Development 124, 2603-2613 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV9519

Limb proprioceptive deficits without neuronal loss in transgenic mice overexpressing neurotrophin-3 in the developing nervous system

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SUMMARY

The role of neurotrophin-3 (NT3) during sensory neuron development was investigated in transgenic mice overexpressing NT3 under the control of the promoter and enhancer regions of the *nestin* gene, an intermediate filament gene widely expressed in the developing nervous system. Most of these mice died during the first postnatal day, and all showed severe limb ataxia suggestive of limb proprioceptive dysfunction. Tracing and histological analyses revealed a complete loss of spindles in limb muscles, absence of peripheral and central Ia projections, and lack of cells immunoreactive to parvalbumin in the dorsal root ganglion (DRG). Despite these deficits, there was no neuronal loss in the DRG of these mice. At birth, transgenic DRG showed increased neuron numbers, and displayed a normal proportion of neurons expressing substance P, calcitonin gene-related peptide and the NT3 receptor trkC. Transgenic dorsal roots exhibited an increased number of axons at birth, indicating that all sensory neurons in transgenic mice projected to the dorsal spinal cord. Despite the absence of central Ia afferents reaching motorneurons, several sensory fibers were seen projecting towards ectopic high levels of NT3 in the midline of transgenic spinal cords. These findings suggest novel roles for NT3 in differentiation of proprioceptive neurons, target invasion and formation of Ia projections which are independent from its effects on neuronal survival.

Key words: sensory neurons, target innervation, 1a afferent, neurotrophin-3, mouse

INTRODUCTION

Neurotrophins are a family of soluble growth factors required for the survival, differentiation and maintenance of different subsets of peripheral and central neurons (Davies, 1994; Lewin and Barde, 1996; Persson and Ibáñez, 1993). During the period of target innervation, limiting amounts of neurotrophic factors regulate neuronal numbers by allowing survival of only some of the innervating neurons, the remaining being eliminated by programmed cell death (Barde, 1989). Increasing evidence indicates that neurotrophins also influence the proliferation, survival and differentiation of precursors of a number of neuronal lineages. Although neurotrophins are potent inducers of neurite outgrowth, they appear not to be important for guiding axons to their normal targets, but for establishing proper innervation patterns within target tissues (Ernfors et al., 1995; Hoyle et al., 1993; Zhang et al., 1994a). In the adult, neurons continue to be dependent on trophic factor support, which may be provided by the target or by the neurons themselves (Acheson et al., 1995). Thus, the neurotrophins are important signalling molecules for the development and maintenance of neurons, acting throughout development on many types of neuronal populations.

NT3 is a member of the neurotrophin family that displays multiple biological activities on peripheral and central neurons. Several in vitro and in vivo studies have demonstrated that NT3 is required for the survival of a subset of sensory neurons in DRG innervating the skeletal musculature. NT3 is very effective at supporting limb muscle sensory neurons in vitro (Horylee et al., 1993). It is expressed in skeletal muscle spindles (Copray et al., 1994), and injections of blocking anti-NT3 antibodies into the limb reduces the survival of muscle sensory neurons (Oakley et al., 1995). NT3 is also expressed in developing DRG (Schecterson and Bothwell, 1992) and in spinal cord motor neurons (Ernfors and Persson, 1991), the central target of Ia neurons, but, in contrast to the limb, injections of anti-NT3 antibodies in the spinal cord do not affect the survival of Ia neurons (Oakley et al., 1995).

Inactivation of the *NT3* gene in mice leads to the elimination of NT3-dependent Ia afferents (Ernfors et al., 1994; Fariñas et al., 1994; Tessarollo et al., 1994). Interestingly, the *NT3* mutation not only abolishes the development of proprioceptive neurons, but additionally, up to 70% of the sensory DRG neurons are lost in *NT3*–/– mice, consistent with the widespread expression of the NT3 receptor, trkC, during the period of DRG gangliogenesis. This loss appears to be due to the death of sensory precursor cells during the proliferative stage of DRG gangliogenesis (El-Shamy and Ernfors, 1996). Moreover, treatment with an anti-NT3 monoclonal antibody during early

chicken development also results in substantial cell loss during gangliogenesis (Gaese et al., 1994). Interestingly, a recent study indicates that NT3 may also play a role as an antiproliferative signal during normal development of DRG neuroblasts (Ockel et al., 1996). Exogenous application of NT3 during sensory neurogenesis decreases the number of sensory neurons in chick DRG and nodose ganglia, suggesting a premature cessation of proliferation during gangliogenesis. No particular subsets of sensory neurons seem to be selectively affected by early NT3 treatment, indicating that all sensory precursors may be equally sensitive to NT3 during gangliogenesis.

The experimental evidence accumulated so far indicates that NT3 plays a complex role during peripheral sensory development, affecting both proliferating neuroblasts during sensory gangliogenesis and Ia neurons during target innervation. In particular, it has been difficult to separate the effects of NT3 on cell survival from possible actions during neuronal differentiation and target innervation. To gain additional insight into the developmental role of NT3, we have generated transgenic mice overexpressing NT3 under the control of the promoter and enhancer regions of the *nestin* gene, an intermediate filament gene widely expressed in the developing nervous system. Unexpectedly, we found that NT3 overexpression results in a phenotype that is similar to that of NT3-deficient mice, but which is due to the failure of neurons to acquire the proprioceptive phenotype in spite of the presence of a normal complement of trkC-expressing neurons in DRG.

MATERIALS AND METHODS

Generation of *NesPIXpNT3* mice

The NesPIXpNT3 construct consisted of a region extending 5.8 kb upstream from the initiation codon of the mouse *nestin* gene (Zimmerman et al., 1994), followed by a 1 kb fragment from the third exon of the rat *NT3* gene containing the complete NT3 protein coding sequence (Ernfors et al., 1990), a 300 bp long SV40 polyadenylation signal, and 5.4 kb of *nestin* gene downstream sequence, including introns 1, 2 and 3 (Zimmerman et al., 1994). The construct was injected into fertilized mouse oocytes which were subsequently transplanted into pseudopregnant females. The offspring was screened for founders by PCR using primers 5′-TCAACCCCTAAAAGCTCC-3′, corresponding to upstream *nestin* gene sequences, and 5′-GGCAGGGTGCTCTGGTAA-3′, complementary to sequences within the *NT3* gene, which generated a 263 bp product.

Immunocytochemistry

Postnatal day (P) 0 mice were fixed by immersion for 7-14 hours in 4% paraformaldehyde, cryoprotected by overnight immersion in 10% sucrose in phosphate-buffered saline (PBS, 0.5 M NaCl, 0.01 M phosphate buffer, pH 7.3). The spine was then dissected out, rapidly frozen, and 10 μm sections were cut on a cryostat. Sections were preincubated in dilution buffer (PBS, 3% goat serum, and 0.3% Triton X-100) for 1 hour, followed by overnight incubation with the different antisera in dilution buffer. Rabbit anti-SP antiserum (Incstar) was used at a 1:1000 dilution, rabbit anti-CGRP antiserum (Amersham) was used at a 1:500 dilution, and mouse monoclonal anti-PV antibody (Sigma) at a 1:300 dilution. Sections that had been incubated with anti-CGRP and anti-SP antisera were subsequently washed four times in PBS, incubated for 2 hours with rhodamine-conjugated goat anti-rabbit secondary antiserum, washed three times in PBS, and covered by a 9:1 solution of glycerol:PBS. Sections that had been incubated with the anti-PV antibody were processed using the avidin-biotin complex method, as previously described (Kucera and Walro, 1992).

