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# BDNF and NT-4/5 Exert Neurotrophic Influences on Injured Adult Spinal Motor Neurons

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**Adult motor neurons, like their immature antecedents, express the mRNA for the signaling receptor for brain-derived neurotrophic factor (BDNF) and for neurotrophin-4/5 (NT-4/5). However, while both BDNF and NT-4/5 support the survival of axotomized developing spinal motor neurons *in vitro* or *in vivo*, it is not known whether these factors continue to influence spinal motor neurons in adulthood.**

**The present study tests if BDNF or NT-4/5 modulate the reactive responses of adult spinal motor neurons to nerve injury. We utilize sciatic nerve transection to axotomize the spinal motor neurons that form the retrodorsal lateral nucleus (RDLN) and show that, after axotomy, RDLN motor neurons lose ChAT immunoreactivity and also reexpress $p75^{lngfr}$, the low affinity receptor for all neurotrophin family members. Treatment with BDNF or NT-4/5 alters these effects of sciatic nerve transection. Both BDNF and NT-4/5 attenuate the loss of ChAT expression in axotomized RDLN motor neurons; thus, as compared to vehicle treatments, BDNF and NT-4/5 produce statistically significant increases in the optical density of ChAT immunostaining. Furthermore, BDNF and NT-4/5 also significantly increase the RDLN reexpression of $p75^{lngfr}$ after sciatic nerve transection. Interestingly, essentially identical increases in RDLN ChAT and $p75^{lngfr}$ immunostaining are produced by sciatic nerve crush injuries in the absence of exogenous neurotrophin treatment. These data show that treatment with exogenous BDNF and NT-4/5 changes the response of adult spinal motor neurons to sciatic nerve transection. Furthermore, these neurotrophins elicit reactive responses in axotomized motor neurons that mimic those produced by endogenous agents in regenerating crushed peripheral nerve.**

***[Key words: neurotrophins, brain-derived neurotrophic factor, neurotrophin-4/5, motor neurons, nerve transection, nerve crush, spinal cord***

Recombinant BDNF and NT-4/5 promote the survival of axotomized immature spinal motor neurons (Sendtner et al., 1990, 1992; Yan et al., 1992; Henderson et al., 1993; Koliatsos et al., 1993; Wong et al., 1993). Studies of neurotrophins and neurotrophin receptor mRNA distribution in the adult suggest that BDNF and NT-4/5 may also influence adult spinal motor neurons. Mature spinal motor neurons express TrkB, a protein tyrosine kinase, which functions as the signalling receptor for both BDNF and NT-4/5 (Soppet et al., 1991; Squinto et al., 1991; Koliatsos et al., 1993). On the other hand, peripheral targets of spinal motor neurons, such as the skeletal muscles of the lower limb, express the mRNA for BDNF and NT-4/5 (Maisonpierre et al., 1990a,b; Ip et al., 1992; Koliatsos et al., 1993). Furthermore, nerve injury upregulates the mRNA for BDNF, NT-4/5, as well as NGF, in nontarget sites such as the nonneuronal cells in the degenerating distal stump of the transected sciatic nerve (Heumann et al., 1987; Meyer et al., 1992; Funakoshi et al., 1993). Since injured adult spinal motor neurons demonstrate avid retrograde transport of all neurotrophin family members (DiStefano et al., 1992; Curtis et al., 1993, Soc Neurosci Abstr 606.2) these cells have the capacity to utilize distal degenerating nerve as a potentially new source of trophic factors. In this regard, it is of interest that exposure to the local environment of degenerating nerve influences a range of responses of injured peripheral and central neurons (Ramon y Cajal, 1928; Richardson and Ebendal, 1982; Aguayo et al., 1982). However, it is not known if neurotrophins are the functionally important bioactive agents in degenerating peripheral nerves, nor is it known if the responses of adult spinal motor neurons to nerve injury are, in fact, affected by neurotrophins.

The present study tests whether treatment with exogenous BDNF or NT-4/5 affects the reactive responses of adult motor neurons to sciatic nerve transection. We have monitored immunocytochemical indices of motor neuron reactions to nerve injury in the retrodorsal lateral nucleus (RDLN) as this is an easily recognized cluster of cells in the ventral horn of the lumbar spinal cord and the peripheral axons of these cells are all cut by sciatic nerve transection. Our main finding is that one week of treatment with pharmacological doses of recombinant BDNF or NT-4/5 alters the reactions of RDLN motor neurons to axotomy: both neurotrophins produce statistically significant increases in the density of both ChAT and $p75^{lngfr}$ immunostaining. Furthermore, essentially identical injury responses occur in the RDLN motor neurons of untreated nerve-crush lesioned animals. These data suggest that treatment with recombinant BDNF or NT-4/5 may in part substitute for the bioactivities that are present in regenerating crushed peripheral nerve.

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## Materials and Methods

*Surgery.* The experimental tissues were obtained from adult male Sprague–Dawley rats (200–300 gm: Zivic-Miller, Zelienople, PA). Rats were anesthetized with 170 mg/kg chloral hydrate (Sigma, St. Louis, MO) and 35 mg/kg sodium pentobarbital (A. J. Buck & Son, Owens Mills, MD). The right sciatic nerve was exposed and transected 0.5 cm distal to the obturator tendon and a 0.5 cm segment of nerve was resected to prevent reapposition of proximal and distal nerve stumps. Nerve crush lesions were performed at the same level by clamping the sciatic nerve, twice, in a blade holder (#105-95; Tiemann, Plainview NY) for 10 sec. All rats were permitted a 1 week survival time after nerve lesion. All experimental protocols were performed with the review and approval of the Institutional Animal Care and Use Committee.

*Intranerve treatment.* Direct infusions of the transected nerves with trophic factor or vehicle were accomplished (Fitzgerald et al., 1985) by anastomosis of the proximal nerve stump to a silastic chamber that was connected to an osmotic pump (Alzet-2001; Alza Corp., Palo Alto, CA) via polyvinyl tubing (VWR; Piscataway, NJ).

The effects of recombinant BDNF (Leibrock et al., 1989; Hohn et al., 1990) on the phenotype of injured adult motor neurons are based on data from three experiments. In the first two experiments (16 rats total: 8 treated and 8 controls) the osmotic pumps were loaded either with vehicle (saline, 3 rats; or phosphate-buffered saline, 5 rats) or recombinant human BDNF (supplied by Amgen-Regeneron Partners) diluted either with saline (3 rats) or phosphate-buffered saline (5 rats) to a final concentration of 0.5 mg/ml. This concentration of BDNF provided a dose of approximately 12 μg/d.

In a third experiment the loading concentration of BDNF was varied between animals in order to compare the effects of three different doses (19 rats: 13 treated and 6 controls). One set of animals was treated with vehicle alone (saline, 6 rats). A second set was infused with BDNF at the relatively low concentration of 0.05 mg/ml, corresponding to a dose of 1.2 μg/d (4 rats). A third set was infused at the normal concentration of 0.5 mg/ml (5 rats). A last set was infused at the relatively high concentration of 2.5 mg/ml, which corresponds to a dose of 60 μg/d (4 rats).

The effects of recombinant NT-4/5 (Hallböök et al., 1991) on the phenotype of injured adult motor neurons are based on data from three experiments (24 rats total: 12 treated and 12 controls). The osmotic pumps were loaded with the appropriate vehicle (50 mM acetate, 50 mM saline, 7 rats; or, phosphate-buffered saline, 5 rats). Other pumps were loaded with recombinant human NT-4/5 (Regeneron) diluted to a final concentration of 0.5 mg/ml either with acetate-saline (9 rats) or with phosphate-buffered saline (3 rats). This concentration of NT-4/5 provided a dose of 12 μg/d.

*Systemic treatment.* These studies required the use of relatively large quantities of recombinant protein. Thus we restricted our examination to the effects of BDNF on the phenotype of injured adult motor neurons and the data is based on one experiment (16 rats total: 12 treated and 4 controls). The control group of animals (4 rats) were treated by subcutaneous injection of vehicle (saline). Animals in the treatment group received one of four doses of BDNF; 0.01 mg/kg (4 rats), 0.1 mg/kg (4 rats), 1.0 mg/kg (4 rats), and 20 mg/kg (4 rats). Each rat received four injections; the first injection was made 1–2 hr after the nerve transection and subsequent injections were made every other day.

*Crush versus cut lesions.* This experiment compared the effects of nerve-cut lesion (4 rats) to nerve-crush lesion (5 rats) in the absence of any vehicle or neurotrophin treatment. All animals were allowed 1 week postlesion survival times.

*Intact rats.* The immunostaining patterns in three normal, unlesioned rats were examined after 1 week of daily subcutaneous injections of saline.

*Immunocytochemistry.* At the end of the week of treatment, all rats were overdosed with chloral hydrate-pentobarbital. They were perfused transcardially with heparinized saline followed by phosphate-buffered paraformaldehyde (4% w/vol: Fisher, Fairlawn, NJ). The lumbar (L) spinal cord segments from L4–L6 were identified by tracing spinal nerves from their union in the sciatic nerve to their dorsal root insertion sites. The cord was then blocked and the L4–L6 segments were scored with a scalpel blade so that a shallow nick was made in the left dorsal horn. The tissue was postfixed in the same fixative overnight and equilibrated in 30% (w/v) sucrose buffered with phosphate buffer (Fisher) as a cryoprotectant.

