

Differential Expression of mRNAs for Neurotrophins and Their Receptors after Axotomy of the Sciatic Nerve

Hiroshi Funakoshi,* Jonas Frisén,‡ Gisela Barbany,* Tönis Timmusk,* Olof Zachrisson,* Valerie M. K. Verge,§ and Håkan Persson*†

*Department of Medical Chemistry, Laboratory of Molecular Neurobiology, ‡Department of Anatomy, and §Department of Histology and Neurobiology, Karolinska Institute, Stockholm, Sweden

Abstract. The neurotrophin family includes NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Previous studies have demonstrated that expression of NGF and its low-affinity receptor is induced in non-neuronal cells of the distal segment of the transected sciatic nerve suggesting a role for NGF during axonal regeneration (Johnson, E. M., M. Taniuchi, and P. S. DeStefano. 1988. *Trends Neurosci.* 11:299–304). To assess the role of the other neurotrophins and the members of the family of Trk signaling neurotrophin receptors, we have here quantified the levels of mRNAs for BDNF, NT-3, and NT-4 as well as mRNAs for *trkA*, *trkB*, and *trkC* at different times after transection of the sciatic nerve in adult rats. A marked increase of BDNF and NT-4 mRNAs in the distal segment of the sciatic nerve was seen 2 wk after the lesion. The increase in BDNF mRNA was mediated by a selective activation of the BDNF exon IV

promoter and adrenalectomy attenuated this increase by 50%. NT-3 mRNA, on the other hand, decreased shortly after the transection but returned to control levels 2 wk later. In Schwann cells ensheathing the sciatic nerve, only *trkB* mRNA encoding truncated TrkB receptors was detected with reduced levels in the distal part of the lesioned nerve. Similar results were seen using a probe that detects all forms of *trkC* mRNA. In the denervated gastrocnemius muscle, the level of BDNF mRNA increased, NT-3 mRNA did not change, while NT-4 mRNA decreased. In the spinal cord, only small changes were seen in the levels of neurotrophin and *trk* mRNAs. These results show that expression of mRNAs for neurotrophins and their Trk receptors is differentially regulated after a peripheral nerve injury. Based on these results a model is presented for how the different neurotrophins could cooperate to promote regeneration of injured peripheral nerves.

SCHWANN cells in the distal part of the transected sciatic nerve secrete a variety of factors that facilitate regeneration of the injured nerve fibers (Cajal, 1928; David and Aguayo, 1981; Fawcett and Keynes, 1990). The best characterized of these factors is NGF, which in the peripheral nervous system supports the survival of sympathetic and neural crest-derived sensory neurons (Levi-Montalcini, 1987). NGF mRNA and protein are not detected in the intact adult rat sciatic nerve but their synthesis is markedly induced in nonneuronal cells of the distal segment of the axotomized nerve (Heumann et al., 1987a,b). Similarly, the level of mRNA for the low-affinity NGF receptor ($p75^{NGFR}$)¹ is in-

creased in nonneuronal cells in the distal segment of the injured nerve (Heumann et al., 1987b). The addition of macrophages, known to invade the site of injury in vivo, to cultured pieces of sciatic nerve induces NGF mRNA synthesis suggesting that the increase is mediated by factors secreted by macrophages (Heumann et al., 1987b). In agreement with this, recombinant interleukin-1 beta (IL-1 β) increases NGF mRNA in nonneuronal cells of the rat sciatic nerve (Lindholm et al., 1987).

The purification and molecular cloning of a second neurotrophic factor, brain-derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989) led to the discovery of a family of neurotrophic factors collectively known as the neurotrophins. Besides NGF and BDNF, the neurotrophin family includes neurotrophin-3 (NT-3) (Hohn et al., 1990; Maisonpierre et al., 1990; Ernfors et al., 1990; Rosenthal et al., 1990; Kaisho et al., 1990; Jones and Reichardt et al., 1990) and neurotrophin-4/5 (NT 4/5) (Hallböök et al., 1991; Berkemeier et al., 1991; Ip et al., 1992). The neurotrophins show ~50% amino acid sequence identities which are clustered in conserved regions intersected by variable regions, combinations of which define the biological

Please address all correspondence to Dr. H. Funakoshi, Department of Medical Chemistry, Laboratory of Molecular Neurobiology, Karolinska Institute, Box 604 00, S-104 01, Stockholm, Sweden.

The current address of Valerie M. K. Verge is the Department of Anatomy, College of Medicine, Saskatoon, SK, Canada S7N 0W0.

[†]Dr. Håkan Persson deceased 16 May 1993.

1. *Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglia; IL-1 β , interleukin-1 beta; NT-3, neurotrophin-3; NT-4, neurotrophin-4; NT-4/5, neurotrophin-4/5; $p75^{NGFR}$, the low-affinity NGF receptor; TrkA, $p140^{trk}$; TrkB, $p145^{trkB}$; TrkC, $p145^{trkC}$.

specificity of each neurotrophin (Ibáñez et al., 1991). The survival of neurons in the sympathetic and nodose ganglia is promoted by specific neurotrophins, while all four neurotrophins support the survival of embryonic sensory neurons in cell culture (Barde, 1989; Hohn et al., 1990; Maisonpierre et al., 1990; Hallböök et al., 1991; Berkemeier et al., 1991). All four neurotrophins bind to p75^{NGFR} with similar affinities (Kd 10⁻⁹M) (Rodriguez-Tebar et al., 1990, 1992; Ernfors et al., 1990; Hallböök et al., 1991). The specificity of the neuronal subpopulations responsive to the different neurotrophins is provided by the selective interaction between the different neurotrophins and members of the Trk family of tyrosine kinase receptors either alone (Klein et al., 1991a) or together with p75^{NGFR} (Hempstead et al., 1991). NGF binds to and activates p140^{trk} (herein called TrkA) (Kaplan et al., 1991; Klein et al., 1991a). BDNF and NT-4 interact with p145^{trkB} (herein called TrkB) (Soppet et al., 1991; Squinto et al., 1991; Klein et al., 1989, 1991b, 1992; Ip et al., 1992), while NT-3 interacts with p145^{trkC} (herein called TrkC) and to a lesser extent also with TrkA and TrkB (Cordon-Cardo et al., 1991; Lamballe et al., 1991; Klein et al., 1991b; Squinto et al., 1991).

The sciatic nerve contains a mixture of sensory and motor axons and transection of the nerve increases the synthesis in the neuromuscular junction of acetylcholine receptors, the cell-adhesion molecule N-CAM and other proteins important for synaptogenesis (Changeux, 1991; Gatchalian et al., 1989; Daniloff et al., 1989). Moreover, peripheral neurite outgrowth after injury is facilitated by the extracellular matrix, probably in particular laminin (Wang et al., 1992) and fibronectin, and the expression of fibronectin is increased in the Schwann cells after transection of mature peripheral neurons (Lefcort et al., 1992). Target-derived trophic factors could also play a role in the regenerative response and in agreement with this possibility, denervation of skeletal muscle induces neurite-outgrowth and survival-promoting activities for motoneurons in extracts of denervated muscle (Henderson et al., 1983; Hill and Bennet, 1986; Houenou et al., 1991). Interestingly, administration of BDNF to the transected sciatic or facial nerves in newborn rats prevents the death of the axotomized motoneurons (Yan et al., 1992; Sendtner et al., 1992). Moreover, BDNF rescues *in vivo* developing chick motoneurons from naturally occurring cell death (Oppenheim et al., 1992). Hence, certain neurotrophins could function as target-derived neurotrophic factors for developing spinal cord motoneurons and promote survival of motoneurons after injury.

The aim of the present study was to obtain information on the role of the neurotrophins for regeneration of injured peripheral nerves by first comparing the spatial and temporal expression of mRNAs for neurotrophins and trk receptors in muscle, sciatic nerve, and the spinal cord after transection of the sciatic nerve in adult rats. The results show that expression of these mRNAs is differentially regulated in the three tissues and suggest that the neurotrophins could act in a concerted fashion, both locally within the lesioned nerve, and at a distance in the target-fields, to promote regeneration of peripheral nerves.