Histological analysis of muscle spindles and nerve axons

For histology of muscle spindles and nerve cross sections, hind limbs and spinal cords from P0 animals were fixed by immersion in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, and embedded in Eponate 12. In addition, adult animals and some P0 animals were perfused with the same fixative, dissected and postfixed for 2 hours, and then treated as above. Transverse sections of plastic-embedded tissues were cut on an ultramicrotome at a thickness of either 1.0 or 0.09 μm. The 1.0 μm thick sections were stained with tolouidine blue and used to locate muscle spindles. The 0.09 μm sections were stained with lead citrate and uranyl acetate and used to examine the number of nerve fibers in the soleus muscle nerve or the number of axons in dorsal roots from newborn animals.

In situ hybridization

In situ hybridization for *trk*C mRNA was performed on 10 μm cryostat sections from P0 animals that had been fixed and cryoprotected as described above. Sections were postfixed for 5 minutes in 4% paraformaldehyde, rinsed twice in PBS and twice in distilled water, delipidated with 0.2 M HCl for 10 minutes, acetylated for 20 minutes with 0.25% acetic anhydride in 0.1 M ethanolamine and dehydrated with ethanol. After drying, the sections were incubated overnight in a humidified chamber with 180 ml hybridization buffer per slide (hybridization buffer is 50% formamide, 20 mM Tris-HCl (pH 7.6) 1 mM EDTA (pH 8.0), 0.3 M NaCl, 0.1 M dithiothreitol, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly(A) RNA (Sigma), 1× Denhardt's solution, and 10% dextran sulfate) containing 2.5×10^6cpm labelled probe/ml. After hybridization, the sections were washed once in 1× SSC at 48°C for 40 minutes, treated with RNase (10 mg/ml) in 0.5 M NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA at 37°C for 30 minutes, and washed twice with 0.5× SSC and twice with 0.1× SSC for 10 minutes each at 60°C. Finally, sections were dehydrated with ethanol, dried and then dipped in KODAK NT-B2 autoradiography emulsion. After a 4 week exposure at –20°C, the slides were developed, stained lightly with cresyl violet and mounted in Permount. *trk*C riboprobes, sense and antisense, were labelled with ^{35}S-UTP by in vitro transcription from a 600 bp mouse *trk*C fragment (Lamballe et al., 1994) subcloned into pBS-KS (Stratagene) with T3 or T7 RNA polymerases, respectively.

NT3 mRNA in situ hybridization was performed on 10 μm cryostat sections from freshly frozen embryonic day (E13.5) embryos. Sections were thawed on silane-treated glass slides and quickly refrozen until usage. Thawed air-dried sections were incubated with hybridization cocktail containing an end-labelled 48-mer antisense oligonucleotide complementary to the mRNAs of rat and mouse NT3. The in situ hybridization protocol with labelled oligonucleotides has been described previously (Ernfors et al., 1990).

DiI tracing

P0 mice were fixed for 7-14 hours in 4% paraformaldehyde. Cervical and lumbar portions of spinal cord with attached dorsal root ganglia were dissected, the pia membrane carefully removed, and the ventral roots were cut. The spinal cords were pinned down on Sylgard gel Petri dishes with insect pins and submerged in 4% paraformaldehyde. Crystals of DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; Molecular probes) were applied to individual C4-C6 DRG, and the spinal cords were incubated for 7-10 days at 42°C. The spinal columns were rinsed several times in PBS, followed by embedding in 2% agar at 50°C. Cross sections (100 μm) of the spinal cord were cut on a vibratome and viewed with rhodamine filter on a Nikon microscope.

Neurite outgrowth bioassay and NT3 ELISA

Neurite outgrowth assays were performed with explanted E13.5 wild-type DRG cultured in a collagen gel matrix as previously described (Ernfors et al., 1990). Quantification of NT3 protein by ELISA was performed as previously described (Acheson et al., 1995).

RESULTS

Generation of transgenic mice and sites of transgene expression

The *nestin* gene encodes an intermediate filament protein abundantly expressed in neuronal precursors and developing neurons throughout the peripheral and central nervous systems. In the mouse, *nestin* gene expression starts at embryonic day (E) 7.5 in the neural plate (Dahlstrand et al., 1995), and increases up to E10.5, at which time *nestin* mRNA is observed in the neural tube, migrating neural crest cells and in the myotomes of the somites. At E12.5, expression is abundant in brain, neural crest, early muscle and spinal cord. At prenatal embryonic stages, expression levels decrease, and the adult nervous system is largely devoid of *nestin* expression (Dahlstrand et al., 1995; Lendahl et al., 1990). Previous studies have shown that introns 1 and 2 from the *nestin* gene are required for the correct spatiotemporal pattern of nestin expression in developing muscle and CNS, respectively (Zimmerman et al., 1994). In a construct with the bacterial *lacZ* gene as reporter (referred to as *NesPIXpLacZ*), these sequences have been shown to recapitulate the endogenous pattern of nestin expression in transgenic mice (Zimmerman et al., 1994). In multiple primary injections of the *NesPIXpLacZ* construct, strong X–gal labelling was detected in the spinal cord of several transgenic embryos from E12 to E15, primarily in cells surrounding the central canal (T. R. and C. F. I., unpublished).

We have now produced a construct in which the *nestin* gene promoter and enhancer sequences drive transcription of the protein coding region of the *NT3* gene. This construct (*NesPIXpNT3*) was used to generate transgenic mice. Expression of *NT3* mRNA in developing wild-type and transgenic embryos generated by independent injections of the *NesPIXpNT3* construct was analyzed by in situ hybridization using a probe which recognized both endogenous and overexpressed *NT3* mRNA. The pattern of *NT3* mRNA expression resembled that previously seen for *lacZ* in *NesPIXpLacZ* transgenic animals. At E11 and E13.5, elevated levels of transgenic *NT3* mRNA were mainly restricted to the spinal cord, where it concentrated in the midline in cells surrounding the central canal (Fig. 1A,B,D,E,I and K). Endogenous expression of *NT3* mRNA in the ventral motor columns was maintained in *NesPIXpNT3* embryos at levels compar-