Spinal cord sections (50 μm) were cut on a freezing-sliding microtome and transferred serially into individual wells of a tissue culture plate and stored at −20°C in cryoprotectant (Watson et al., 1986). The anterior-posterior extent of the RDLN could be identified by microscopic inspection of wet-mounted sections. Four to six sections per rat at the level of the RDLN were pooled into a netted well (Becton-Dickinson, Franklin Lakes, NJ). For each experiment, sections from trophic factor-treated and the vehicle-treated rats were placed in adjacent netted wells and stained together in a large petri dish so that the staining conditions and solutions would be identical for sections from treated and control animals.

The primary antibodies were goat anti-choline acetyltransferase (ChAT) (1:20,000 dilution; Chemicon, Temecula, CA) and monoclonal antibody 192 (MAB 192; hybridoma cells were a gift from Dr. Eric Shooter, Stanford University), which recognizes the low affinity receptor for neurotrophins ($p75^{lngfr}$) (1:10,000 dilution; Chandler et al., 1984). Either antibody was applied for 2 d at room temperature in a diluent comprised of 10% (v/v) serum (GIBCO Life Technologies, Grand Island, NY), 1% Triton (Sigma, St. Louis, MO), and 0.2% (w/v) sodium azide (Fisher). After extensive washing, the sections were incubated with appropriate biotinylated peroxidase conjugated secondary antibody; anti-goat secondary antibody was diluted 1:1,000 and anti-mouse antibody was diluted 1:500 (Vector, Burlingame, CA). This was followed by incubation in avidin–biotin (Vector) and visualization of the bound complex with nickel intensified diaminobenzidine, as described previously (Friedman et al., 1992).

The criteria for further analysis of ChAT-stained sections from individual animals was the observation of qualitatively equivalent staining of the dorsal lateral nucleus ipsilateral and contralateral to the sciatic nerve lesion in a minimum of three sections. As the axons of the dorsal lateral nucleus motor neurons are not injured by the sciatic nerve lesion these neurons serve as technical controls for antibody access to the lesioned side. The criteria for analysis of MAB 192 staining was strong staining of the dorsal horn ipsilateral to the nerve lesion.

*Analysis of staining.* The intensity of staining of motor neurons in the RDLN was estimated for immunoreactive ChAT and $p75^{lngfr}$, by quantitative analysis of the stained sections which met the inclusion criteria discussed above (typically four sections per rat and no less than three sections). Individual coronal sections of cord were imaged and analyzed by a person who was blinded to the treatment group. The spinal cord sections were imaged at low magnification (2.5× objective) on a Zeiss Axioplan microscope using bright-field optics, so that the entire section filled the field. The image was projected to a video camera, digitized (512×480 pixels) and stored on a floppy disc; Loats Systems, MD) for subsequent analysis on a workstation (SparcStation 2; Sun Microsystems, Mountainview, CA) using software coded with Interactive Display Language (Research Systems, CO). All sections stained with a single antibody within a single experiment were digitized in one seating. In addition, the transmitted light through the slide, away from the section was captured to allow for correction of unevenness in incident light illumination of the section.

In sections stained for $p75^{lngfr}$ the reaction product is consistently present on the lesioned side and tends to demarcate both motor neurons and intervening neuropil within the entire RDLN. However, staining is relatively weak on the intact side. For this case, we focus only on the lesioned side and measure the average optical density (OD) in a sample area that just encompasses the RDLN. To correct for optical absorption by the tissue and thus determine the average OD per pixel for stained neurons, we subtract the average optical density determined within the ventral funiculi, an essentially unstained region. For each animal, we analyzed three to six sections. The mean value of the corrected OD across sections for a given animal was taken as the unit of measure and averaged over all animals in each treatment group, all costained (see above), to compute a group mean denoted as $p75^{lngfr}$. The dominant source of error in $p75^{lngfr}$ arose from variability between animals, as opposed to variability between sections for the same animal. Note that the variations in staining density in separately run experiments precludes comparisons of $p75^{lngfr}$ values between experiments.

Sections stained for ChAT appeared qualitatively different from those stained for $p75^{lngfr}$ immunoreactivity. First, the reaction product was concentrated in neuronal somata and the intervening neuropil is lightly and variably stained. This suggests that an appropriate measure of staining is the OD of the soma relative to the neighboring neuropil, rather than a distant region of the section. To determine this, we calculate the number of pixels whose OD is above a threshold level as that level is increased. This number will exhibit a sharp decrease from a high initial

value to a lower final value when the increase in threshold excludes the contribution of regions containing weakly stained neuropil. So long as the sample area included only the RDLN somata, the OD per pixel of the stained motor neurons relative to that of the neuropil was essentially independent of the sample boundaries. Second, reaction product in sections stained for ChAT was consistently present in the RDLN on the intact side. This allowed us to normalize the reaction product on the lesioned side relative to that on the intact side at the level of individual sections; see Appendix for details. As in the case for the $p75^{lngfr}$ immunoreactivity, the mean value of the normalized OD across sections for a given animal was taken as the unit of measure and averaged over all animals in each treatment group to compute a group mean denoted as ChAT(R/L). Because the immunoreactivity to ChAT is normalized on a section by section basis, it is possible to compare ChAT (R/L) between different experiments where BDNF or NT-4/5 were administered. The group means obtained within an individual experiment, for both ChAT (R/L) and for $p75^{lngfr}$(OD), were analyzed by ANOVA and the results tested for significance with the FISHER PLSD using STATVIEW software (Macintosh).

*Bioassay of neurotrophins.* Osmotic minipumps were removed from rats treated via the intranerve route and residual trophic factor was retrieved for *in vitro* bioassays. BDNF was assayed by scoring neurite outgrowth of chick embryo dorsal root ganglia that had been explanted to culture dishes (Davies et al., 1986). NT-4/5 was assayed for bioactivity using a 4 d survival assay with MG87 fibroblasts that had been transfected with the rat TrkB receptor as described in Fandl et al. (1994). These assays demonstrated essentially full bioactivity of trophic factor that was retrieved from the osmotic pumps used in the 33 neurotrophin-treated cases presented here.

## Results

### *Phenotypic changes in RDLN motor neurons produced by sciatic nerve transection in the adult rat*

The present study introduces the use of the RDLN (Navaratam and Lewis, 1970; Burt, 1975; Schroder, 1980) in the spinal cord as a convenient model system to assay agents that may attenuate the effects of peripheral axotomy on spinal motor neurons in the adult rat. The RDLN motor neurons form a column in lower lumbar and upper sacral spinal cord segments. In cross-section, the RDLN can be distinguished even in unstained sections, when viewed with a microscope at low magnification, as the dorsal and lateral-most cluster of motor neurons in the ventral horn. This stereotypic cytoarchitecture provides a site to evaluate experimental manipulations of spinal motor neurons, as the same set of motor neurons can be consistently identified in individual animals (Fig. 1*A*). The RDLN motor neurons innervate the intrinsic muscles of the ipsilateral foot (Leslie et al., 1991) via axons that travel within the tibial division of the sciatic nerve and its peripheral branches. Thus our transection lesions of the sciatic nerve consistently sever all of the axons of the neurons in the ipsilateral RDLN.

The lesioned RDLN motor neurons survive the acute effects of axotomy (Fig. 1*B*) but undergo two distinctive phenotypic changes within 1 week after unilateral sciatic nerve transection. First, in normal adult rats monoclonal antibody 192, which recognizes the low affinity neurotrophin receptor known as $p75^{lngfr}$, occasionally stains a few RDLN motor neurons (Fig. 1*C*). However, transection of the sciatic nerve in the adult markedly increases $p75^{lngfr}$ immunoreactivity in the lesioned spinal motor neurons. Thus, 1 week after axotomy the RDLN is demarcated by $p75^{lngfr}$ associated immunostaining (Fig. 1*D*). Second, in normal rats, antibodies that recognize ChAT (Houser et al., 1983) consistently stain RDLN motor neurons with a dense reaction product that fills somata and shows variable extension into the dendritic processes (Fig. 1*E*). However, transection of the sciatic nerve reduces the ChAT immunoreactivity in RDLN motor neurons. One week after axotomy, the large motor neurons in the injured RDLN are pale and are stained only slightly more darkly than the diffuse background staining of the adjacent neuropil (Fig. 1*F*). Neighboring motor neurons that reside in the dorsolateral nucleus are not injured by sciatic nerve lesion and these cells retain strong ChAT immunoreactivity.