Materials and Methods

Animal Treatments and Surgery

For transection of the sciatic nerve, adult male Sprague-Dawley rats (Alab, Sweden, 200–250 g body weight) were anesthetized with sodium pentobar-

bital (50 mg/kg, *i.p.*) and both right and left sciatic nerves were transected distal to the obturator tendon. Both the proximal and distal ends of the axotomized nerve were reflected to minimize nerve regeneration. This lesion is known to induce only a partial deficit in mobility of the lower limbs, allowing for free movement of the animal inside the cage. At the indicated times after axotomy, the animals were sacrificed by decapitation and the distal and proximal segments (7–8 mm of each) of the sciatic nerve, the lumbosacral part of the spinal cord and the gastrocnemius muscle were dissected. Where indicated, four different parts of the lesioned nerve were analyzed (distal/distal, distal/proximal, proximal/distal, and proximal/proximal) (see Fig. 8 A). For control, normal adult male Sprague-Dawley rats were sacrificed by decapitation and the dorsal root ganglia (DRG) (L4 and L5), and the same tissues as above, were collected. All dissected tissues were immediately frozen on dry ice and stored at -70°C before preparation of RNA.

Adult male Sprague-Dawley rats were adrenalectomized bilaterally under pentobarbital anaesthesia. After adrenalectomy, the drinking water was supplemented with 3% saline to compensate for the loss of salt. Three days after the bilateral adrenalectomy, the right sciatic nerve was transected as described above. The animals were sacrificed 14 d after the axotomy and sciatic nerve segments were collected from both the control side (left side) and the axotomized side (right side). The tissues were immediately frozen on dry ice and stored at -70°C before preparation of RNA.

RNA Preparation

Total RNA from muscle and spinal cord was purified by the guanidine isothiocyanate/CsCl method as described by Chirgwin et al. (1979). Total RNA from the sciatic nerve and DRG was purified by the acid guanidine isothiocyanate/phenol-chloroform extraction method as described by Chomczynski and Sacchi (1987). The recovery of RNA was measured spectrophotometrically. The quality and quantity was analyzed by electrophoresis of 5 µg RNA in a formaldehyde-containing 1% agarose gel stained with ethidium bromide, followed by UV illumination of the gel.

RNase Protection Analysis

RNase protection assays were performed with a RPAII Ribonuclease Protection Assay Kit (Ambion, Austin, TX). Anti-sense cRNA probes for BDNF and NT-4 were prepared as previously described (Timmusk et al., 1993b). To prepare the NT-3 antisense cRNA probe, a 320-bp EcoRV/Scal fragment from a rat NT-3 cDNA clone (Ernfors et al., 1990) was inserted into pBSKS plasmid (Stratagene, La Jolla, CA). The construct was linearized with BamHI and transcribed with T3 RNA polymerase. The anti-sense cRNA probe detecting all TrkB transcripts was obtained by transcribing a linearized pBSKS plasmid containing a 485-bp insert encompassing nucleotides 1,030–1,515 in the rat *trkB* sequence of Middlemas et al. (1991). To prepare an anti-sense cRNA probe specific for mRNA encoding the full-length TrkB receptor, a 410-bp Aval/AvaI fragment covering the protein tyrosine kinase domain of the TrkB receptor was inserted into pBSKS, linearized, and transcribed with T7 RNA polymerase. For the *trkC* antisense cRNA, a 438-bp fragment encompassing nucleotides 1,164–1,602 in the rat *trkC* sequence of Merlio et al. (1992) was cloned into pBSKS, linearized, and transcribed with T7 RNA polymerase. Anti-sense cRNA probes specific for rat BDNF exon I, II, III, and IV mRNAs were prepared as previously described (Timmusk et al., 1993a). All probes were labeled with α-³²P-CTP. The probes were hybridized at 45°C for 16 h to 10 or 20 µg of total RNA from gastrocnemius muscle or 10 µg of total RNA from the indicated sciatic nerve segments, spinal cord, and DRG (L4 and L5) at the indicated time after axotomy. The assay was performed as described by the manufacturer. Protected cRNA fragments were separated on 4% polyacrylamide gels under denaturing conditions and the gels were exposed to X-ray film at -70°C with intensifying screen. To quantify the relative levels of mRNAs for the neurotrophins and their Trk receptors in control and axotomized animals, only exposures within the linear range of the densitometer were used for analysis. The optical density values of the autoradiograms were measured by image analysis using a Dual-Wavelength Flying-spot Scanner CS-9000 (Shimadzu Corporation, Kyoto, Japan). The results are quantified relative to the same amount of total RNA from the mentioned tissues and therefore are independent of changes in cell number or size of tissue sample.

In Situ Hybridization

Synthetic 48-mer oligonucleotides complementary to mRNAs for rat p75^{NGFR} (Ernfors et al., 1989), *trkA*, *trkB*, and *trkC* (Merlio et al., 1993) were labeled at their 3' end with α-³⁵S-dATP (New England Nuclear, Boston, MA) using terminal deoxynucleotidyltransferase (IBI) to a specific ac-

tivity of 10^9 cpm/ μ g. The *trkB* and *trkC* probes are complementary to regions of these two mRNAs encoding parts of the extracellular and transmembrane domains and therefore detect mRNAs encoding both truncated and full-length forms of these receptors (Middlemas et al., 1991). Cryostat sections (14 μ m) were hybridized overnight at 42°C with 10^7 cpm of labeled probe in 1 ml of hybridization solution as described previously (Verge et al., 1992). Control slides were hybridized as above in the presence of excess (400 \times) unlabeled probe or in the presence of an unrelated probe (growth hormone releasing factor). After hybridization, the slides were washed four times in $1\times$ SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) for 15 min at 55°C, rinsed briefly in distilled water, and dehydrated in ascending concentrations of ethanol. The sections were then dipped in Kodak NTB2 emulsion diluted 1:1 in distilled water and exposed in the dark. The sections were counter stained with toluidine blue.

Results

Expression of mRNAs for Neurotrophins and Members of the Trk Family after Transection of the Sciatic Nerve

RNAse protection assays were used to quantify the relative levels of mRNAs for neurotrophins and Trk receptors at different times after transection of the sciatic nerve in adult rats. Three different tissues were included in the analysis: the gastrocnemius muscle; the target-area of the axotomized motoneurons; the distal and proximal segments of the lesioned nerve; and the spinal cord containing the cell bodies of the axotomized motoneurons. Two, or where indicated, three independent experiments were performed and the relative levels of mRNA were quantified by densitometric scanning of autoradiograms obtained after the hybridizations.

Brain-derived Neurotrophic Factor

In the gastrocnemius muscle, BDNF mRNA decreased slightly ($\sim 40\%$ lower than control) 6 and 12 h after the tran-

section but reached twofold elevated levels one and two weeks after the transection (Fig. 1 A).

Low levels of BDNF mRNA, close to the border of the detection limit, were seen in the intact adult rat sciatic nerve (Fig. 1 B). An increase was seen in the distal segment one week after the lesion and sixfold higher levels were seen at 2 wk compared to 1 wk after the lesion.

Since glucocorticoids have been suggested to regulate BDNF mRNA expression in the brain (Barbany and Persson, 1992), we tested if the increase of BDNF mRNA in the lesioned sciatic nerve was affected by adrenalectomy. Transections in animals adrenalectomized three days before the axotomy resulted in a twofold decrease in BDNF mRNA in the distal segment of the injured nerve compared to transection in nonadrenalectomized animals (Fig. 1 B). The adrenalectomy alone did not change the expression of BDNF mRNA in the intact nerve.

In the spinal cord, BDNF mRNA decreased slightly 6 and 12 h after transection, increased by $\sim 30\%$ at 3 d, and declined to control level one week after the lesion (Fig. 1 C).