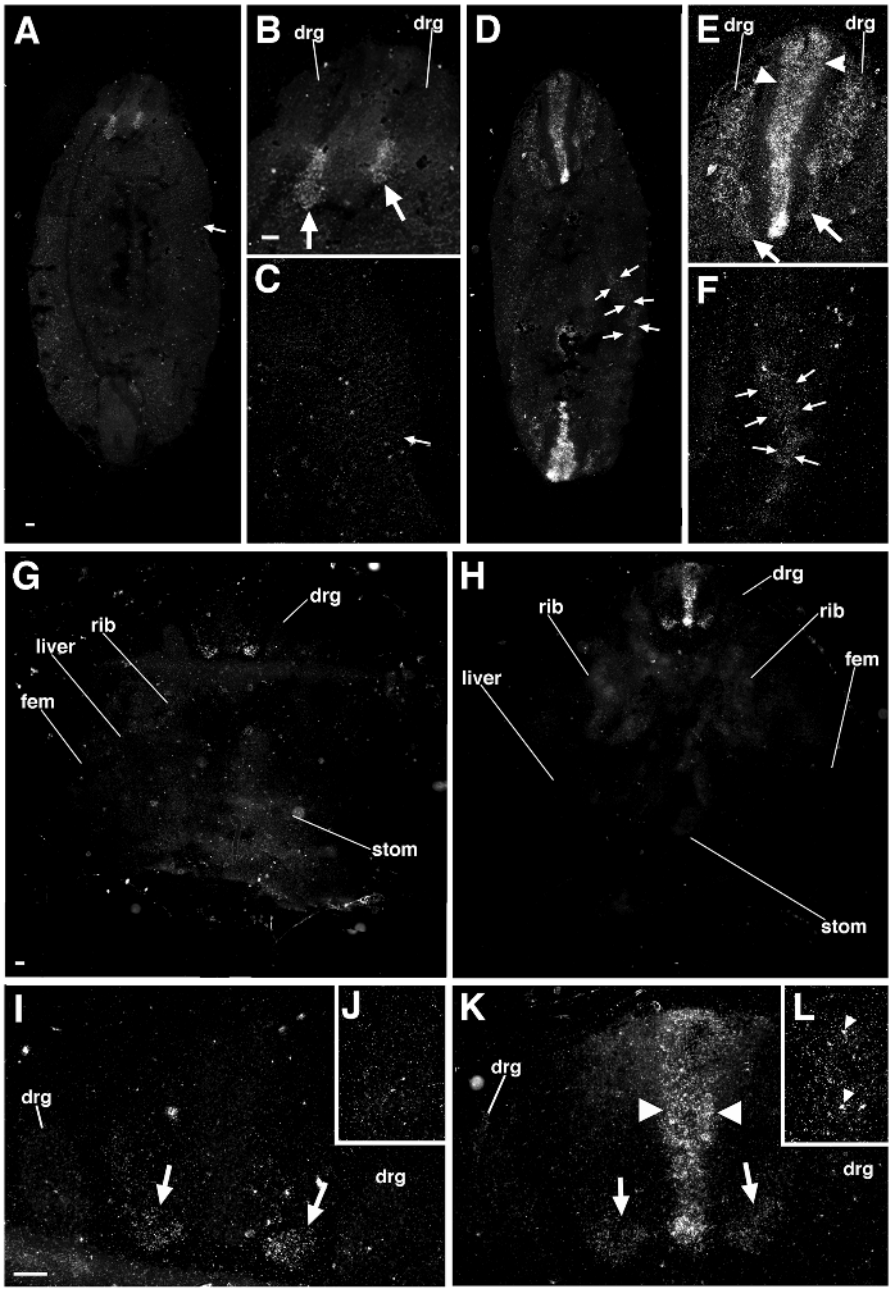

Fig. 1. *NT3* mRNA expression in wild-type and *NesPIXpNT3* mouse embryos. Dark-field in situ hybridization analysis of *NT3* mRNA expression in E11 wild-type (A-C), E11 *NesPIXpNT3* (D-F), E13.5 wild-type (G,I,J) and E13.5 *NesPIXpNT3* (H,K,L) mouse embryos. (A,D) Whole mounts of E11 embryos. Note that, due to the curvature of the embryo, the spinal cord is cut twice in these transversal sections. (B,E) Close up of the spinal cords of E11 embryos. Note prominent labelling over the central canal in the transgenic embryo (arrowheads). Arrows point to endogenous NT3 mRNA expression in the motor columns. The location of DRG is indicated. (C,F) Close up of muscle primordia showing diffuse labelling in the transgenic embryo. Small arrows mark equivalent positions in panels A and C and D and F, respectively. (G,H) Whole mounts of E13.5 embryos in transversal section. fem, cartilage primordium of femur; rib, cartilage primordium of Th12 rib; stom, stomach. (I,K) Close up of the spinal cords of E13.5 embryos. Note again prominent labelling over the central canal in the transgenic embryo (arrowheads). Lateral motor columns are labelled in both wild-type and transgenic (arrows). DRG are indicated. (J,L) Close up on DRG. Small arrowheads point to two labelled cells in transgenic DRG. Scale bars, 100 μm.

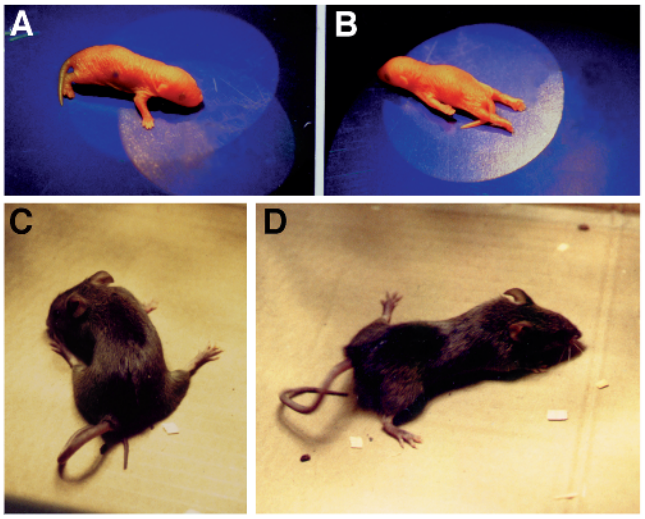

Fig. 2. Newborn and adult *NesPIXpNT3* mice display ataxia and extensor posturing. (A) control mouse, (B,C,D) *NesPIXpNT3* transgenic mice. The *NesPIXpNT3* mice had a clearly recognizable phenotype characterized by severe ataxia, lack of limb coordination and extensor posturing.

able to those seen in wild-type animals (Fig. 1E,K). Low levels of *NT3* mRNA were also seen in DRG of E11 and E13.5 transgenic, but not wild-type, mice (Fig. 1B,E,J and L). Diffuse expression of transgenic *NT3* mRNA could also be seen in muscle primordia at E11 (Fig. 1F), although at levels many fold lower than those seen in spinal cord. No overexpression of *NT3* mRNA could be seen in transgenic skin (Fig. 1D,F and H).

Early postnatal lethality, posture abnormalities and deficits in limb coordination in NT3 overexpressing mice

At birth, most of the transgenic offspring of females transplanted with oocytes injected with the *NesPIXpNT3* construct appeared clumsy and ataxic, and lacked limb coordination (Fig. 2B). When stimulated by a light tail pinch, they stiffened intermittently with all four limbs in an extensor posture, a response that also arose spontaneously as they attempted to move. Extensor posturing is a feature of deafferented limbs (Ranson, 1931). Although most of them died at P1, a few of these animals, still ataxic and with deficiencies in limb coordination, survived up to 4 months (Fig. 2C,D). Variations in the severity of this phenotype may have arisen from differences in sites of transgene integration and levels of transgene expression. In multiple transgene injections, only 10% of the animals were detected as *NesPIXpNT3* gene carriers 2 weeks after birth, compared to 30% obtained with the *NesPIXpLacZ* construct. The few adult *NesPIXpNT3* mice recovered displaying the ataxic phenotype proved unable to generate any offspring. Five normal-looking adult *NesPIXpNT3* carriers were tested for transgene transmission. Three of these animals did not transfer the *NesPIXpNT3* transgene to their offspring. The transgenic offspring of the fourth animal appeared normal, and from the results of the analysis described below, we conclude that the NT3 transgene was not expressed in this line. At birth, all of the transgenic offspring of the fifth adult *NesPIXpNT3* carrier displayed the characteristic ataxia seen in transgenic newborns obtained from independent injections of the *NesPIXpNT3* construct, and all died at P1. The same result was obtained in subsequent litters recovered from the same founder. The absence of phenotype in the founder animal suggests that it was a mosaic carrying the *NesPIXpNT3* transgene in only some of its somatic cells in addition to the germ line.