### *BDNF treatment modifies the phenotypic changes of RDLN motor neurons lesioned by sciatic nerve transection*

The effects of 1 week of treatment with solutions of neurotrophin or vehicle were examined in RDLN neurons that were axotomized by transection of the sciatic nerve. One method of administration utilized a localized delivery of solutions to the cut end of the nerve, which commenced immediately after the transection was performed. The proximal nerve stump was anchored within a silastic tube that was connected to an osmotic minipump to allow better local delivery of the treatment solution to the lesioned nerve. In the first set of these experiments, the pumps were loaded either with BDNF or with vehicle control solutions. A second method of administration utilized a widespread, systemic delivery of solutions. Vehicle or different doses of BDNF were injected subcutaneously, starting within 1–2 hr after closure of the surgery site and repeated every other day for 1 week for a total of four injections.

Local delivery of BDNF to the injured nerve influenced the levels of $p75^{lngfr}$ immunoreactivity in lesioned RDLN motor neurons. In animals treated with vehicle, the $p75^{lngfr}$ immunoreactivity of lesioned RDLN motor neurons was darker than the staining of the contralateral intact RDLN (Fig. 2*A*). This is consistent with the changes in staining, described above, in animals that have transected sciatic nerves but have not been experimentally treated with vehicle solutions. However, in animals treated with BDNF (12 μg/d), the $p75^{lngfr}$ immunoreactivity of lesioned RDLN motor neurons is even darker than the analogous staining in the vehicle treated animals (Fig. 2*A,B*). Systemic delivery of BDNF to the injured nerve also influenced the levels of $p75^{lngfr}$ immunoreactivity in lesioned RDLN motor neurons. At the effective dose of 20 mg BDNF/kg, the $p75^{lngfr}$ immunoreactivity of lesioned RDLN motor neurons is markedly darker than this staining in vehicle treated animals (Fig. 2*E,F*). However, this dose of subcutaneously administered BDNF did not induce the reexpression of $p75^{lngfr}$ immunostaining in intact adult motor neurons since the uninjured, contralateral RDLN motor neurons showed no increase in $p75^{lngfr}$ immunoreactivity (Fig. 2*F*).

Local as well as systemic delivery of BDNF, at the doses described above, also influenced the levels of ChAT immunoreactivity in lesioned RDLN motor neurons (Fig. 3). Axotomized RDLN motor neurons in animals treated with vehicle showed a marked reduction of ChAT immunoreactivity (Fig. 3*A*). In contrast, in animals treated with BDNF, the effects of nerve transection were attenuated and the axotomized RDLN neurons were more darkly stained than they were in the vehicle-treated animals (Fig. 3*B*).

We devised a method to quantify ChAT staining intensity which allowed us to compare the staining levels within and between individual experiments (see Materials and Methods and Appendix). Optical density measures were made of the ChAT staining in the lesioned RDLN on the right side and also in the contralateral intact RDLN on the left side of the same section. This procedure was used to generate a staining ratio, ChAT [Right(R)/Left(L)], as a measure of the asymmetry of the average staining intensity in the right and left RDLNs (see Materials and Methods and Appendix). In intact rats ($n$ = 3) where the

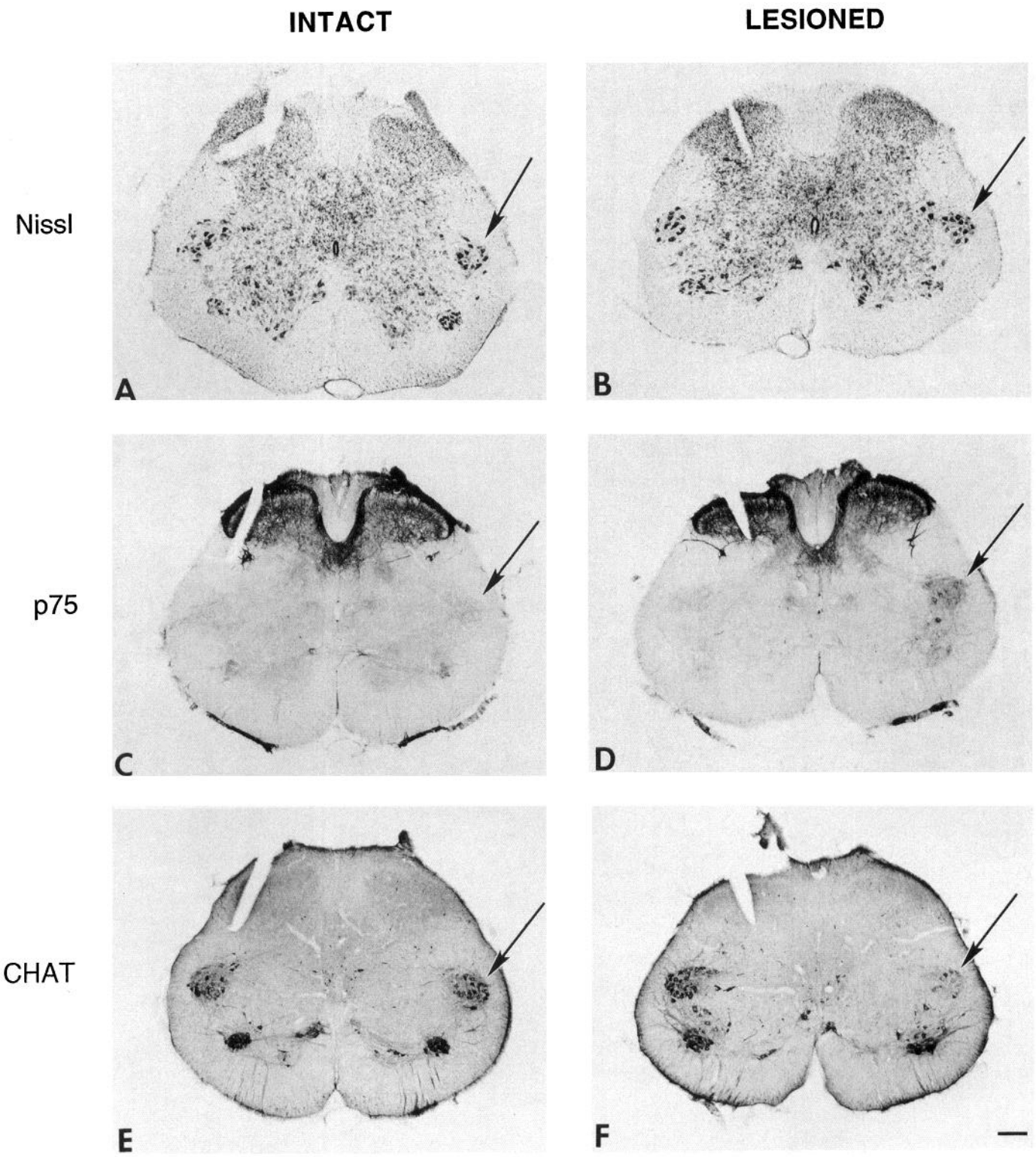


*Figure 1.* Photomicrographs of coronal sections of lumbar spinal cord through the level of the retrodorsal lateral nucleus (RDLN) from intact adults rats (*A,C,E*) and from adult rats after right sciatic nerve transection (*B,D,F*). *A,* Cresyl violet stained section showing cell somata in intact left and right RDLN in a normal adult male rat. The right RDLN (*arrow*) can be identified in this and in all other sections by its position opposite to the score mark made in the dorsal horn on the left side. *B,* Cresyl violet stained section of spinal cord section after transection of right sciatic nerve. There is no obvious reduction in cell number among axotomized RDLN motor neurons on the right side (*arrow*). *C,* Spinal cord sections stained with MAB 192 to show sites of $p75^{lngfr}$ immunoreactivity. Immunostaining in sections from an intact animal is restricted to the dorsal horn and to the sexually dimorphic motor neuron cell groups of the dorsolateral and bulbocavernosus nuclei. The right RDLN (*arrow*) shows only faint staining. *D,* MAB 192 staining of spinal cord section 1 week after right sciatic nerve transection. The motor neurons of the right RDLN (*arrow*) reexpress $p75^{lngfr}$ immunostaining, normally present in these cells only during early developmental stages (Yan and Johnson, 1988). *E,* Section stained for ChAT immunoreactivity illustrates the symmetry in staining intensity in the left and right RDLN (*arrow*) in intact rats. *F,* Section from a spinal cord 1 week after right sciatic nerve transection. The axotomized right RDLN neurons (*arrow*) show a marked reduction in ChAT immunostaining with only a few cells showing moderate staining. Scale bar, 200 μm.

ChAT intensity in the RDLNs on both sides is qualitatively similar, the ChAT (R/L) staining ratio was 1.02 ± 0.13 (average ± SD) while in vehicle-treated animals where the lesioned RDLNs on the right side are much more lightly stained than on the contralateral left side the ChAT (R/L) values were approximately 0.5 (Table 1, Fig. 4*A*).

The ChAT (R/L) values for groups of animals treated with BDNF confirmed that our observations of individual cases could be generalized to groups of treated animals (Fig. 4*A*, Table 1). Thus the individual animals that were treated with doses of BDNF that were too low to produce an observable change in injured RDLN motor neuron phenotype also yielded ChAT (R/L) ratios that, as a group, were not statistically significantly different from controls (Fig. 4*A*; e.g., intranerve dose of 1.2 μg/d, or four systemic doses of 0.01, 0.1, or 1.0 mg/kg). On the other hand the doses that visibly affected the levels of ChAT staining (12 and 60 μg/d, intranerve and 20 mg/kg, systemic) all produced statistically significant preservation of prelesion levels of ChAT, as these ChAT (R/L) ratios were significantly different from the vehicle-control values.