The rat BDNF gene consists of four short 5'-exons and one 3'-exon encoding the preproBDNF protein with a separate promoter upstream of each 5'-exon (Timmusk et al., 1993a). The probe used in Fig. 1 is from the 3'-exon and therefore detects BDNF mRNAs transcribed from all four promoters. BDNF exon specific probes were used to determine if the increase of BDNF mRNA in the lesioned sciatic nerve was due to a differential activation of the BDNF promoters. Only BDNF exon IV mRNA was detected in the distal part of the sciatic nerve two weeks after the transection (Fig. 2).

Neurotrophin-3

In the gastrocnemius muscle the level of NT-3 mRNA did not

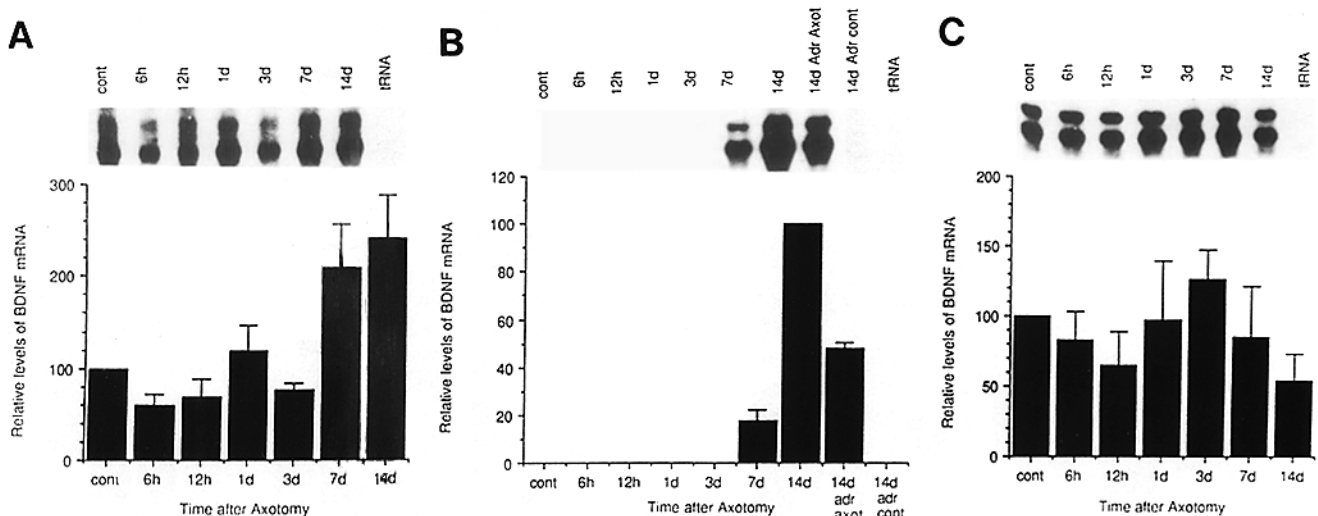


Figure 1. BDNF mRNA expression in gastrocnemius muscle (A), sciatic nerve (B), and spinal cord (C) after transection of the sciatic nerve in adult rats. Total cellular RNA (10 μ g for sciatic nerve and 20 μ g for spinal cord and muscle) prepared at different times after transection of the adult rat sciatic nerve was hybridized to a 32 P-labeled anti-sense BDNF cRNA probe. In the lesioned sciatic nerve, RNA was prepared from the distal segment of the nerve. RNAse protection assays were performed as described in Materials and Methods and the protected probe was electrophoresed in a denaturing acrylamide gel followed by exposure to X-ray film. Hybridization to tRNA was used as a negative control. The upper part of the figure shows autoradiograms obtained after the hybridizations. The autoradiograms were scanned in a densitometer and the optical density values were used to measure the relative levels of BDNF mRNA among the different samples. The levels of BDNF mRNA in the control gastrocnemius muscle and spinal cord were arbitrarily set at 100. For the sciatic nerve, the level of BDNF mRNA in the distal segment 2 wk after the transection was arbitrarily set at 100. All values are mean \pm SEM of two independent experiments. *cont.*, control; *adr.*, RNA prepared from adrenalectomized animals; *ad_x*, axo, RNA prepared from the distal segment of the transected sciatic nerve in adrenalectomized animals.

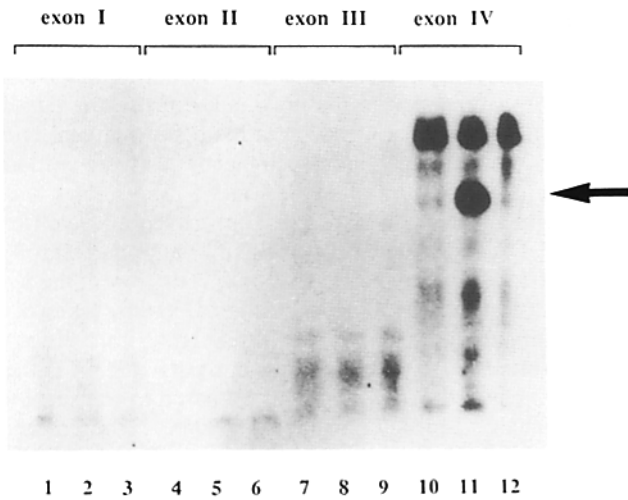


Figure 2. Expression of BDNF exon specific mRNAs in the transected sciatic nerve. RNase protection assays were performed with total RNA (10 μ g) from control sciatic nerve (1, 4, 7, and 10) or from the distal segment of the adult rat sciatic nerve 2 wk after axotomy (2, 5, 8, and 11). Anti-sense cRNA probes specific for the indicated BDNF 5'-exons were used. Hybridization to tRNA was used as a control (3, 6, 9, and 12). Arrow indicates the position of the protected fragment using the exon IV specific probe.

change at any of the time points tested after transection of the sciatic nerve (Fig. 3 A).

In contrast to BDNF, NT-3 mRNA was clearly detected in the intact sciatic nerve from adult rats (Fig. 3 B). The level of NT-3 mRNA decreased ninefold in the distal segment of the lesioned nerve 6 and 12 h after the transection, and then returned progressively to control levels 2 wk after the injury.

A biphasic response was seen in the spinal cord with a twofold decrease 12 h after the lesion, a progressive increase to control level at three days followed by a twofold decrease at 2 wk (Fig. 3 C).

Neurotrophin-4

NT-4 mRNA increased approximately twofold in the gastrocnemius muscle 6 h after transection of the sciatic nerve but returned to control level 12 h after the lesion. A 4.5-fold decrease was seen at one day and the level decreased further reaching 11-fold lower than control 2 wk after the lesion (Fig. 4 A).

In the distal segment of the lesioned sciatic nerve, NT-4 mRNA decreased 6 and 12 h after the axotomy but increased progressively thereafter with eightfold higher levels than in control 2 wk after the lesion (Fig. 4 B).

In the spinal cord, a slight decrease was seen 6 h after the lesion returning to control levels at 12 h (Fig. 4 C).

TrkB

Two different anti-sense cRNA probes were used to detect *trkB* mRNA. The first probe is complementary to *trkB* mRNA encoding part of the transmembrane and of the extracellular domains of the TrkB receptor. This probe (referred to as the all *trkB* probe) detects *trkB* transcripts encoding both full-length and truncated TrkB receptors (Klein et al., 1989; Middlemas et al., 1991). The second *trkB* probe is from the part of the *trkB* sequence encoding the tyrosine kinase domain of the TrkB receptor and is therefore specific for *trkB* transcripts encoding a full-length TrkB receptor.

Both *trkB* probes revealed no, or only very low, levels of *trkB* mRNA in gastrocnemius muscle from intact or denervated animals (data not shown).

In the intact and axotomized adult rat sciatic nerve, *trkB* transcripts were only detected with the all *trkB* probe. The level of all *trkB* transcripts in the proximal segment of the injured nerve increased progressively after the transection reaching twofold higher levels at one day followed by a decline to control levels at 3 d (Fig. 5 A).