Absence of muscle spindles and peripheral and central Ia afferents in *NesPIXpNT3* mice

The abnormalities observed in the *NesPIXpNT3* animals resembled those seen in mice carrying null mutations in the *NT3* gene (Ernfors et al., 1994; Fariñas et al., 1994; Tessarollo et al., 1994) or in the NT3 receptor, trkC (Klein et al., 1994), which suggested that they might have been caused by the same de-

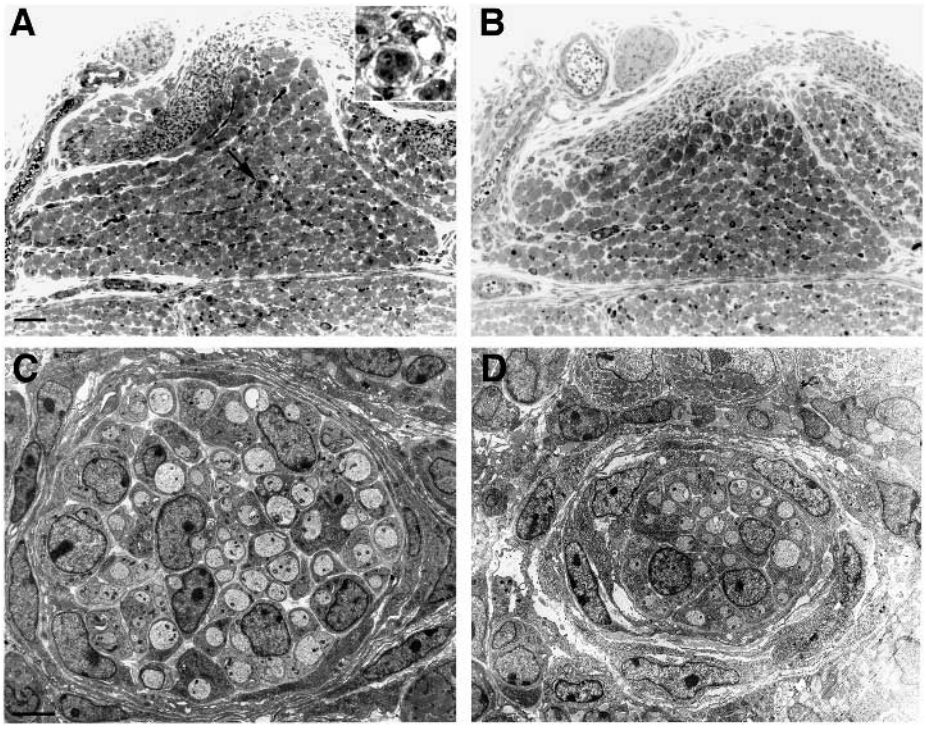

Fig. 3. Deficiencies in the peripheral part of the limb proprioceptive sensory system in *NesPIXpNT3* mice. Low-magnification micrograph of a semithin section through the soleus muscle of control (A) and *NesPIXpNT3* (B) newborn mice. A muscle spindle is seen at the centre of the picture (arrow and inset). No muscle spindles were detected in the *NesPIXpNT3* mice. (C,D) EM micrograph of a cross section through the main soleus muscle nerve. *NesPIXpNT3* mice (D) have reduced diameter and number of fibers in soleus muscle nerve as compared to control mice (C). Scale bar in A and B, 40 μm, in C and D, 5 μm.

ficiencies. *NT*3 and *trk*C–/– mice display a loss of all limb proprioceptive components, including central and peripheral Ia projections, their cell bodies, muscle spindles, and γ motor neurons. Thus, we examined muscle spindles in soleus muscle of newborn *NesPIXpNT3* mice and wild-type littermate controls from the offspring of the mosaic founder animal. No muscle spindles could be found in *NesPIXpNT3* mice, while control animals had a normal complement of muscle spindles (Fig. 3A,B, and Table 1). Since the development of these structures is dependent on Ia innervation, we examined cross sections of nerves entering the soleus muscle, by electron microscopy. At birth, *NesPIXpNT3* animals had on average 37.0±1.0 nerve fibers per nerve cross section, a 50% reduction compared to control mice (Fig. 3C,D and Table 1). The absence of muscle spindles and the deficiency in the number of axons entering the soleus muscle persisted in two adult transgenic animals obtained from independent injections of the *NesPIXpNT3* construct which had survived up to 4 months (data not shown), suggesting that these deficits were not due to a developmental delay. Interestingly, a similar reduction has also been observed in newborn *NT3*–/– mice (Kucera et al., 1995). These results indicated that the phenotype observed in *NesPIXpNT3* mice was in part due to deficits in limb proprioception.

Next, we investigated whether the central projection of the limb proprioceptive system was also affected in *NesPIXpNT3* mice. In wild-type animals, retrograde tracing with the lipophilic carbocyanide dye, DiI, showed a normal pattern of afferent innervation in the spinal cord. Ia afferents transverse the spinal grey matter as coarse bundles after entering the dorsal horn, and terminate in layer IX of the ventral spinal cord, where they make monosynaptic contacts with spinal cord motor neurons (Fig. 4A). In contrast, no Ia afferents reached the ventral spinal cord in *NesPIXpNT3* mice (Fig. 4B,C,E), although no differences could be seen in the dorsal horn layers I-IV. Newborn transgenic mice obtained from multiple primary injections of the *NesPIXpNT3* construct also lacked Ia fibers in the ventral spinal cord (data not shown), indicating that these defects were not restricted to one transgenic line, and were indeed due to overexpression of the *NesPIXpNT3* transgene. Interestingly, several fibers in *NesPIXpNT3* mice were seen to characteristically reach towards the midline, where the NT3 transgene was expressed at the highest levels (Fig. 1D). This was observed at both the cervical (Fig. 4B) and lumbar levels (Fig. 4C,E) in *NesPIXpNT3* mice but never in normal littermate controls (Fig. 4A and data not shown). At closer examination, fibers projecting towards the midline appeared of large calibre and terminating in synaptic-like bouton structures (Fig. 4D,F).

No neuronal loss in DRG of *NesPIXpNT3* mice

Deficits in proprioceptive afferents and muscle spindles in both *NT*3–/– and *trk*C–/– mice are accompanied by a marked cell loss in DRG. At birth, DRG from *NesPIXpNT3* mice look either normal or slightly enlarged when compared to controls (Fig. 5A,B). We then counted and compared the number of neurons in cresyl violet-stained sections of L2 DRG from newborn *NesPIXpNT3* transgenics and wild-type littermate controls. Neuron number appeared increased in transgenic DRG by 36% (n=10) as compared to controls (Table 2). No significant difference was detected in the number of neurons in newborn nodose ganglia (data not show), in which approximately 50% of the neurons are NT3-dependent (Ernfors et al., 1994). In contrast to newborn animals, neuronal counts in L4 DRG from two adult *NesPIXpNT3* mice that had survived up to 4 months showed a decrease of 20% compared to wild-type littermate controls