Average optical densities were determined for the neurons in the RDLN that were stained to reveal $p75^{lngfr}$ immunoreactivity (see Materials and Methods and Appendix); for these cases stain ratios (R/L) could not be measured because of the weak, variable staining present on the normal intact side. Thus for the statistical analysis of $p75^{lngfr}$ immunostaining optical density data, for each experiment, we limit our comparisons to values obtained from costained sections that were run in that particular experiment. Local intranerve administration of BDNF at doses of 1.2, 12, and 60 μg/day all produced significant increases in $p75^{lngfr}$(OD) relative to the vehicle-control group (Fig. 4*B*). Furthermore, the magnitude of these effects increased with dose as the $p75^{lngfr}$(OD) value for the 60 μg/d group was significantly greater than for the 1.2 μg/d group. It should be noted that although the lower dose of BDNF (1.2 μg/d) reached statistical significance for $p75^{lngfr}$(OD), there was no significant effect of this dose on ChAT (R/L) values.

Systemic administration of BDNF at doses of 0.01, 0.1, and 1.0 mg/kg produced no significant difference relative to the values for the vehicle control group. However, the $p75^{lngfr}$(OD) value for the high dose treatment group (20 mg/kg) was significantly greater than this value for the vehicle controls (Fig. 4*B*).

*Effects of NT-4/5 on axotomized RDLN motor neurons*

Local delivery of NT-4/5 to the injured nerve influenced the levels of both $p75^{lngfr}$ and ChAT immunoreactivity in lesioned RDLN motor neurons. The animals that were treated with vehicle (either acetate saline or phosphate-buffered saline) showed the expected phenotype after axotomy of increased $p75^{lngfr}$ immunoreactivity and diminished ChAT levels in the lesioned RDLN (Fig. 2*A*, 3*A*). However, local intranerve administration of NT-4/5 (12 μg/d) stimulated a further increase in $p75^{lngfr}$ immunoreactivity in the RDLN (Fig. 2*A,B*). Treatment with NT-4/5 also helped preserve ChAT staining in lesioned RDLN motor neurons (Fig. 3*B*).

Quantitation of the above effects indicate that the differences between the control and NT-4/5 treated groups in staining density are statistically significant (Table 1). Of interest, the ChAT (R/L) values obtained for the NT-4/5 treatment groups are not statistically different from these values measured in groups of animals treated with comparable doses of BDNF (i.e., intranerve administration of 12 μg/d).

*Sciatic nerve crush reproduces the effects of neurotrophin treatment on axotomized RDLN motor neurons*

The effects of sciatic nerve crush or cut on the phenotype of RDLN motor neurons were compared in a series of untreated rats subjected either to sciatic nerve transection ($n = 4$) or to sciatic nerve crush ($n = 5$). The $p75^{lngfr}$ staining is darker after crush than after nerve cut (Fig. 5*A,B*). In addition, animals with nerve crush show greater preservation of ChAT immunoreactivity in the lesioned RDLN motor neurons (Fig. 5*C,D*). Optical densitometry corroborates these observations as the differences between the crush and cut group values for $p75^{lngfr}$(OD) and ChAT(R/L) are statistically significant (Table 1). Qualitatively, the effects of nerve crush reproduce the effects of pharmacological doses of single neurotrophins (BDNF or NT-4/5) in animals with sciatic nerve transection lesion. Furthermore, the degree of ChAT preservation and the group ChAT(R/L) value associated with nerve crush is not statistically different from the ChAT(R/L) values obtained in animals treated by local intranerve administration of either BDNF or NT-4/5 (12 μg/d).

## Discussion

*Summary*

As in development, adult motor neurons have the capacity to respond to the neurotrophins BDNF and NT-4/5. Even qualitative inspection of immunostained sections of spinal cord from rats treated for one week with BDNF, NT-4/5, or vehicle shows that the neurotrophins change the reactive phenotype of axotomized RDLN motor neurons. This visual impression is confirmed by comparisons of the optical densitometric measures which reveal statistically significant increases in immunostaining density of $p75^{lngfr}$ and ChAT in the neurotrophin treatment groups. The reproduction of these changes in phenotype by nerve-crush lesion alone suggests that endogenous factors, possibly BDNF and/or NT-4/5, can also modulate the reaction of motor neurons to nerve injury.

*BDNF and NT-4/5 effects on immunocytochemically detected ChAT*

Intranerve administration of NT-4/5 and BDNF at 12 μg/d produces similar changes in ChAT immunoreactivity in adult spinal

