulation of sympathetic neuroblasts during gangliogenesis. Thus, there may be at least two populations of sympathetic neuroblasts at this stage: NT-3 dependent and NT-3 independent. It is possible that the dependence of sympathetic neurons on different trophic support during differentiation reflects a commitment of the neuroblasts to lineages with particular functions in the postnatal mouse. It is also conceivable that NT-3 together with other factors support most of the embryonic SCG neurons.

Many target tissues of sympathetic innervation previously shown to express NGF mRNA, such as the vas deferens, salivary glands, heart, spleen duodenum and iris (Bandtlow et al., 1987; Ernfors et al., 1990, 1992; Heumann et al., 1984; Shelton and Reichardt, 1984a,b) contained a normal complement of sympathetic innervation in the Nt-3−/− mice. This result indicates that although the lack of NT-3 leads to an absence of about 50% of the sympathetic neurons, it does not lead to a marked reduction of sympathetic innervation of these target organs. It is interesting to note that sympathetic neurons are capable of markedly increasing the number of terminals, and can expand within the target, into novel territories and transplanted extra targets (reviewed by Purves et al., 1988). Furthermore, the density of sympathetic innervation in these targets has been shown to correlate with the levels of NGF mRNA expression (Heumann et al., 1984; Shelton and Reichardt, 1984b). Thus, the extent of sympathetic innervation of these targets appears to be limited by the availability of NGF. These results suggest that the early loss of sympathetic neurons in Nt-3−/− mice is compensated for in many targets by enlarging the target area of the remaining neurons.

We found that the ablation of the Nt-3 gene led to a failure of sympathetic fibers to invade the pineal gland and the ear. Our results do not exclude the possibility that there is a selective loss of a subset of neurons that would normally project to these targets. However, it appears unlikely that the innervation deficit is the result of the early loss of neurons in these mice because of the remarkable capacity of remaining sympathetic neurons to expand into new territories, as well as the normal complement of innervation of the sweat gland which, like the pineal gland and ear, is innervated postnatally. Instead, it is possible that the deficit of innervation reflects a failure of fibers to invade the targets. In agreement with this, sympathetic fibers were shown to be present in the proximity of the ear by retrograde tracing, and administration of NT-3 to the external ear of the mutant mice led to sympathetic innervation of the ear. Furthermore, NT-3 promoted neurite growth and induced trkC mRNA expression in explanted neonatal sympathetic ganglia. The expression of NT-3 mRNA, but not NGF mRNA, in the pineal gland (Ernfors et al. 1992) suggests a target-derived trophic role for NT-3 in pineal gland innervation.

NT-3 appears to be required selectively for the innervation of the pineal gland and external ear since no deficits were seen in innervation of the iris and submandibular gland, which are supplied by the same nerves as the pineal gland and ear. Thus, our results are consistent with a postnatal role of NT-3 in vivo, acting as a target-derived factor required for sympathetic innervation of specific targets.

We showed that trkC mRNA expression is induced in cultured sympathetic neurons by NT-3. The levels of trkC mRNA in sympathetic neurons cultured with NGF or without any neurotrophin may be below the detection limit for our RNase protection assay. Low levels of trkC mRNA expression have previously been reported in the neonatal ganglion from the rat (DiCicco-Bloom et al., 1993).

It is interesting to note that the prenatal loss of sympathetic neuroblasts in the NT-3 mutant mice plateaus at E17.5. This is the stage at which SCG neurons down regulate trkC mRNA expression and instead begin to express trkA and respond to NGF (Dechant et al., 1993; Birren, 1993; DiCicco-Bloom,
1993). Thus, the early survival effect of NT-3 is likely to be mediated by an interaction of NT-3 with the trkC receptor, and not the trkA receptor. Several forms of full-length trkC receptors containing insertions in the tyrosine kinase domain have been reported (Garner and Large, 1994; Tsouflias et al., 1993; Valenzuela et al., 1993). Activation of receptors with inserts, like full-length receptors, by NT-3 leads to tyrosine phosphorylation. However, the activation of the trkC FL− and trkC FL+ receptors leads to different responses in measurements of proliferation, neurite outgrowth and survival of cultured fibroblasts and PC12 cells (Garner and Large, 1994; Tsouflias et al., 1993; Valenzuela et al., 1993). Although the mammalian and avian trkC FL+ receptors that have been characterised exhibit different functional capacities, it is interesting to note that activation of the chick trkC FL+ receptor by NT-3 induces process outgrowth from rat PC12 cells, but does not stimulate the survival of serum-deprived PC12 cells (Garner and Large, 1994). The predominant induction of trkC FL+ receptors by NT-3 in cultured sympathetic ganglia suggests a postnatal role of NT-3 which is mediated by binding to receptors containing insertions in the kinase domain. It is possible that the different forms of trkC specify different functions of NT-3 during embryonic and postnatal development of the sympathetic nervous system.

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