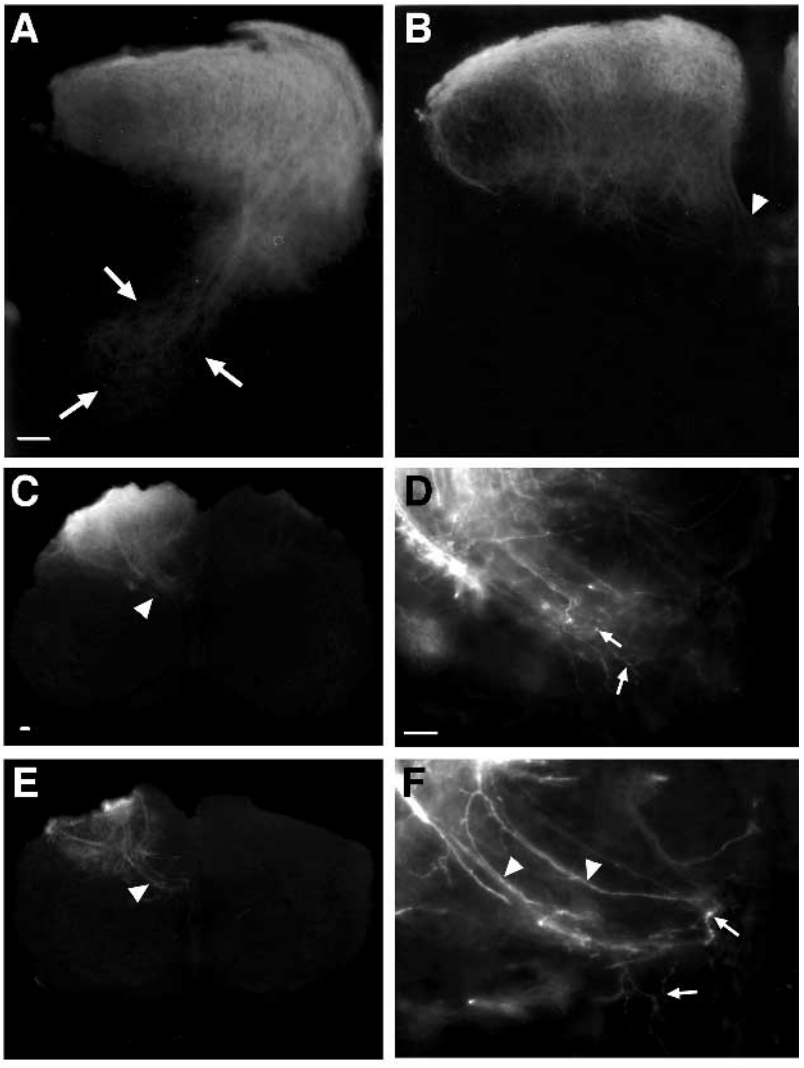

Fig. 4. Loss of central Ia afferents in *NesPIXpNT3* mice demonstrated by DiI tracing. (A,B) Micrographs of sections through the cervical spinal cord after DiI tracing. Control mice (A) showed a normal complement of Ia fibers (arrows), in contrast to *NesPIXpNT3* mice (B). (C,E) Sections through the lumbar spinal cord after DiI tracing in *NesPIXpNT3* mice showing absence of Ia fibers. Arrowheads indicate fibers extending towards high NT3 levels in the midline. (D,F) Higher magnification of C and E, respectively, showing thick fibers (arrowheads in F) projecting towards the midline terminating in synaptic-like bouton structures (small arrows). Scale bars, 40 μm.

Table 1. Counts of muscle spindles in soleus muscle and axons in soleus muscle nerve in *NesPIXpNT3* and wild-type mice

	Wild type	*NesPIXpNT3*
Number of spindles per muscle at birth (mean ± s.e.m.)	11.1±0.8 (n=9)	0.0±0.0 (n=3)
% of control	100	0
Number of axons per muscle nerve at birth (mean ± s.e.m.)	77.7±3.8 (n=9)	37.0±1.0 (n=3)
% of control	100	47.6

Transverse sections were cut from the ankle to the knee of the hind legs at a thickness of either 1.0 or 0.09 μm. For the muscle spindle counts, every 20th section (1 μm) was collected, stained with tolouidine blue and the number of spindles in the soleus muscle was determined. Nerve axons in the main soleus muscle were counted in EM micrographs of cross sections (0.09 μm) of Soleus muscle nerves.

(Table 2). Absolute neuronal counts in newborn and adult DRG were obtained from cryo and plastic sections, respectively, by different quantification methods and cannot be directly compared with each other. Together, these data suggest that expression of the *NesPIXpNT3* transgene during embryonic development rescued a certain number of sensory neurons or their precursors from developmental programmed cell death, while downregulation of the *nestin* promoter in the *NesPIXpNT3* transgene after birth deprived NT3-dependent neurons in transgenic DRG from trophic support.

In addition, DRG from *NesPIXpNT3* mice were abnormal in overall morphology throughout their lifespan. In wild-type animals, DRG appeared as separate entities. In contrast, DRG of *NesPIXpNT3* mice were linked together into a longitudinal chain by narrow bridges of tissue containing sensory neurons, without gaps between individual ganglia (Fig. 5C,D). In addition, the dorsal root of mutant animals often exited the DRG at different points and joined outside the ganglion to form the dorsal root proper (not shown).

Table 2. Total number of cells, and proportion of cgrp, sp and *trk*c mrna positive cells in the lumbar DRG of *NesPIXpNT3* and wild-type mice

	Wild type	*NesPIXpNT3*
Number of neurons in L2 DRG at birth (mean ± s.e.m.)	3971±380 n=10	5392±600 n=10
% of control	100	136
Number of neurons in adult L4 DRG (mean ± s.e.m.)	8536±364 n=2	6864±368 n=2
% of control	100	80
Proportion of CGRP positive cells in newborn L2-L4 DRG (% ± s.e.m.)	20.6±0.9 n=2	19.7±2.7 n=2
Proportion of SP positive cells in newborn L2-L4 DRG (% ± s.e.m.)	18.4±0.78 n=2	19.5±0.5 n=2
Proportion of TrkC mRNA positive cells in newborn L2-L4 DRG (% ± s.e.m.)	15.9±0.95 n=3	16.0±5.57 n=3

Transversal sections through the dorsal root ganglia were taken at 10 μm. For the cell counts, neurons with a clear nucleus and nucleoli were counted at every fifth section. The narrowest point of the bridge between two adjacent ganglia was taken as the boundary between the ganglia. CGRP and SP positive cells were counted as a percentage of total cell number. *trkC* mRNA positive cells were counted as a percentage of total cell number in sections hybridized in situ with a ^{35}S-UTP-labelled probe complementary to mouse *trkC* mRNA.

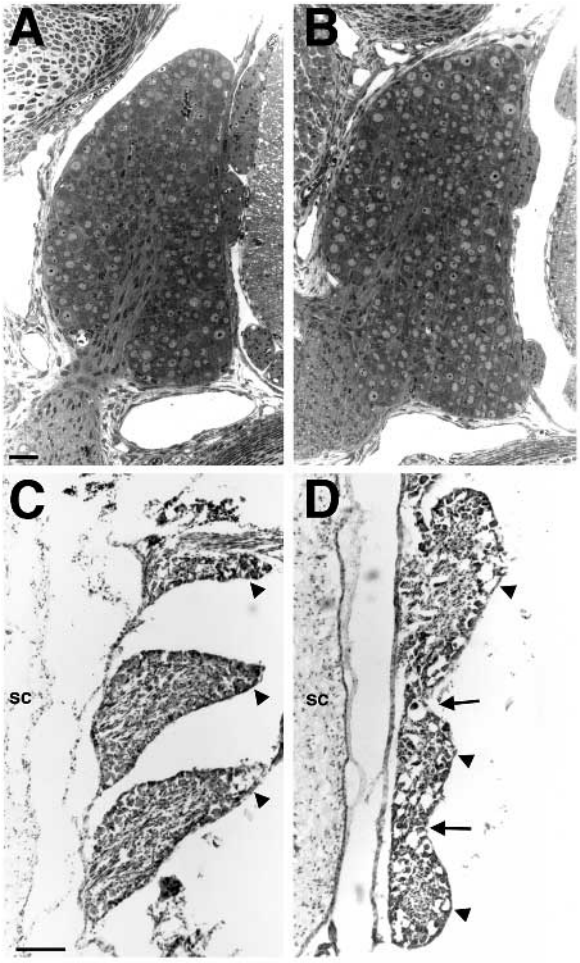

Fig. 5. Morphology of DRG from newborn wild-type and *NesPIXpNT3* mice. Micrographs of semithin sections through wild-type (A) and *NesPIXpNT3* (B) newborn DRG. Longitudinal cryosections of lumbar DRG of newborn wild-type (C) and *NesPIXpNT3* mice (D) stained with cresyl violet. Lumbar DRG (arrowheads) of *NesPIXpNT3* mice were linked together into a longitudinal chain by narrow bridges (arrows) of tissue containing sensory neurons (D), in contrast to DRG of control animals (C), in which gaps between individual ganglia were apparent. SC, spinal cord.