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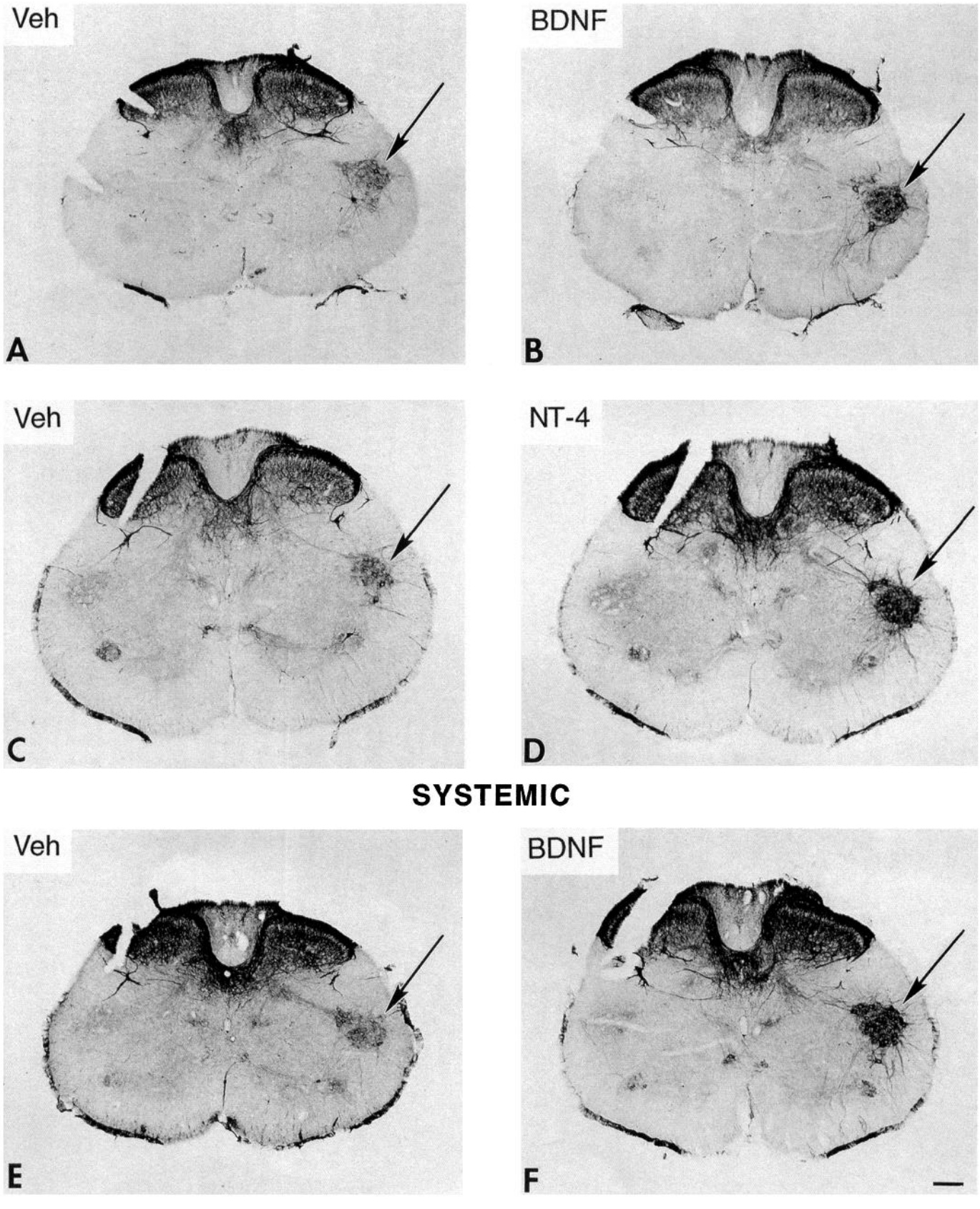
*Figure 2.* Photomicrographs of lumbar spinal cord sections from adult rats 1 week after right sciatic nerve transection stained with MAB 192 to show sites of $p75^{lngfr}$ immunoreactivity. *A* and *B*, Spinal cord sections from a vehicle (saline)treated rat (*A*) and from a BDNF-treated rat (12 μg/d, intranerve administration) (*B*). These sections were stained simultaneously and in the same staining solutions. Axotomized RDLN neurons treated with BDNF (*B*) are stained more darkly than after vehicle treatment (*A*). *C* and *D*, Spinal cord sections from a rat treated with acetate buffer vehicle (*C*) or with NT-4/5 (*D*) (12 μg/d, intranerve administration). NT-4/5 treatment results in denser $p75^{lngfr}$ immunoreactivity than vehicle treatment. *E* and *F*, Spinal cord sections from a rat treated systemically with (saline) vehicle every other day (*E*) (sc, subcutaneous administration; four injections) or with BDNF (*F*) (sc, four injections at 20 mg BDNF/kg) during the week following sciatic nerve transection. BDNF-treated axotomized RDLN neurons (*F*) show higher levels of $p75^{lngfr}$ immunoreactivity than the vehicle-treated RDLN (*E*). Note that systemic BDNF did not induce reexpression of $p75^{lngfr}$ immunoreactivity in intact RDLN neurons (*F*). Scale bar, 200 μm.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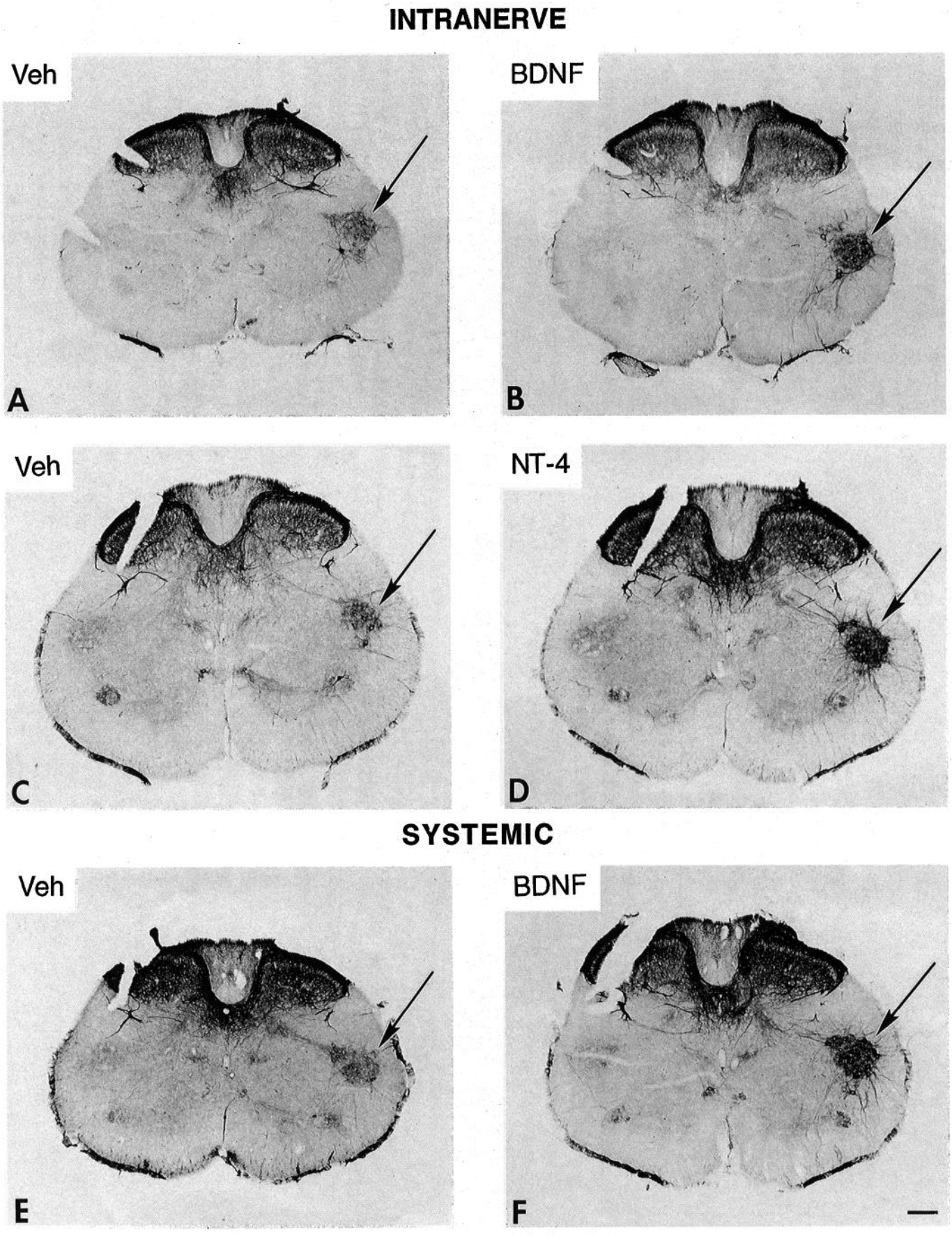
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**Table 1. Effects of trophic factors on the phenotype of injured RDLN motor neurons**

| | Pump solution | $p75^{lngfr}$ (OD) | CHAT (R/L) Stain Ratio |
|---|---|---|---|
| Right sciatic nerve cut | | | |
| Exp I | BDNF (5) | 0.41 ± 0.04 ($p$ = 0.0002)* | 0.69 ± 0.15 ($p$ = 0.03)* |
| | VEH (4) (PBS) | 0.28 ± 0.05 | 0.38 ± 0.12 |
| Exp II | BDNF (3) | 0.25 ± 0.02 ($p$ = 0.02)* | 0.75 ± 0.05 ($p$ = 0.007)* |
| | VEH (3) (SAL) | 0.16 ± 0.04 | 0.41 ± 0.10 |
| Exp III | BDNF (5) | 0.30 ± 0.03 ($p$ = 0.0001)* | 0.78 ± 0.16 ($p$ = 0.03)* |
| | VEH (6) (SAL) | 0.20 ± 0.04 | 0.54 ± 0.19 |
| Exp IV | NT-4/5 (3) | — | 0.73 ± 0.30 |
| | VEH (1) (ACET) | — | 0.40 |
| Exp V | NT-4/5 (6) | 0.34 ± 0.05 ($p$ = 0.002)* | 0.79 ± 0.13 ($p$ = 0.008)* |
| | VEH (6) (ACET) | 0.24 ± 0.03 | 0.56 ± 0.12 |
| Exp VI | NT-4/5 (3) | 0.27 ± 0.03 ($p$ = 0.006)* | 1.04 ± 0.11 ($p$ = 0.004)* |
| | VEH (5) (PBS) | 0.23 ± 0.01 | 0.65 ± .18 |
| Right sciatic nerve crush (5) | | 0.40 ± 0.03 ($p$ = 0.001)* | 0.72 ± 0.07 ($p$ = 0.0004)* |
| Right sciatic nerve crush (5) | | 0.29 ± 0.03 | 0.38 ± 0.09 |

Values are mean ± SD. $p$ values with * are significantly different (5% significance level) from vehicle controls, that were run in the same experiment, as determined by ANOVA and the Fisher PLSD test. Numbers in parentheses indicate the number of sampled animals. All neurotrophins were administered for 1 week via osmotic pumps loaded to deliver nominal doses of 12 μg/d. Diluents: PBS, phosphate-buffered saline; SAL, saline; ACET, acetate saline. VEH refers to treatment with the diluent without added neurotrophin.

motor neurons after nerve transection. These effects are consistent with data that shows that NT-4/5 and BDNF can be potent and equivalent ligands for full length TrkB, which is the high affinity receptor for both neurotrophin family members (Klein et al., 1991; Soppet et al., 1991; Squinto et al., 1991; Ip et al., 1992, 1993; Wong et al., 1993). For example, BDNF and NT-4/5 produced comparable increases in ChAT activity in cultures of fetal motor neurons (Henderson et al., 1993; Wong et al., 1993; A. C. Kato and R. M. Lindsay, unpublished observations) but the effects of BDNF and NT-4/5 were not additive, an indication that these TrkB ligands act through common receptor mechanisms (Wong et al., 1993). Importantly, motor neurons continue to express full length TrkB into adulthood (Koliatsos et al., 1993).

We note that Chiu and coworkers (1993) do not see reductions in immunocytochemically detected ChAT in adult spinal motor neurons after sciatic nerve lesions. The discrepancy between their data and those in the present study may stem from differences in the lesion sites and/or in the anti-ChAT antibodies that were used. This interpretation is supported by a recent report that corroborates our observations of a downregulation of ChAT immunoreactivity in axotomized lumbar spinal motor neurons in the adult rat (Clatterbuck et al., 1994). Clatterbuck and coworkers (1994) also examined the effects of BDNF on the reactive responses of adult motor neurons but this group did not observe any changes in injured motor neuron phenotype: it is likely that this reflects the difficulty of providing a sufficient dose of BDNF using osmotic minipumps that are not directly anchored to the cut end of the nerve via an anastomosed reservoir, as described by Fitzgerald and her coworkers (1985). In the absence of such a direct anastomosis, the delivered trophic factor is likely to be rapidly diluted and excreted as are other small molecules.