In the spinal cord, the full-length *trkB* probe revealed slightly decreased levels of *trkB* transcripts 12 h and one day after the lesion. A slight increase was seen at 3 d declining

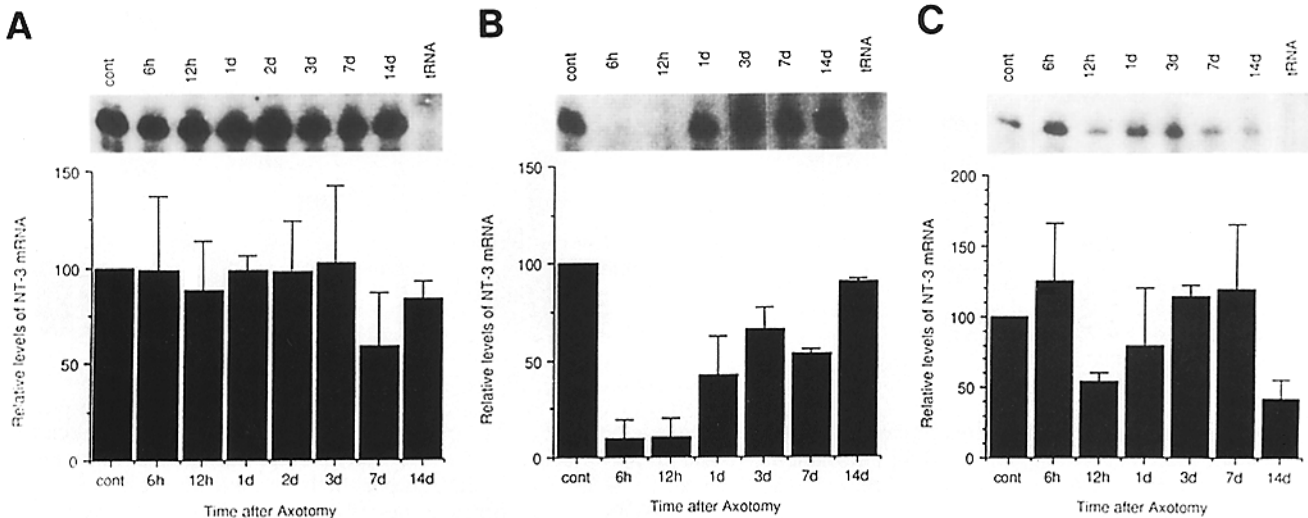


Figure 3. NT-3 mRNA expression in gastrocnemius muscle, (A) sciatic nerve (B), and spinal cord (C) after transection of the sciatic nerve in adult rats. RNase protection assay was used to measure the level of NT-3 mRNA in the indicated samples as described in Fig. 1. The amount of NT-3 mRNA in the control samples was arbitrarily set at 100. The values are mean \pm SEM from two (sciatic nerve) or three (muscle and spinal cord) independent experiments.

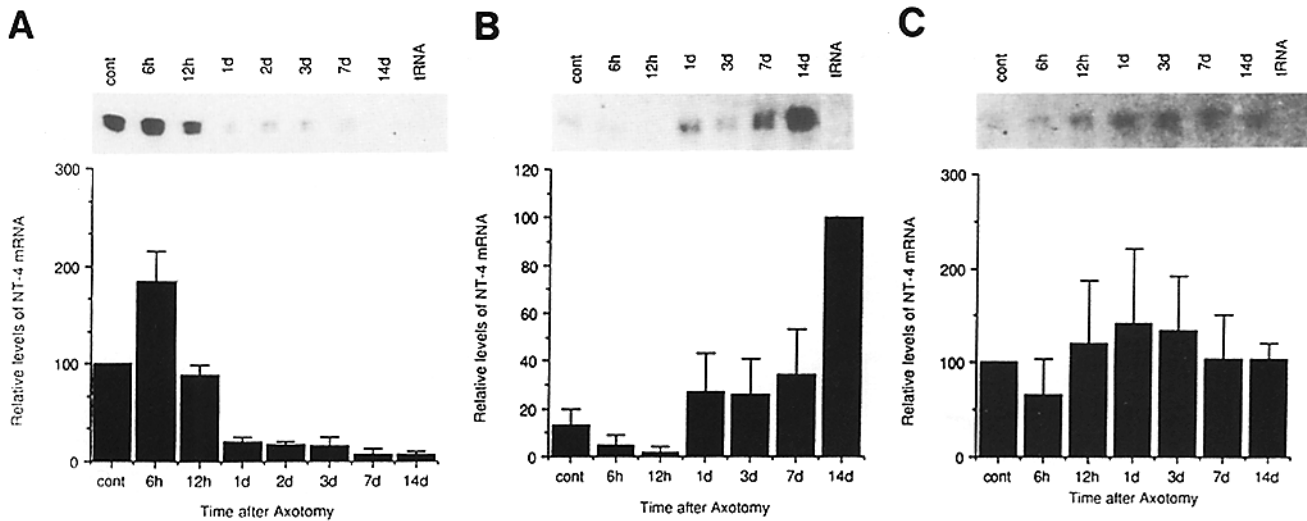


Figure 4. NT-4 mRNA expression in gastrocnemius muscle (A), sciatic nerve (B), and spinal cord (C) after transection of the sciatic nerve in adult rats. RNase protection assays were used to measure the level of NT-4 mRNA in the indicated samples as described in Fig. 1. The level of NT-4 mRNA in control gastrocnemius muscle and spinal cord were arbitrarily set at 100. For the sciatic nerve, the level of NT-4 mRNA in the distal segment 2 wk after the transection was arbitrarily set at 100. The values are mean \pm SEM of two (sciatic nerve and spinal cord) or three (muscle) independent experiments.

to control level 2 wk after the lesion (Fig. 5 B). The level of all *trkB* mRNA decreased twofold one day after the transection with a twofold increase at 3 d followed by a threefold decrease 2 wk after the lesion (Fig. 5 C).

Comparison of All *trkB* and Full-length *trkB* mRNA Expression in Neuronal and Nonneuronal Tissues

Relatively high levels of all *trkB* mRNA were detected in all neuronal and nonneuronal tissues examined, except in the gastrocnemius muscle where only low levels were seen. In

contrast, full-length *trkB* mRNA was detected only in the neuronal tissues, spinal cord, and the dorsal root ganglia (Fig. 6).

TrkC

The anti-sense cRNA probe for *trkC* is complementary to *trkC* mRNA encoding part of the transmembrane and of the extracellular domains of the TrkC receptor and therefore detects *trkC* transcripts encoding both full-length and putative truncated forms of the TrkC receptor.