We then investigated the possibility that *NesPIXpNT3* transgene overexpression may have affected the proportion of different neuronal subpopulations within DRG of transgenic animals. Using immunohistochemistry, we examined the expression of parvalbumin (PV), a metabolic marker of proprioceptive neurons, as well as calcitonin gene-related peptide (CGRP) and substance P (SP), two markers of nociceptive neurons. PV immunoreactivity was absent in DRG of newborn *NesPIXpNT3* mice (Fig. 6A,B). No differences were seen, however, in the number or labelling intensity of CGRP and SP containing neurons between *NesPIXpNT3* mice and wild-type littermate controls (Fig. 6C-F and Table 2). Because expression of PV is believed to depend on several functional properties of proprioceptive neurons, such as firing rate and target innerva-

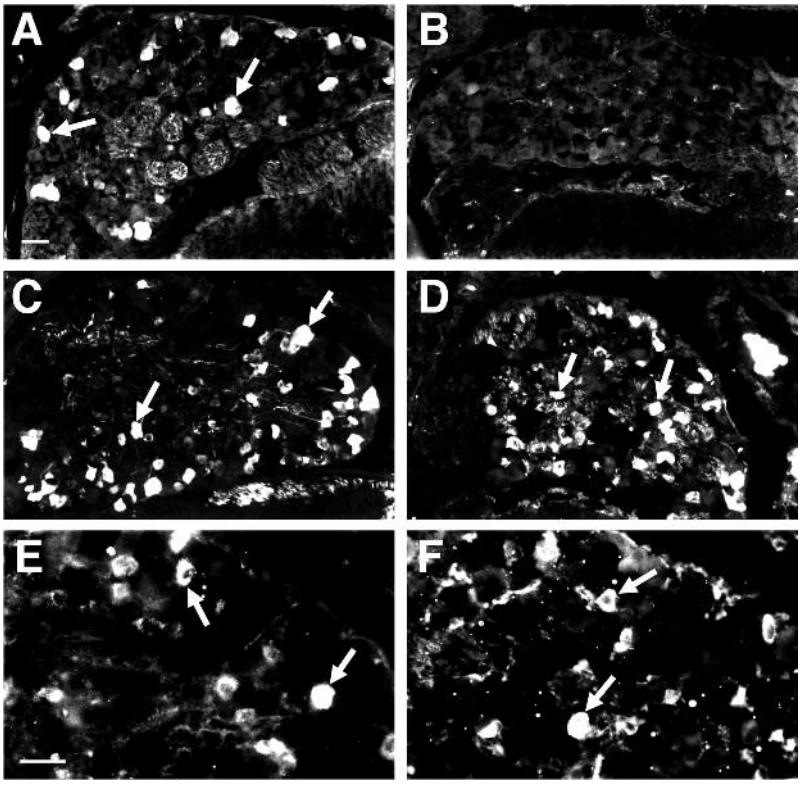

Fig. 6. No PV, but normal complement of SP and CGRP immunoreactive cells in DRG of *NesPIXpNT3* mice. PV immunocytochemistry (A,B), CGRP immunocytochemistry (C,D), and SP immunocytochemistry (E,F) of wild-type (A,C,E) and *NesPIXpNT3* (B,D,F) newborn DRG. Several PV immunoreactive neurons were seen in sections from wild-type, but not transgenic, DRG. No differences were seen, however, in the number or labelling intensity of CGRP and SP containing neurons between *NesPIXpNT3* mice and wild-type littermate controls. Arrows indicate labelled cells. Scale bars, 40 μm.

tion, its absence in transgenic DRG correlated with the lack of proprioceptive projections in these animals. We then examined expression of the NT3 receptor trkC, which is considered a good marker for NT3 responsive Ia neurons in normal postnatal DRG. Despite the absence of PV immunoreactivity, *trk*C mRNA-expressing neurons were present in normal proportion in DRG from *NesPIXpNT3* mice (Fig. 7 and Table 2). Taken together, our results suggest that *trk*C-expressing neurons in transgenic DRG failed to acquire functional properties of normal proprioceptive neurons, perhaps due to their inability to innervate their peripheral and central targets.

A role for NT3 in the formation of central Ia projections

NT3 overexpression might have prevented Ia target innervation via a number of different mechanisms. In explanted chicken sympathetic ganglia, nerve growth factor (NGF)-induced neurite outgrowth shows a bell-shaped dose-response curve,

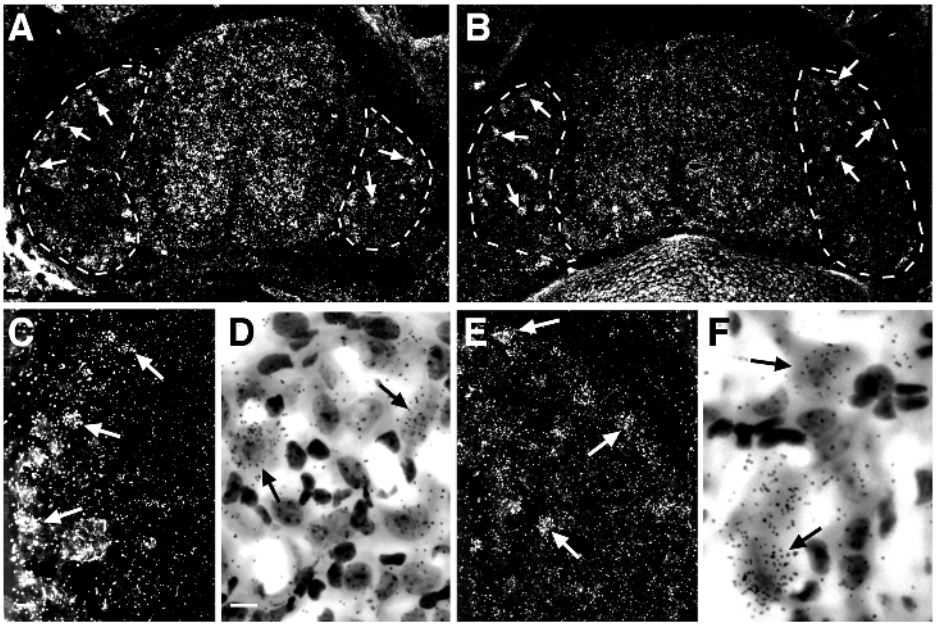

Fig. 7. Normal proportion of *trk*C *mRNA* containing cells in *NesPIXpNT3* mice. Dark-field (A-C,E) and bright-field (D,F) micrographs of spinal cord and DRG sections hybridized in situ with a ^{35}S-UTP-labelled riboprobe complementary to murine *trk*C mRNA. *NesPIXpNT3* mice (B,E,F) show *trk*C mRNA positive cells at a similar frequency to wild-type animals (A,C D). Scale bars, 40 μm. Arrows indicate labelled cells.

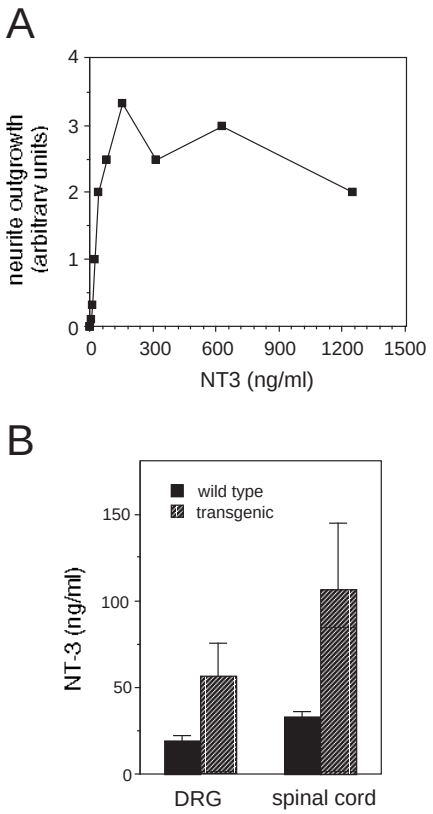

Fig. 8. (A) Dose-response relationship of NT3-induced neurite outgrowth in explanted wild-type E13.5 mouse DRG. Results are expressed in arbitrary units as mean ± s.d. (*n*=4). (B) ELISA determination of NT3 protein concentration in DRG and spinal cord of wild-type (solid bars) and *NesPIXpNT3* (hatched bars) mice. Protein concentration is expressed in ng of NT3 per ml of tissue. Tissue volumes were calculated from the dimensions of dissected pieces of tissue prior to homogenization. Results are expressed as mean ± s.e.m. (*n*=6).