### *BDNF and NT-4/5 modulation of injury provoked reexpression of $p75^{lngfr}$*

The reexpression of $p75^{lngfr}$ that is provoked by nerve lesion in adult motor neurons (Raivich and Kreutzberg, 1987; Yan and Johnson, 1988; Ernfors et al., 1989; Koliatsos et al., 1991) is further up-regulated by treatment of injured motor neurons with either BDNF or NT-4/5. Despite the clear modulation of $p75^{lngfr}$ staining by BDNF, it is unlikely that BDNF is the signal that induces $p75^{lngfr}$ reexpression in injured adult motor neurons. The acute phase of injury-provoked reexpression of $p75^{lngfr}$ immunoreactivity can be prevented by nerve ligation or by pharmacological blockers of retrograde axoplasmic transport which suggests that the onset of $p75^{lngfr}$ expression depends on a retrogradely transported signal (Wood et al., 1990; Greeson et al., 1992). However, the injury-provoked reexpression of $p75^{lngfr}$ staining in spinal motor neurons is detectable within 24 hr of sciatic nerve crush or cut injury (Rende et al., 1992) and thus

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*Figure 3.* Photomicrographs of lumbar spinal cords stained to show ChAT immunoreactivity. *A* and *B*, Spinal cord sections from a vehicle (saline) treated rat (*A*) and from a BDNF-treated rat (12 μg/d, intranerve administration) (*B*). Section in *A* shows a marked reduction in ChAT staining in the right RDLN (*arrow*) 1 week after right sciatic nerve transection. RDLN neurons contralateral to the transection are densely ChAT stained. Section in *B* from BDNF-treated rat shows that BDNF attenuates the reduction in ChAT staining (*arrow*). *C* and *D*, Spinal cord sections from a rat treated with acetate buffer vehicle (*C*) or with NT-4/5 (*D*) (12 μg/d, intranerve administration). NT-4/5, like BDNF, also results in greater retention of ChAT staining than does the vehicle treatment (compare *arrows C* vs *D*). *E* and *F*, Spinal cord sections from a rat treated systemically with (saline) vehicle every other day (*E*) (sc, systemic administration, four injections) or with BDNF (*F*) (sc, four injections at 20 mg BDNF/kg) during the week following sciatic nerve transection. BDNF-treated RDLN neurons (*arrows*) retained greater levels of ChAT immunoreactivity 1 week after right sciatic nerve transection then did the RDLN in vehicle treated animals (compare *arrows E* and *F*). Scale bar, 200 μm.

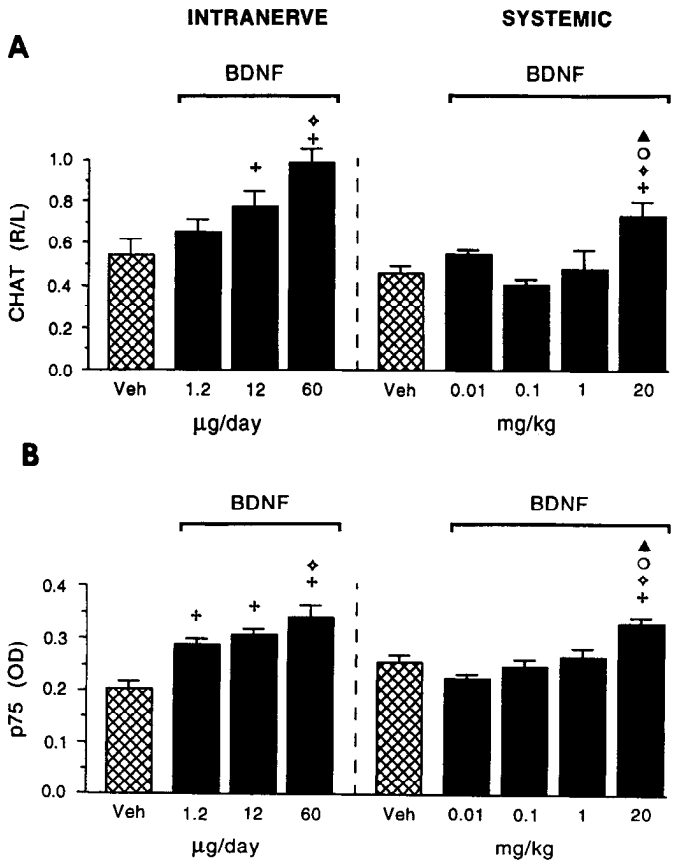


*Figure 4.* *A*, Effects of treatment with BDNF on ChAT (right/left) staining intensity. Intranerve treatment: this data was obtained from ChAT immunostained sections of lumbar spinal cord from rats treated either with vehicle or with three doses of BDNF which include 1.2 µg/d, 12 µg/d, and 60 µg/d. The two higher doses of BDNF resulted in ChAT (*R/L*) group ratios that were significantly higher than the vehicle-group value ($p < 0.05$). Key for intranerve treatment panels in *A* and *B*: *plus*, significantly different from vehicle control; *diamond*, significantly different from 1.2 µg/d. Systemic treatment: three doses (0.01 mg/kg, 0.1 mg/kg, 1 mg/kg) of subcutaneously administered BDNF failed to produce a significant change in the lesion-provoked losses in ChAT (*R/L*) group ratios relative to the vehicle group. However, the ChAT (*R/L*) value for the group treated with a dose of 20 mg BDNF/kg was significantly greater than this value for the vehicle group ($p < 0.05$). Key for systemic treatment panels in *A* and *B*: *plus*, significantly different from vehicle control; *diamond*, significantly different from 1 mg/kg; *open circles*, significantly different from 0.1 mg/kg; *solid triangles*, significantly different from 0.01 mg/kg). *B*, Effects of BDNF treatment on p75$^{lngfr}$(*OD*) staining intensity. Intranerve treatment: animals were treated with three different doses of BDNF: 1.2 µg/d ($n = 4$), 12 µg/d ($n = 5$), and 60 µg/d ($n = 4$). All three doses of BDNF resulted in average p75$^{lngfr}$(OD) values that were significantly greater than these values in vehicle treated rats ($p < 0.05$). The p75$^{lngfr}$(OD) at the lowest dose of BDNF (1.2 µg/d) is significantly less than that for the highest dose (60 µg/d) which indicates that the magnitude of the effects of BDNF are dose related ($p < 0.05$). Systemic treatment: effects on p75$^{lngfr}$. Animals were treated via subcutaneous administration of four different doses of BDNF: 0.01 mg/kg; 0.1 mg/kg, 1.0 mg/kg, and 20.0 mg/kg. The three lower doses of BDNF resulted in p75$^{lngfr}$(OD) values that were not significantly different from each other nor were they different from the vehicle group value. The highest dose used (20 mg BDNF/kg) resulted in a p75$^{lngfr}$(OD) group value that was significantly greater than the vehicle control group ($p < 0.05$).

occurs far in advance of the upregulation of BDNF in degenerating peripheral nerve distal to the lesion site (Meyer et al., 1992), as elevations in BDNF mRNA are first detected between 3 and 7 d postinjury. Furthermore, the systemic administration of BDNF, at the doses used in the present study, failed to induce a reexpression of p75$^{lngfr}$ in intact motor neurons contralateral to the nerve transection (see Fig. 2*F*). It is even less likely that NT-4/5 administration is directly responsible for triggering the initial injury-associated reexpression of p75$^{lngfr}$, since the upregulation of p75$^{lngfr}$ appears to be a prerequisite for NT-4/5 retrograde transport in motor neurons (Curtis et al., 1993, Soc Neurosci Abstr 606.2).

The increase of p75$^{lngfr}$ immunoreactivity associated with neurotrophin treatment, at first glance could appear to represent an exacerbation of the response of motor neurons to injury since injury is associated with the reexpression of this protein. However, in the context of the normal responses of motor neuron to nerve-crush lesion an alternative view is possible. Thus, the elevation of p75$^{lngfr}$ expression in neurotrophin-treated animals may signify a potentiation of responses that lead to motor neuron recovery from injury since similar effects are observed after nerve-crush injury.

P75$^{lngfr}$ acts as a low affinity receptor for all neurotrophin family members (Rodriguez-Tébar et al., 1990; Hallböök et al., 1991; Squinto et al., 1991). Recent studies have demonstrated that there are likely to be cooperative effects between p75$^{lngfr}$ and TrkA, the signaling receptor for NGF (Hempstead et al., 1991). Interestingly, the upregulation of p75$^{lngfr}$ may also have consequences for the signaling capacities of TrkB. Although earlier studies demonstrated that BDNF stimulation of TrkB in the absence of p75$^{lngfr}$ can produce a functional response in a fibroblast cell line (Glass et al., 1991), more recent *in vitro* data demonstrates that p75$^{lngfr}$ can function as an auxiliary molecule for full length TrkB; fibroblasts (NIH 3T3 cells) that coexpress full-length TrkB together with p75$^{lngfr}$ are rendered newly responsive to their endogenous levels of BDNF, while expression of either full-length TrkB or of p75$^{lngfr}$, by themselves, does not unmask this autocrine loop (Hantzopoulos et al., 1994). Thus while adult motor neurons normally express TrkB (Koliatsos et al., 1993), their increased upregulation of p75$^{lngfr}$ may further enhance their sensitivity to the TrkB ligands, BDNF and NT-4/5.