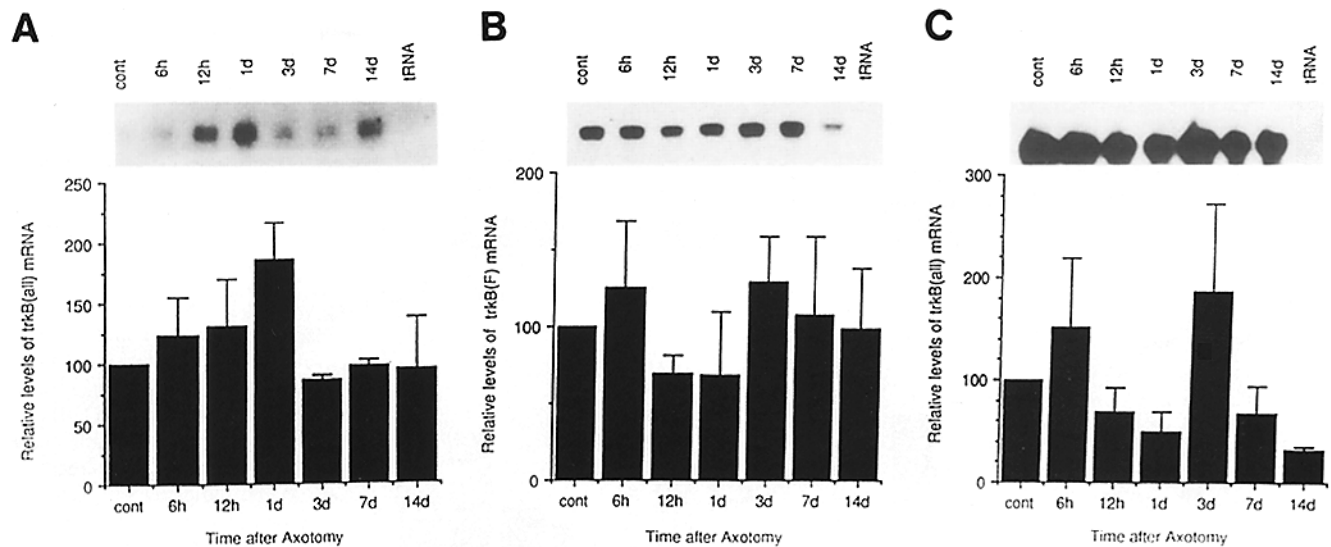


Figure 5. Expression of transcripts encoding truncated and full-length TrkB receptors in the sciatic nerve and spinal cord after axotomy. RNase protection assays were used to measure the level of *trkB* transcripts encoding truncated and full-length TrkB receptors in the indicated samples as described in Fig. 1. (A) *trkB* truncated transcripts in the proximal segment of the injured sciatic nerve. *trkB* truncated (B) or full-length (C) transcripts in the spinal cord. The amount of *trkB* transcripts in the control samples was arbitrarily set at 100. The values are mean \pm SEM from two independent experiments.

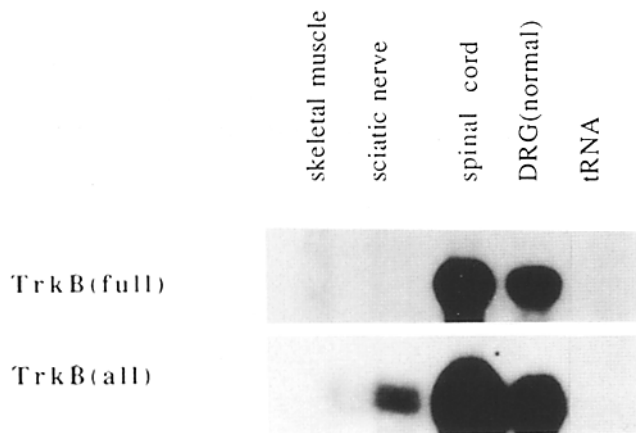


Figure 6. Truncated and full-length *trkB* mRNAs in gastrocnemius muscle, sciatic nerve, spinal cord, and DRG. RNase protection assays were performed using 10 μ g of total RNA from the indicated tissues dissected from nonoperated adult rats. cRNA probes specific for mRNAs encoding full-length TrkB receptors or all forms of TrkB receptors were used as described in Materials and Methods. (*Skeletal muscle*) Normal gastrocnemius muscle, sciatic nerve; (*normal sciatic nerve, spinal cord*) normal lumbosacral part of spinal cord; (*DRG*) normal dorsal root ganglia (L4 and L5).

trkC probe revealed only very low levels of *trkC* mRNA in gastrocnemius muscle from intact or denervated animals (data not shown).

In the proximal segment of the injured sciatic nerve, *trkC* mRNA decreased twofold 6 h after the injury, returned to control levels at one day, with a twofold increase at 2 wk (Fig. 7 A).

In the spinal cord, *trkC* mRNA decreased twofold 12 h after the axotomy, and then increased progressively to slightly higher levels compared to control at two weeks (Fig. 7 B).

Regional Distribution of *trkB* and *trkC* mRNAs in the Axotomized Sciatic Nerve

To determine the distribution of *trkB* and *trkC* mRNAs in

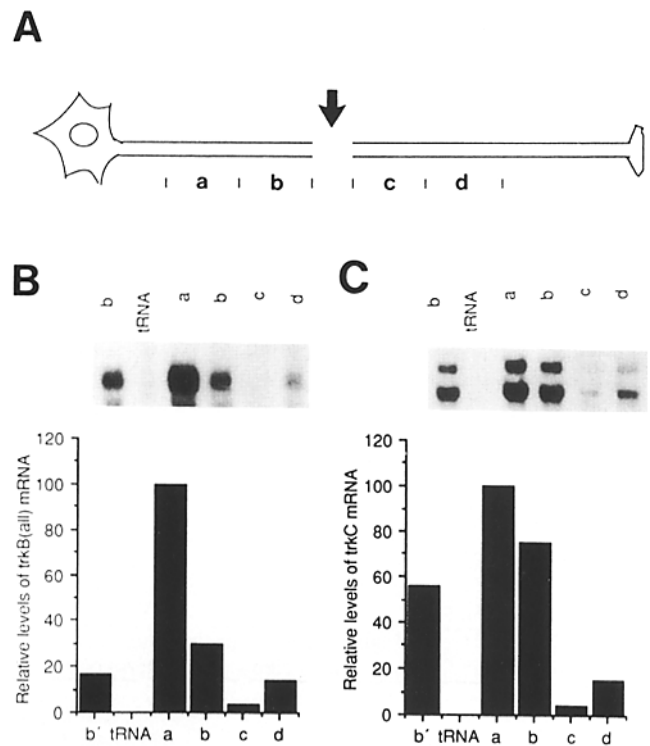


Figure 8. (A) Schematic representation of the location of different parts of the axotomized sciatic nerve. (a) Proximal/proximal segment; (b) proximal/distal segment; (c) distal/proximal segment; (d) distal/distal segment. (B) TrkB and (C) TrkC mRNA expression in the sciatic nerve. (a) Proximal/proximal segment, (b and b') proximal/distal segment. (c) Distal/proximal segment; (d) distal/distal segment; a, b, c, and d are from the injured nerve 1 day (B) and 14 days (C) after the axotomy while b' is from the intact nerve.

different parts of the transected sciatic nerve, four different segments of the lesioned sciatic nerve (Fig. 8 A) were analyzed for all *trkB* and *trkC* mRNAs at one day and 2 wk after the lesion, respectively. For both mRNAs the highest levels

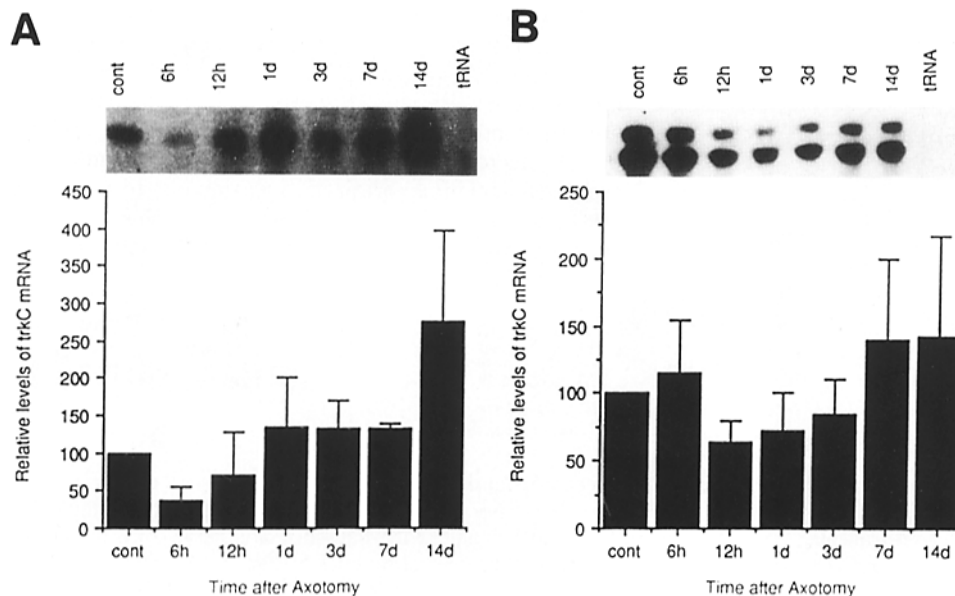


Figure 7. *trkC* mRNA expression in sciatic nerve (A) and spinal cord (B) after axotomy. RNase protection assays were used to measure the level of *trkC* transcripts encoding full-length and putative truncated TrkC receptors in the indicated samples as described in Fig. 1. The amount of *trkC* mRNA in the control samples was arbitrarily set at 100. The values are mean \pm SEM of two (sciatic nerve) or three (spinal cord) independent experiments.