with complete inhibition at supramaximal concentrations (Trupp et al., 1995). We therefore examined the dose-response relationship of NT3-induced neurite outgrowth in explanted wild-type embryonic mouse DRG at the time of central target innervation (Kucera et al., 1995). NT3 stimulated outgrowth from E13.5 DRG up to a concentration of 156 ng/ml, after which the response saturated (Fig. 8A). No significant inhibitory effect by supramaximal concentrations of NT3 was seen below 1 μg/ml (Fig. 8A). An ELISA specific for NT3 was then used in order to quantify the level of NT3 protein in DRG and spinal cords of E13.5 *NesPIXpNT3* embryos generated from independent injections of the *NesPIXpNT3* construct. NT3 protein levels were found to be 55±18 and 104±38 ng/ml (*n*=6) in DRG and spinal cord, respectively, of E13.5 *NesPIXpNT3* embryos. The elevated levels of NT3 protein in transgenic DRG could have been produced locally (see Fig. 1), although we think they are more likely to be the result of diffusion from the high levels of NT3 produced in spinal cord. In general, the level of overexpression of NT3 protein in transgenic tissue represented only a relatively modest, approximately 3-fold, increase over normal NT3 levels in wild-type animals (Fig. 8B), and corresponded to concentrations of NT3 in transgenic tissue below the level of saturation of NT3-induced neurite outgrowth in embryonic mouse DRG. Combined, these data suggest that NT3 overexpression in *NesPIXpNT3* mice did not prevent Ia target innervation by overall inhibition of DRG axonal outgrowth.

Table 3. Number of axons in lumbar dorsal roots of newborn and adult *NesPIXpNT3* and wild-type mice

	Wild type	*NesPIXpNT3*
Total number of axons in newborn L2 dorsal roots (mean ± s.e.m.)	7844±230 *n*=3	11568±589 *n*=3
% of control	100	147
Number of myelinated axons in adult dorsal roots (mean ± s.e.m.)	2403.5±1.5 *n*=2	1513.66±85.7 *n*=2
% of control	100	63

Number of axons in newborn L2 dorsal roots was quantified in cross sections by electron microscopy. Myelinated axons in dorsal roots were counted in semithin sections.

It follows from this observation that, if all neurons in DRG from *NesPIXpNT3* mice projected to the spinal cord, newborn transgenic dorsal roots should exhibit an increased number of axons similar to the increase seen in neuron number. We therefore quantified, by electron microscopy, the total number of axons in cross sections of L2 dorsal roots of newborn *NesPIXpNT3* mice and wild-type littermate controls. This analysis revealed an increase of 47% compared to controls in the number of axons in transgenic L2 dorsal roots at birth (Table 3). This increase was in accordance with the increase in neuron number observed in L2 DRG of newborn transgenic mice (Table 2). The discrepancy between axon and neuron number could be due to differences in counting procedures, although the possibility that some P0 DRG neurons send more than one axon cannot be ruled out. As neuron numbers decreased in DRG of adult *NesPIXpNT3* mice (Table 2), so did axon numbers in their corresponding dorsal roots. In two surviving adult *NesPIXpNT3* mice, dorsal roots showed a 50% depletion of myelinated axons compared to wild-type animals of the same age (Fig. 9 and Table 3). Taken together, these data indicate that most, if not all, of the neurons in transgenic DRG did project to the dorsal spinal cord, but that prospective Ia afferents never reached the ventral part of the cord.

DISCUSSION

The most striking aspect of the *NesPIXpNT3* mice was a marked similarity of their phenotype to that of *NT3*- and *trk*C-deficient mice (Ernfors et al., 1994; Fariñas et al., 1994; Klein et al., 1994; Tessarollo et al., 1994). The majority of *NesPIXpNT3* mice die at birth, similar to the *NT3* null mutant. In addition, *NesPIXpNT3*, *NT3*–/– and *trk*C–/– mice all exhibit a kind of limb ataxia that is associated with dysfunction of the limb proprioceptive system. Thus, three different genetic defects may result in one striking phenotype by either excess or defect in NT3 signalling. Unlike *NT3*–/– or *trk*C–/– mice, however, there was no neuronal loss in the DRG of *NesPIXpNT3* mice at birth. The absence of PV expression, a marker of mature Ia neurons (Carr and Nagy, 1993), in transgenic DRG exhibiting a normal complement of trkC-expressing cells suggests that NT3 over-

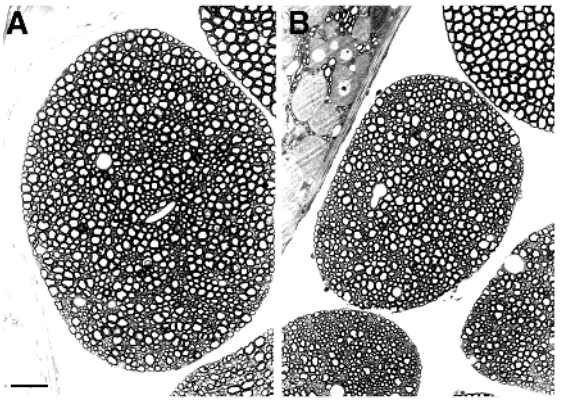

Fig. 9. Reduction in the number of myelinated Ia dorsal root afferents in adult *NesPIXpNT3* mice. Micrographs of semithin sections through dorsal roots (DR) of control (A) and *NesPIXpNT3* (B) adult mice. The number of myelinated axons in DR of adult *NesPIXpNT3* mice was 63% of that in wild-type animals. Scale bar, 40 μm.

expression somehow prevented Ia neuron differentiation while maintaining their survival. Moreover, the normal counts of CGRP and SP positive cells suggest that the neurons that should have become Ia did not adopt an alternative phenotype, but merely failed to develop into proprioceptive neurons. The deficits in Ia differentiation could be secondary to the lack of Ia projections. It is therefore possible that trkC-expressing cells in transgenic DRG failed to acquire physiological properties of Ia neurons as a consequence of their inability to innervate targets in skeletal muscle and spinal cord.

What is the evidence for a role of neurotrophins in the formation of projections? Overexpression of NGF in the sympathetic ganglia of DBH-NGF transgenic mice did not prevent sympathetic axons from reaching peripheral tissues, although terminal sympathetic innervation within tissues was greatly decreased (Hoyle et al., 1993). Moreover, lack of brain-derived neurotrophic factor (BDNF) and NT3 has been shown not to affect ingrowth of nerve fibers into the vestibular epithelium, although fibers in BDNF mutants failed to invade the target and to maintain afferent and efferent innervation (Ernfors et al., 1995). These observations are consistent with the notion that neurotrophin gradients are not required to guide axons over long distances, but are necessary for target invasion and for the establishment of the normal density and pattern of innervation within target tissues. In a recent study, systemic administration of NT3 delayed the growth of dorsal root axons in spinal cord grey matter when applied at early developmental stages (Zhang et al., 1994a). This inhibitory effect was not seen if NT3 was applied at later stages, after Ia axons had already penetrated the grey matter of spinal cord. Our data indicates that NT3 does not have a general inhibitory effect on the growth of axons. Quantification of the number of axons in transgenic dorsal roots at birth showed no loss of sensory axonal projections to the spinal cord. Thus, it would appear that the axons from prospective Ia neurons of transgenic mice were able to enter the dorsal spinal cord, but then failed to reach ventrally towards the motor neurons, indicating that NT3 prevented Ia fibers from innervating the ventral spinal cord by a mechanism other than inhibiting axonal outgrowth all together.