### *Exogenous BDNF or NT-4/5 may potentiate effects of endogenous neurotrophins on injured spinal motor neurons*

Previous work has shown that nerve cut and nerve crush injuries produce quantitative differences in the acute changes in motor neuron phenotype even at short survival times, when both lesions result in a disconnection of motor neurons from their target muscles. Nerve crush results in greater acute elevations in the levels of p75$^{lngfr}$ mRNA reexpression as compared to nerve transection for both facial and hypoglossal motor neurons (Saika et al., 1991; Hayes et al., 1992). Furthermore, as compared to nerve transection, nerve crush results in smaller reductions in ChAT immunostaining in adult cranial motor neurons (Armstrong et al., 1991; Borke et al., 1993). The present study extends these findings to the RDLN spinal motor neurons where nerve crush injury both augments p75$^{lngfr}$ immunostaining and also reduces the loss in ChAT immunostaining relative to losses observed after nerve cut. Clearly, the exposure of injured motor neurons to trophic factors produced in degenerating nerve will differ after nerve-cut and nerve-crush injuries. Motor neurons subjected to crush lesions will extend axons into degenerating nerve which may be a relatively rich source of BDNF and NT-4/5 since the mRNA for these neurotrophins is expressed at high levels (Meyer et al., 1992; Funakoshi et al., 1993). In contrast to the effects

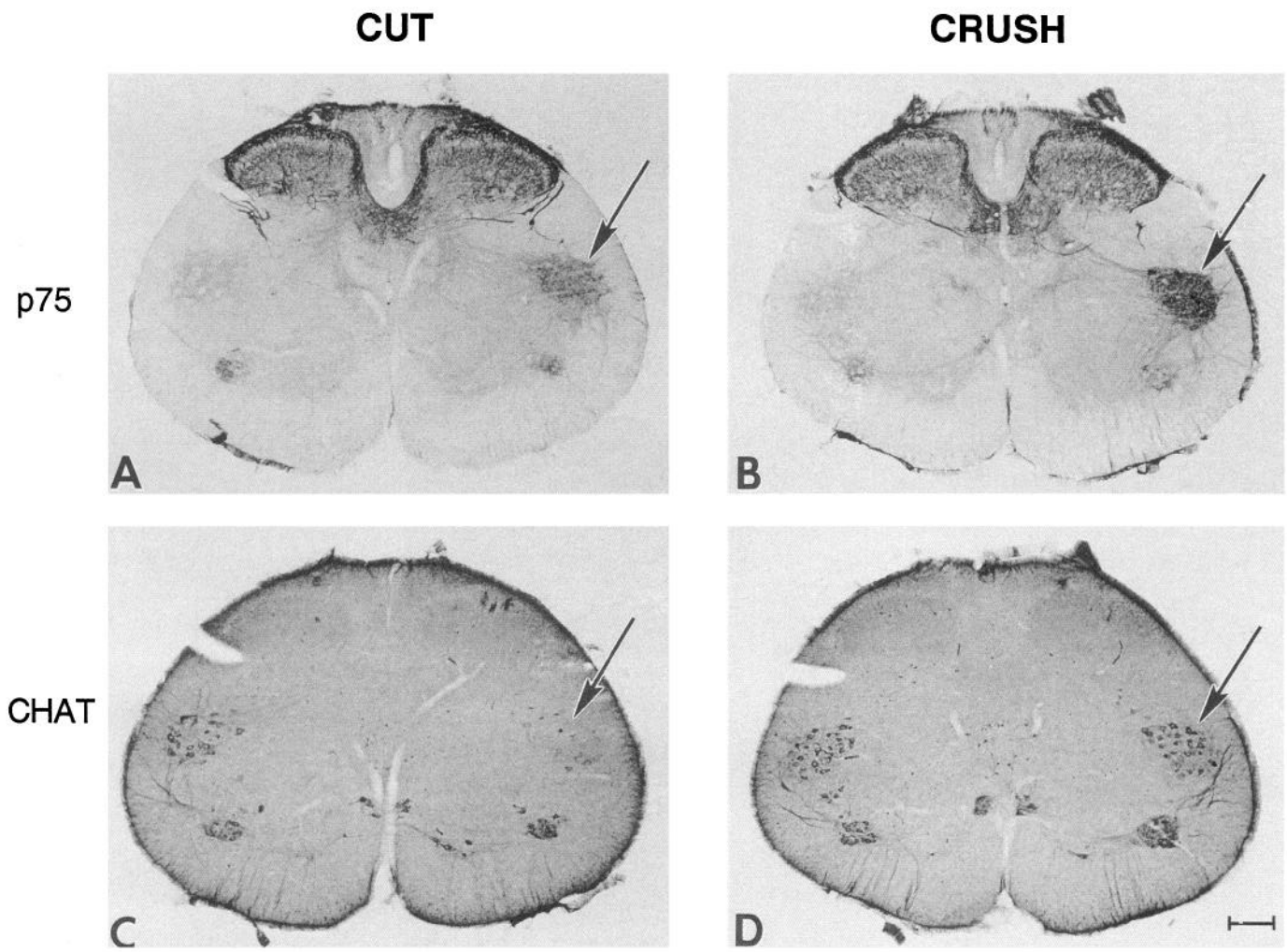


*Figure 5.* Photomicrographs of costained sections from adult rats with sciatic nerve cut or crush lesions. *A* and *B*, Immunoreactivity of $p75^{ngfr}$ is upregulated in injured RDLN motor neurons (*arrows*) after either sciatic nerve transection (*A*) or after sciatic nerve crush (*B*) but the motor neurons in animals with nerve crush lesions are more darkly stained. *C* and *D*, Immunoreactivity of ChAT in injured RDLN motor neurons (*arrows*) is reduced after sciatic nerve transection (*C*) but this reduction is much less after nerve crush lesion (*B*) even though both lesions result in acute disconnection of RDLN neurons from their muscle targets. Scale bar, 200 μm.

of nerve-crush, after a nerve-transection injury, the surviving axonal segments of motor neurons are restricted to the proximal nerve stump which is a site that does not upregulate its expression of BDNF mRNA (Meyer et al., 1992) after this injury. Therefore, depending on the type of injury, the supplies of endogenous native neurotrophins may be limited; this raises the possibility that treatment *in vivo* with recombinant neurotrophins may act to supplement the effects of endogenous neurotrophic factors. In conclusion, this study suggests that the expression of BDNF and of NT-4/5 in the adult is likely to be of functional significance for injured adult motor neurons. At a minimum, administration of these factors reveals the plasticity of the reactive responses of axotomized adult motor neurons. Furthermore, the idea that these changes are positive is supported by their similarity to the reactive responses observed in regenerating motor neurons. Whether these effects of BDNF and NT-4/5 could also contribute to successful axon regrowth remains to be determined.

## Appendix

The relative staining in the RDLNs is determined from an analysis of stained sections, as follows.

(1) A video image of the coronal section of cord is digitized (Materials and Methods). Each pixel in the image is denoted IMAGE $(I,J)$.

(2) A video image of the incident illumination is digitized through a blank region of the slide holding the section. Each pixel in the image of the incident light is denoted INCIDENT $(I,J)$.

(3) The optical density of each pixel, denoted OD $(I,J)$, is calculated as

$$\mathrm{OD}\ (I,J) = -\log_{10}\left[\frac{\mathrm{IMAGE}(I,J)}{\mathrm{INCIDENT}(I,J)}\right]. \qquad (1)$$

Further numerical analysis proceeds in one of two ways depending on the nature of the staining.

*$p75^{ngfr}$ immunoreactivity.* When reaction product is present only on the lesioned side, as with sections stained for $p75^{ngfr}$, a box is defined that encompasses the RDLN on the lesioned side. The average optical density of the reaction product is calculated, following standard procedures, as follows.