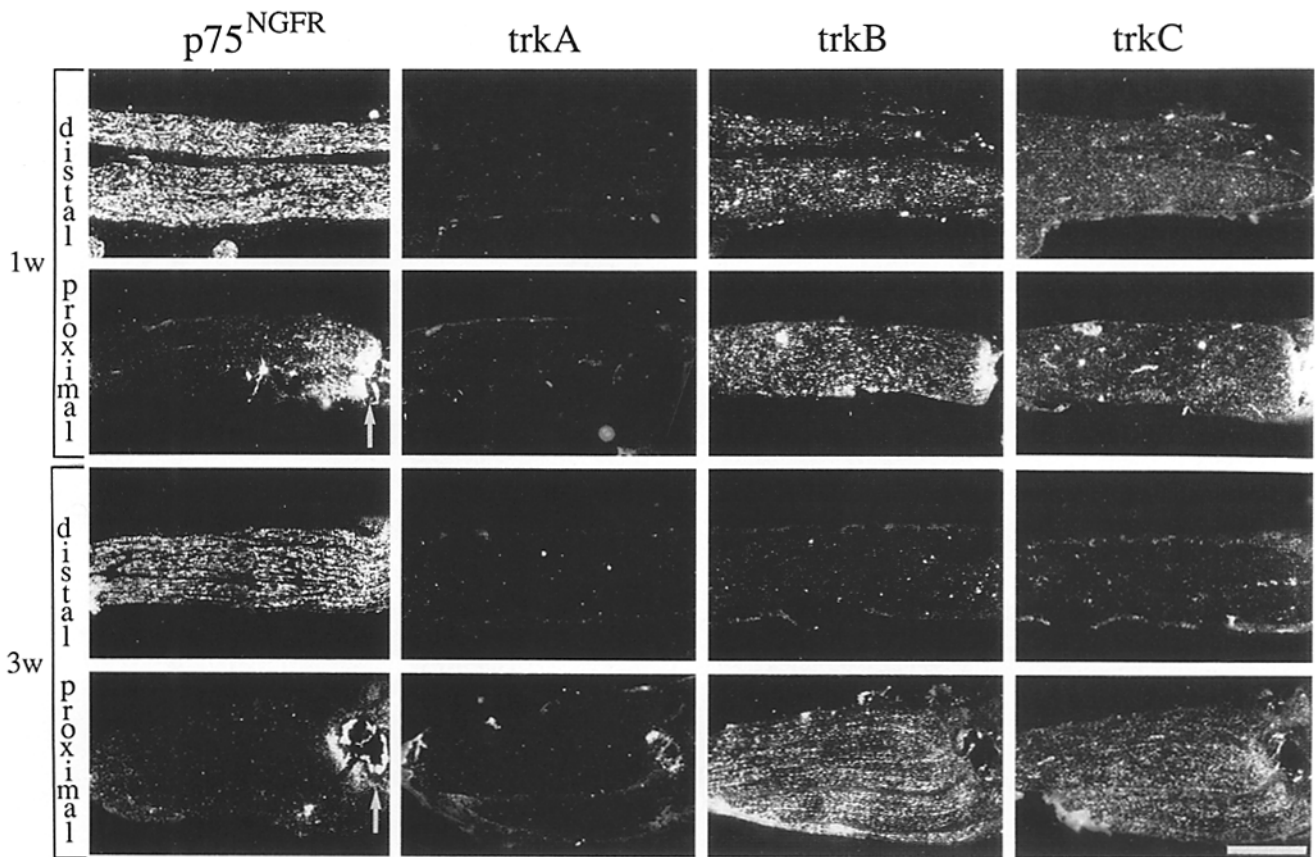


Figure 9. Dark-field micrographs showing the detection of $p75^{NGFR}$, $trkA$, $trkB$, and $trkC$ mRNAs in the transected sciatic nerve by in situ hybridization. The upper two rows show adjacent sections of the distal and proximal stump one week after the lesion. The lower two rows demonstrate the expression in adjacent sections distal and proximal to the nerve stump three weeks after the lesion. Low levels of $p75^{NGFR}$ mRNA were detected proximal to the transection one and three weeks after the transection. This was in contrast to the high expression in the distal denervated part of the nerve. $trkA$ mRNA was not detected in the sciatic nerve at any time point. $trkB$ and $trkC$ mRNAs were detected in many cells in the sciatic nerve proximal to the transection. Distal to the injury, $trkB$ and $trkC$ mRNAs were slightly decreased after one week, and had decreased to almost undetectable levels after three weeks. Bar, 1,000 μm . Arrows point at the site of ligation.

were found in the proximal/distal and proximal/proximal parts of the axotomized nerve where the levels were 1.5–5-fold higher than in the intact nerve. In contrast, both mRNAs decreased in the distal/proximal and distal/distal segments to levels significantly lower than in the intact nerve (Fig. 8, B and C).

Cellular Localization of Neurotrophin Receptor mRNAs in the Injured Sciatic Nerve

The expression of $p75^{NGFR}$, $trkA$, $trkB$, and $trkC$ mRNAs in the intact and axotomized sciatic nerve was also analyzed by in situ hybridization. $p75^{NGFR}$ and $trkA$ mRNAs were not detected in the intact sciatic nerve. In contrast, $trkB$ and $trkC$ mRNAs were detected at high levels in Schwann cells in the intact nerve (data not shown). After axotomy, $p75^{NGFR}$ mRNA was not detected in the proximal segment of the sciatic nerve, except in Schwann cells close to the ligature (Fig. 9). These cells, as well as the majority of Schwann cells in the denervated distal sciatic nerve segment, expressed high levels of $p75^{NGFR}$ mRNA one and three weeks after the lesion (Fig. 10). $trkA$ mRNA was not detected in the injured sciatic nerve proximal or distal to the lesion. The levels of $trkB$ and $trkC$ mRNAs in Schwann cells proximal to the tran-

section slightly increased to the levels in the intact nerve one week after the transection, but decreased in the distal part of the nerve (Fig. 9). Analysis of emulsion autoradiographs revealed that in the proximal segment of the injured nerve the labeling for $p75^{NGFR}$, $trkB$, and $trkC$ mRNAs were confined to Schwann cells (Fig. 10). At three weeks, $trkB$ and $trkC$ mRNAs were still abundantly expressed proximal to the ligature. In contrast, in the distal segment, $trkB$ and $trkC$ mRNAs decreased further at 3 wk to low or undetectable levels. All specific hybridization was abolished in the presence of an excess of unlabeled probe, but no change was seen when an unrelated probe was used for competition.