During development, NT3 expression in the spinal cord is normally restricted to the motor columns (Ernfors and Persson, 1991). Could NT3 be playing a chemotropic role during the innervation of motor neurons by Ia afferents? NT3 appears not to be required for the ventral projection of central Ia afferents because injection of anti-NT3 blocking antibodies in the spinal cord had no effect on these projections (Oakley et al., 1995). In addition, a preliminary study showed that the level and pattern of expression of netrin-1 and netrin-2, two axon guidance molecules present in embryonic spinal cord (Kennedy et al., 1994; Serafini et al., 1994), were normal in *NesPIXpNT3* mice (T. R. and C. F. I., unpublished results). Nevertheless, our data showed that a number of spinal cord afferents in *NesPIXpNT3* mice had projected towards the high NT3 levels in the midline, where they appeared to be forming synaptic-like terminal boutons. Thus, although sensory axons may still be able to navigate correctly in the spinal cord in the absence of NT3, a precise spatial distribution of NT3 within the spinal cord is important for correct target invasion, directional growth and motorneuron innervation. We would therefore like to propose that it was the ectopic expression of NT3, rather than increased NT3 levels, that perturbed Ia target innervation in the transgenic spinal cord. Because the terminals of prospective Ia fibers entering the cord may not normally be exposed to substantial levels of NT3 until they reach the proximity of motor neurons, a premature exposure to NT3 may have either halted or redirected further growth of prospective Ia axons in *NesPIXpNT3* mice by supplying an untimely signal to the terminal. Taken together, the available evidence is consistent with a role for a gradient of NT3 in controlling the formation of central Ia projections during the final stages of motor neuron innervation, perhaps at the level of the growth cone by refining the direction of growth and inducing synaptogenesis. This is in agreement with the results of Oakley et al. (1995) showing that, unlike NT3 from peripheral tissues, spinal cord-derived NT3 appears to be controlling processes other than neuron survival.

Prospective Ia neurons in *NesPIXpNT3* mice failed to differentiate even though they expressed trkC and had access to adequate amounts of NT3, suggesting that factors other than NT3 are the most likely regulators of the proprioceptive neuronal phenotype. Because the axons from these neurons did reach the spinal cord but not the muscles, it could be speculated that the access to muscle-derived factors, or the establishment of normal muscle connections, or both, may be responsible for controlling terminal Ia neuron differentiation. Interestingly, recent findings in developing chick embryos suggest that the target muscle is a source of cues that influence the central connections of the sensory neurons projecting to it (Wenner and Frank, 1995). However, in rodents, the innervation of motor neurons by Ia afferents precedes the establishment of afferent-muscle contacts (Kucera et al., 1995), suggesting that the absence of muscle sensory afferents in *NesPIXpNT3* mice could have been secondary to the loss of central Ia projections, in agreement with the notion that only those sensory neurons that succeed in contacting spinal motor neurons become capable of inducing the formation of muscle spindles and of acquiring the Ia phenotype (Kucera et al., 1995). Thus, ectopic expression of NT3 in *NesPIXpNT3* mice may have aborted Ia neuron differentiation by preventing the formation of Ia projections. A subpopulation of cutaneous sensory neurons have recently been

shown to depend on NT3 (Airaksinen et al., 1996), and upregulation of NT3 expression in skin using a keratin gene promoter construct resulted in a large increase in the number of DRG sensory neurons (Albers et al., 1996). Could the remaining trkC cells seen in DRG of *NesPIXpNT3* mice be cutaneous neurons projecting to the skin? Cutaneous SA neurons appear not to depend on NT3 until after the first postnatal weeks (Airaksinen et al., 1996). Thus, it would seem unlikely that the trkC-expressing cells seen at birth in *NesPIXpNT3* mice represent only cutaneous sensory neurons.

In a recent study, administration of NT3 during DRG gangliogenesis in chick embryos resulted in a marked decrease in cell number, presumably due to antiproliferative effects of NT3 during early gangliogenesis (Ockel et al., 1996). In contrast, NT3 applications at later developmental stages increased neuron numbers by 32%, perhaps by preventing naturally occurring cell death in DRG (Ockel et al., 1996). Our data, showing a 36% increase in DRG neuron number at birth in *NesPIXpNT3* mice, would be consistent with the rescue of NT3-dependent DRG neurons from developmental programmed cell death. However, the fact that the proportion of trkC-expressing neurons in DRG of *NesPIXpNT3* mice was the same as in controls suggests instead that cell counts were increased proportionally among different neuron types in transgenic DRG, presumably by an early action of NT3 on cell precursors. The *nestin* gene promoter targeted expression of the *NesPIXpNT3* transgene also early during development, at the time of proliferation of DRG precursors. Thus, the increased neuron number in transgenic DRG at birth appears to be more consistent with an early role of NT3 as a survival factor for DRG neuron precursors (El-Shamy and Ernfors, 1996).

Our observation of groups of transgenic DRG forming longitudinal chains of interconnected ganglia is very intriguing and could indicate an early developmental action of NT3 during DRG gangliogenesis. The formation of DRG initiates after neural crest cells migrate ventrally along the sides of the neural tube and aggregate in the rostral half of each somite. Many neuronal precursors express the NT3 receptor trkC during ganglion formation in the rat (Ernfors and Persson, 1991). In the chick, migrating neural crest cells express trkC, (Kahane and Kalcheim, 1994; Zhang et al., 1994b), and display biological responses to NT3 in culture (Kalcheim et al., 1992; Pinco et al., 1993). Thus, the DRG chains observed in *NesPIXpNT3* mice would be consistent with an incomplete rostral aggregation or migration of neural crest cells caused by NT3 overexpression during DRG condensation (Zimmerman et al., 1994). Alternatively, and because the fusion of DRG was limited to the core of the lumbar region that supplies proprioceptive innervation to the limb (i. e. L2 to L5), this could also have been a consequence of the increased number of neurons in transgenic ganglia.

Deciphering the actual roles played by neurotrophic factors during the ontogenesis of the mammalian nervous system has been complicated by the multiplicity of activities that these molecules display at almost every developmental stage. Ample evidence has established the crucial roles played by the neurotrophins in the regulation of neuronal numbers during sensory neuron development (Lewin and Barde, 1996). By interfering with the timing, site and levels of NT3 expression to which sensory neurons are exposed during normal development, our findings suggest novel roles for NT3 in the formation of Ia projections and the differentiation of proprioceptive neurons which are independent from its effects on survival.

We thank Ann Acheson and Muyeve Meroro, Regeneron Pharmaceuticals Inc., for help with the NT3 ELISA, Mariano Barbacid, Bristol-Myers Squibb, for the mouse *trk*C cDNA clone, Keneth G. Baimbridge, University of British Columbia, for anti-PV antibodies, and Marc Tessier-Lavigne, University of California San Francisco, for cDNA clones for mouse netrin-1 and -2. We also thank Erik Nilsson and Ann-Sofie Nilsson for excellent technical assistance, Lotta Johansson for excellent secretarial assistance, and Annika Ahlsén for additional technical help. The support of Prof. Hugo Lagercrantz is also acknowledged. Financial support was obtained from the Swedish Cancer Society, the Swedish Medical Research Council and the Karolinska Institute (to C. F. I. and U. L.), and from the Veterans Administration, the National Science Foundation and the National Institutes of Health (to J. K.). T. R. was supported by a combined fellowship from the Departments of Medical Biochemistry and Biophysics, and Women and Child Health, Karolinska Institute.

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(Accepted 2 May 1997)