(1) The level of background absorption is estimated by cal-

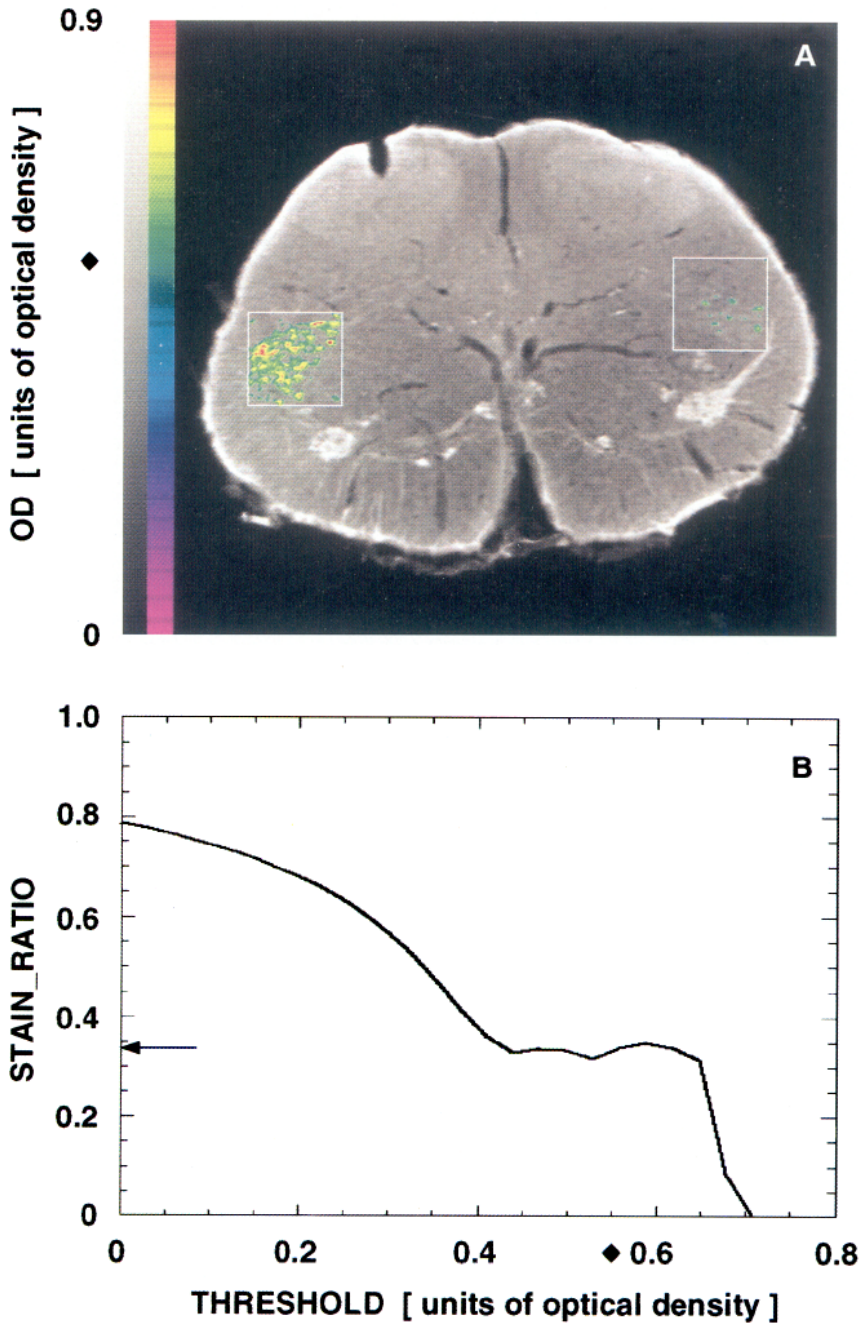


*Figure 6.* Illustration of the methodology used to determine the relative level of ChAT staining in lesioned and in intact RDLN from the same section. *A*, Optical density of the section shown in Figure 3*E* (Eq. 1). The *box on the left* encompasses the intact side: boxes are square, of equal size, and for this example are 35 pixels on a side. Optical densities within each box that exceed a level of 0.55 optical density units (*solid diamond*) are coded in color, rather than shades of gray, to illustrate staining that contributes to the estimate of ChAT (R/L)=STAIN_RATIO (part *B*). *B*, Plot of the function STAIN_RATIO (Eqs. 4–6) versus the threshold level for the RDLN shown in *A*. The plateau region extends approximately from thresholds of 0.45–0.65 optical density units and has an average value of (arrow) ⟨STAIN_RATIO⟩ = 0.34 (Eq. 7). The average threshold across the plateau, that is, THRESHOLD = 0.55 optical density units (*solid diamond*), was used as a threshold for the color code in *A*.

culating the optical density in the white matter of the ventral funiculi, an unstained portion of the tissue section. A box is defined within the anterior columns and the average optical density per pixel within this region, denoted OD_BACK, is defined by

$$\text{OD\_BACK} = \frac{\sum_{(I,J)\text{ in ventral box}} \text{OD}(I,J)}{\sum_{(I,J)\text{ in ventral box}}}. \quad (2)$$

This serves as a reference level.

(2) The specific staining intensity of $p75^{lngfr}$ immunoreactive RDLN motor neurons is given by the average optical density of the tissue within the box that bounds the RDLN, and is calculated as

$$\text{OD\_STAIN} = \frac{\sum_{(I,J)\text{ in lesion box}} \text{OD}(I,J)}{\sum_{\substack{(I,J)\text{ in lesion box} \\ \text{OD}(I,J)>\text{OD\_BACK}}}} - \text{OD\_BACK}. \quad (3)$$

The quantity OD_STAIN measures the intensity of staining in cell bodies and their processes. It is normalized by the area circumscribed by the box (sum in denominator of Eq. 3) and thus is independent of the number of neurons in the box. The dominant source of uncertainty in this measurement is the variation in levels of weaker staining associated with cell processes.

*ChAT immunoreactivity.* When reaction product is present on both sides of the section, such as with sections stained with ChAT, two boxes are defined, one that encompasses the RDLN

on the lesioned side and the other that encompasses the RDLN on the intact side (Fig. 6*A*). A measure of staining on the lesioned versus intact side that is largely insensitive to variations in levels of weaker staining associated with cell processes can be estimated. The objects of our measure are the densely stained cell bodies in the RDLN for both lesioned and intact sides. Rather than make use of a fixed background or threshold level, we use a sliding threshold and calculate the optical density above threshold for both RDLN. The ratio of these optical densities, defined as STAIN_RATIO, is expected to be a "step-like," decreasing function of the threshold level with an approximate plateau region at intermediate values of the threshold. Qualitatively, when the threshold is low all stained material contributes to the optical density and the ratio is dominated by the background optical density of the section. In this limit the value of STAIN_RATIO is close to 1 (Fig. 6*B*). On the other hand, when the threshold exceeds the level of weaker staining associated with cell processes only darkly stained cell somata contribute to the signal and the ratio corresponds to the optical density, above weak staining, of somata in lesioned versus intact RDLN. In this limit STAIN_RATIO is a measure of the relative immunoreactivity per neuron on the two sides. When the threshold exceeds the level of the darkest staining on the lesioned side the numerator of (lesioned/intact) goes to zero and so the value of STAIN_RATIO goes to zero. In detail, the algorithm is as follows.

(1) Step the threshold level, denoted THRESHOLD, starting at 0.0 and continuing until its value exceeds the maximum optical density of a pixel in the box surrounding either RDLN. The step size is typically one-half of the SD in OD_BACK, the background level for neuropil outside the RDLN.

(2) Within the box that encompasses the lesioned RDLN, calculate

$$\text{STAIN\_LESION(THRESHOLD)} = \frac{\sum_{\substack{(I,J)\text{ in lesion box} \\ \text{OD}(I,J)>\text{THRESHOLD}}} \text{OD}(I,J)}{\sum_{\substack{(I,J)\text{ in lesion box} \\ \text{OD}(I,J)>\text{THRESHOLD}}}} - \text{THRESHOLD} \quad (4)$$

for each value of THRESHOLD.

(3) Similarly, within the box that encompasses the intact RDLN, calculate

$$\text{STAIN\_INTACT(THRESHOLD)} = \frac{\sum_{\substack{(I,J)\text{ in lesion box} \\ \text{OD}(I,J)>\text{THRESHOLD}}} \text{OD}(I,J)}{\sum_{\substack{(I,J)\text{ in lesion box} \\ \text{OD}(I,J)>\text{THRESHOLD}}}} - \text{THRESHOLD}. \quad (5)$$

(4) Calculate the ratio of staining, STAIN_RATIO, defined as

$$\text{STAIN\_RATIO(THRESHOLD)} = \frac{\text{STAIN\_LESION(THRESHOLD)}}{\text{STAIN\_INTACT(THRESHOLD)}}. \quad (6)$$

(5) Calculate the average value of STAIN_RATIO(THRESHOLD), denoted ⟨STAIN_RATIO⟩, in the plateau region (arrow in Fig. 6B); that is,

$$\langle\text{STAIN\_RATIO}\rangle = \frac{\sum_{\text{THRESHOLD}_{\min}}^{\text{THRESHOLD}_{\max}} \text{STAIN\_RATIO(THRESHOLD)}}{\text{THRESHOLD}_{\max} - \text{THRESHOLD}_{\min}}, \quad (7)$$

where the values of $\text{THRESHOLD}_{\max}$ and $\text{THRESHOLD}_{\min}$ are determined, starting in the vicinity of THRESHOLD = OD_BACK, to minimize the SD of the estimate of the mean across the plateau. The value determined for ⟨STAIN_RATIO⟩ is accepted only if $\text{THRESHOLD}_{\max} - \text{THRESHOLD}_{\min}$ is at least 2.0 times the SD in the background optical density. This difference in threshold levels is an estimate of the width of the plateau and thus is an estimate of the average optical density above threshold for somata on the lesioned side of the cord.

The measures we report in the Results (Fig. 4, Table 1) are either OD_STAIN for sections stained for $p75^{lngfr}$, referred to as p75 (OD), or ⟨STAIN_RATIO⟩ for sections stained for ChAT, referred to as ChAT (R/L). Except for the placement of boxes that circumscribe the RDLN and background region, the analysis of our sections is completely automated.

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