Discussion

Expression of mRNAs for Neurotrophins and Trk Receptors in the Injured Sciatic Nerve

The data presented here show that expression of neurotrophin mRNAs is differentially regulated in the distal segment of the axotomized sciatic nerve in adult rats. Previous studies have shown a biphasic response in NGF mRNA with a rapid increase peaking at 6 h after the lesion, progressively in-

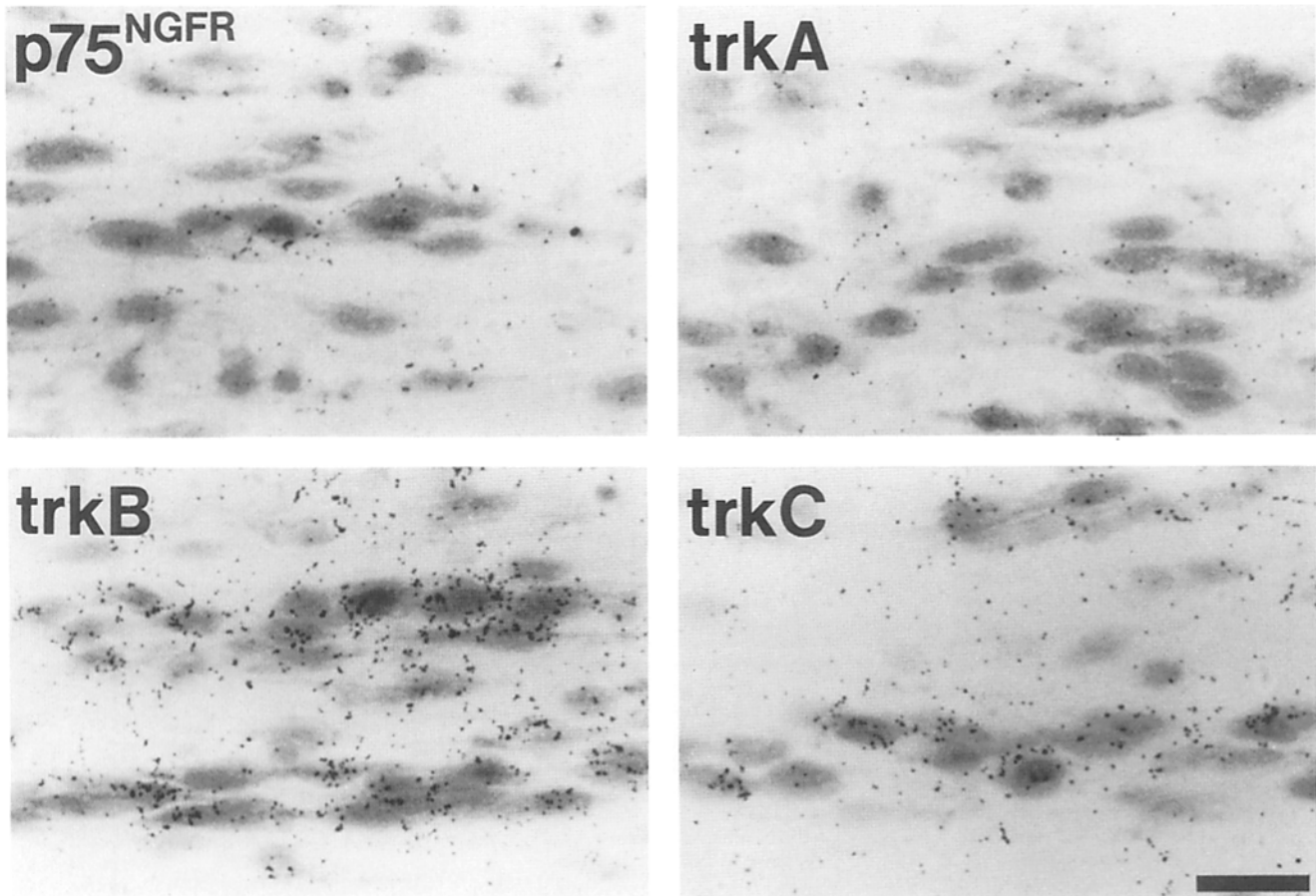


Figure 10. Bright-field emulsion micrographs showing neurotrophin receptor mRNAs expression in the axotomized sciatic nerve proximal to a transection three weeks after the lesion by in situ hybridization. $p75^{NGFR}$ shows $p75^{NGFR}$ mRNA, *trkA* shows *trkA* mRNA, *trkB* shows *trkB* mRNA, and *trkC* shows *trkC* mRNA expression. No specific expression of $p75^{NGFR}$ or *trkA* mRNA is seen, but *trkB* and *trkC* mRNAs were expressed at high levels in Schwann cells. Bar, 20 μm .

creases and attains control levels by 2 wk after the lesion. NT-4 mRNA also decreases shortly after the lesion followed by a decline and a second peak 2–3 d later (Heumann et al., 1987a). In a recent study, Meyer et al. (1992) reported that BDNF mRNA increases in the distal part of the sciatic nerve one week after the lesion with progressively higher levels until 3 wk after the lesion. Similar findings were seen in this study. Our results also show that NT-3 mRNA decreases shortly after the lesion, progressively increases and attains control levels by 2 wk after the lesion. NT-4 mRNA also decreases shortly after the lesion but reached greatly elevated levels 2 wk after the lesion.

A comparison between the relative levels of BDNF, NT-3, and NT-4 mRNAs in the intact adult rat sciatic nerve revealed that the levels of mRNA for NT-3 were the highest, followed by NT-4 and BDNF mRNAs. The low, or undetectable, levels of NGF and BDNF mRNAs could be due to a downregulation of NGF and BDNF mRNA expression by axonal contact. 2 wk after the axotomy, the relative levels of neurotrophin mRNAs changed to the opposite relation seen in the intact nerve, i.e., BDNF mRNA levels were the highest while NT-3 mRNA levels were the lowest. Similar to NGF mRNA (Heumann et al., 1987a,b), NT-3 and NT-4 mRNA showed a biphasic response to the axotomy with an

early, acute response which was different from the second long-term response. In contrast, BDNF mRNA simply goes up from zero to a higher level at days 7 and 14. The peak of NGF mRNA 2 d after transection of the sciatic nerve could be due to an inflammatory response mediated by macrophage released $\text{IL-1}\beta$ which stimulates NGF mRNA expression in pieces of the sciatic nerve in culture (Lindholm et al., 1987). Similarly, an inflammatory response could mediate the increases of BDNF, NT-3, and NT-4 mRNAs 2 wk after the lesion. However, expression of BDNF mRNA in nerve organ cultures or in cultures of Schwann cells is not changed in response to $\text{IL-1}\beta$ indicating that different mechanisms upregulate NGF and BDNF mRNA in the sciatic nerve (Meyer et al., 1992).

The use of BDNF exon specific probes showed that only BDNF exon IV containing mRNA (Timmusk et al., 1993a) was increased in the lesioned sciatic nerve suggesting that the induction was due to a selective activation of BDNF promoter IV. This promoter is preferentially used in peripheral tissues (Timmusk et al., 1993a) which could explain the selective activation in nonneuronal cells in the distal segment of the lesioned nerve.

Adrenalectomy attenuated the increase of BDNF mRNA in the lesioned nerve suggesting that glucocorticoids could

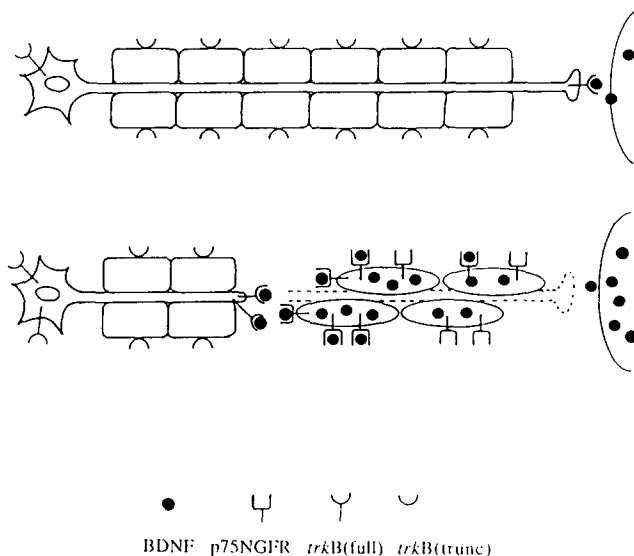


Figure 11. Schematic representation of putative neurotrophin-mediated trophic supports for the injured peripheral nerves. The top panel shows the intact mature sciatic nerve with truncated TrkB receptors present on the surface of Schwann cells and full-length TrkB receptors in the neuron (sensory or motor). A similar scheme may apply to TrkC receptors. A trophic support is provided by the neurotrophin synthesized in the target-fields of the neuron. Putative local autocrine/paracrine modes of trophic interactions (not shown in the figure) could also apply, since BDNF and NT-3 mRNAs are expressed in sensory neurons in the adult DRG (Ernfors et al., 1990b; Ernfors and Persson, 1991). After axotomy of the nerve, expression of $p75^{NGFR}$, BDNF, and NT-4 is upregulated in Schwann cells in the distal segment of the injured nerve while the TrkB receptor is downregulated (see Figs. 8 and 9). Elevated levels of BDNF and NT-4 proteins bind to $p75^{NGFR}$, which concentrates BDNF on the surface of the Schwann cells, presenting them to the full-length TrkB receptor in the proximal part of the injured neuron. As regeneration proceeds (*lower panel*), a target-derived support could resume, enhanced by elevated levels of the neurotrophin in the target-fields as a result of the lesion. This scheme may also apply to NT-3 and TrkC receptors. Note, however, that the levels of NT-3 mRNA did not change in the denervated muscle and NT-4 mRNA decreased in the muscle after the lesion, arguing that the local trophic support from the distal segment of the sciatic nerve is more important for NT-3 and NT-4. See text for further discussion.

play a role in the upregulation of BDNF mRNA in non-neuronal cells of the injured sciatic nerve. Similarly, glucocorticoids have been suggested to regulate BDNF mRNA expression in neurons in the brain (Barbany and Persson, 1992). Comparison of the rat BDNF gene sequence (Timusk et al., 1993a) with known transcriptional regulatory sequences revealed a sequence element with high homology to a consensus binding site for the glucocorticoid receptor (AGAACAN₃TGTTCT; Faisst and Meyer, 1992) 420 bp upstream of the capsite in promoter IV. This sequence could function as a glucocorticoid responsive element that plays a role in the upregulation of BDNF mRNA in the lesioned sciatic nerve.

The presence of neurotrophin receptors in the injured sciatic nerve is a prerequisite for neurotrophin-mediated responses in the axotomized nerve. $p75^{NGFR}$ mRNA (Heumann et al., 1987b) and immunoreactivity (Taniuchi et al.,

1986) increase markedly in the distal segment of the transected sciatic nerve. After axonal contact during regeneration, $p75^{NGFR}$ expression is downregulated to the low level found in the intact sciatic nerve. The induction of $p75^{NGFR}$ expression after the axotomy has been proposed as a mechanism to concentrate NGF on the surface of Schwann cells in the distal part, recruiting the ligand and thereby promoting nerve regeneration (Johnson et al., 1988). This model could be extended to include the other three neurotrophins, since all neurotrophins bind to $p75^{NGFR}$ with similar affinities (Rodriguez-Tebar et al., 1990, 1992; Ernfors et al., 1990; Hallböök et al., 1991) and are all expressed in the distal part of the nerve after axotomy. Interestingly, the levels of *trkB* mRNA encoding truncated forms of the TrkB receptor was downregulated in Schwann cells associated with the distal segment of the transected nerve. This result is in contrast with the increase in the levels of $p75^{NGFR}$ (Taniuchi et al., 1986; and this study), indicating that these two receptor molecules have opposite regulations in Schwann cells of the distal segment of the lesioned nerve. The truncated receptor lacks the tyrosine kinase domain and can therefore not mediate an intracellular signal (Klein et al., 1991). The role of the truncated TrkB receptor is not known, however the decreased levels of truncated TrkB receptors in the distal part of the injured nerve could favor binding of BDNF or NT-4 to an increased number of $p75^{NGFR}$ which then recruits and presents these factors to full-length TrkB receptors present on the terminals of the axotomized neurons (Fig. 11) (Frisen et al., 1993). The level of BDNF mRNA increased in the gastrocnemius muscle 2 wk after denervation. This implies elevated levels of the BDNF protein also in the target-fields of axotomized motoneurons, which could facilitate axonal guidance and regeneration (Fig. 11). Similar trophic interactions as outlined in Fig. 11 may also apply to NT-3, since two weeks after the lesion *trkC* transcripts decreased in the distal segment of the injured nerve. However, the probe used to detect *trkC* mRNA does not distinguish between transcripts for truncated or full-length receptors. The contribution of different forms of the TrkC receptor to this putative trophic support can therefore not be evaluated from the present data.

Expression of Neurotrophin and trk mRNAs in the Spinal Cord and Gastrocnemius Muscle

BDNF, NT-3, and NT-4 mRNAs were detected in the control adult rat spinal cord. The cellular localization of neurotrophin mRNAs in the spinal cord is not known. However, NT-3 mRNA is expressed in embryonic rat spinal cord motoneurons (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992) implying that NT-3 mRNA detected in the adult rat spinal cord could have been synthesized by motoneurons. NT-3 promotes the survival of proprioceptive sensory neurons in cell culture (Hohn et al., 1990) and the finding of NT-3 mRNA in spinal cord motoneurons is consistent with the fact that NT-3 exerts a target-derived trophic support for these neurons. The decreased level of NT-3 mRNA in the spinal cord 2 wk after transection of the sciatic nerve indicates that this trophic support is reduced after axotomy of the sciatic nerve.

A clear example of a differential regulation of neurotrophin mRNA expression was seen in the gastrocnemius muscle after denervation caused by transection of the sciatic

nerve. BDNF mRNA increased 2.5-fold 2 wk after the lesion, NT-3 mRNA did not change significantly, while NT-4 mRNA decreased eightfold 2 wk after the lesion. Recently, Koliatsos et al. (1993) also reported an increase of BDNF mRNA in the gastrocnemius muscle one week after a transection of the sciatic nerve. The marked decrease of NT-4 mRNA could indicate that the expression of NT-4 mRNA in skeletal muscle is dependent upon neuronal stimulation. BDNF has recently been shown to prevent the death of axotomized motoneurons in the sciatic and facial nerves (Yan et al., 1992; Sendtner et al., 1992) and to rescue developing chick motoneurons in vivo from naturally occurring cell death (Oppenheim et al., 1992). Analysis of mRNA levels of RNase protection assay has shown that BDNF mRNA is maximally expressed in skeletal muscle and skin in the early rat embryo (embryonic day 13) with decreasing amounts at later times of development (Timmusk et al., 1993b). This expression pattern is consistent with the hypothesis that BDNF exerts a target-derived trophic support for developing spinal cord motoneurons. A similar expression pattern was seen for NT-4 mRNA (Timmusk et al., 1993b). The increased level of BDNF mRNA after axotomy of the sciatic nerve in both the distal segment of the injured nerve and in the gastrocnemius muscle suggests that elevated levels of BDNF could promote regeneration of axotomized spinal cord motoneurons by both target-derived and local modes of action (Fig. 11). This effect would presumably be mediated by the activation of full-length TrkB receptors present on the surface of the axotomized motoneurons but could also involve p75^{NGFR}, since expression of p75^{NGFR} mRNA is markedly up-regulated in spinal cord motoneurons after a crush lesion of the sciatic nerve (Ernfors et al., 1989, 1993). NT-3 is retrogradely transported within the crushed sciatic nerve to spinal cord motoneurons (DeStefano et al., 1992) and the findings of NT-3 and *trkC* mRNAs in nonneuronal cells of the injured nerve and NT-3 mRNA in the denervated gastrocnemius muscle suggest that NT-3 could promote the regeneration of axotomized spinal cord motoneurons by similar mechanisms as proposed for BDNF. In the case of NT-4, a putative trophic support for spinal cord motoneurons is more likely to occur by a local mechanism, since NT-4 mRNA increased in the injured nerve but decreased markedly in the target area.

In summary, the changes in the levels of neurotrophin mRNAs reported here demonstrate that expression of not only NGF and BDNF but also of NT-3 and NT-4 mRNAs, is regulated after a peripheral nerve injury. Our results are consistent with a model in which the altered levels of different neurotrophin and Trk receptor proteins after lesion cooperate in sequential and concerted ways to enhance, both local and target-derived trophic supports important for the regeneration of injured peripheral neurons.

We would like to dedicate this work to the memory of Professor Håkan Persson whose premature death is a great loss for all of us. We greatly thank Professor Tomas Hökfelt, Carlos Ibáñez, Ernest Arenas, and Ravinder Sehgal for critical comments of the manuscript.

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