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The Biological Responses of Axotomized Adult Motoneurons to Brain-derived Neurotrophic Factor

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Recent studies showed that brain-derived neurotrophic factor (BDNF) prevents developing motoneurons from naturally occurring and axotomy-induced cell death. Here we examined whether adult motoneurons retain responsiveness to BDNF. Consistent with previous studies, we found that adult spinal and brainstem motoneurons expressed the mRNA of BDNF receptor, *trk*B. In addition, the *trk*B immunoreactivities were readily detected in the adult spinal and brainstem motoneurons. We then demonstrated that axotomized adult motoneurons responded to exogenous BDNF. BDNF administered locally markedly attenuated the lesion-induced decrease of ChAT immunoreactivity and activity and enhanced the lesion-induced reexpression of low-affinity NGF receptor immunoreactivity in adult facial motoneurons. Furthermore, we found BDNF administered subcutaneously, intravenously, and into the cerebral ventricle attenuated the lesion-induced decrease of ChAT immunoreactivity in adult facial motoneurons in a dose-dependent fashion. Our data indicate that adult motoneurons retain their responsiveness to BDNF, suggesting that BDNF may be useful as a therapeutic agent for adult motoneuron disease.

***[Key words:* trk*B* receptor, ChAT, low-affinity NGF receptor, p75, facial nerve, axotomy]**

Although NGF, the prototypical neurotrophin, appears not to promote motoneuron survival (Oppenheim et al., 1982; Miyata et al., 1986; Yan et al., 1988; Oppenheim et al., 1992), recent evidence suggests that BDNF (Leibrock et al., 1989), a member of the neurotrophin family, is a trophic factor for motoneurons. Treatment of chick embryos with BDNF prevents the death of approximately one-third of the motoneurons in the lumbosacral spinal cord that normally die during the period of naturally occurring cell death (Oppenheim et al., 1992). In the same study, BDNF prevents the death of lumbosacral spinal motoneurons induced by the deafferentation of these neurons, achieved by the removal of a few segments of thoracic neural tube at embryonic day 2. In neonatal rats, BDNF applied locally in Gelfoam implants reduces the loss of lumbar spinal motoneurons (Yan et al., 1992) and facial motoneurons (Sendtner et al., 1992; Koliatsos et al., 1993; Yan et al., 1993) induced by nerve transection. It is interesting that although BDNF fails to promote the survival of purified embryonic chick motoneurons *in vitro* (Arakawa et al., 1990), BDNF is shown to be a potent survival-promoting factor for cultured rat motoneurons (Henderson et al., 1993).

It appears that BDNF may act on motoneurons in both target-derived and non-target-derived fashions. Northern blot and *in situ* hybridization studies demonstrate that BDNF mRNA is expressed in limb bud and postnatal muscle (Henderson et al., 1993; Koliatsos et al., 1993). BDNF mRNA expression is upregulated in adult muscle by denervation (Koliatsos et al., 1993). BDNF in muscle can act as a target-derived neurotrophic factor for motoneurons. BDNF-like immunoreactivity has been demonstrated in Schwann cells *in vitro* (Acheson et al., 1991; Meyer et al., 1992), while *in vivo* BDNF levels are barely detectable in Schwann cells. However, its level dramatically increases in Schwann cells in the distal region of axotomized sciatic nerve (Meyer et al., 1992). Thus, Schwann cell–derived BDNF might be an important source of this factor during the regeneration of motoneurons. In addition, BDNF mRNA is expressed by a subset of medium- to large-size DRG neurons in rodents during development (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992). It is possible that such BDNF-expressing neurons are proprioceptive neurons that form monosynapses on motoneurons. Motoneurons may receive BDNF from these afferent fibers. The expression of BDNF by a variety of cells in contact with motoneurons and the profound survival-promoting activity of BDNF on developing motoneurons indicate that BDNF may serve as a motoneuron trophic factor.

The biological effects of neurotrophins are believed to be mediated through specific cell surface receptors. One type of receptor for the neurotrophins has been identified as tyrosine kinase receptors coded by the *trk* family of proto-oncogenes. Studies indicate that NGF specifically interacts with *trk*A (Hempstead et al., 1991; Kaplan et al., 1991; Klein et al., 1991a), BDNF with *trk*B (Klein et al., 1991b; Soppet et al., 1991; Squinto et al., 1991), and NT-3 mainly with *trk*C but, at least in some circumstances, also with *trk*A and *trk*B (Lamballe et al., 1991). The expression of specific *trk* receptors by various cell populations is indicative of a cell's responsiveness to a particular neurotrophin. The fact that motoneurons express *trk*B mRNA (Ernfors et al., 1992; Merlio et al., 1992; Yan et al., 1993) is consistent with the biological responsiveness of developing motoneurons to BDNF (Oppenheim et al., 1992; Sendtner et al., 1992; Yan et al., 1992; Koliatsos et al., 1993). In addition to the *trk* family receptors, all the neurotrophins bind to the low-affinity NGF receptor ($p75^{LNGFR}$) with similar affinity (Rodriguez-Tébar et al., 1990; Hallböök et al., 1991; Soppet et al.,

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1991; Squinto et al., 1991), suggesting that the $p75^{LNGFR}$ may act as a common binding molecule for all neurotrophins. The function of the $p75^{LNGFR}$ in mediating the biological effects of neurotrophins is still not clear.

The dependence of neurons on neurotrophic factors is developmentally regulated. Developing sympathetic and sensory neurons require NGF for survival while the requirement for NGF by these neurons declines with maturity (Coughlin et al., 1977; Thoenen and Barde, 1980; Johnson et al., 1986; Ruit et al., 1990). Similarly, the dependence of motoneurons for survival on their target, which presumably provides the neurotrophic factors for motoneurons, is diminished when animals mature (Crews and Wigston, 1990; Snider et al., 1992). Although BDNF promotes the survival of immature motoneurons, it remains to be determined whether it can influence the function of adult motoneurons. The demonstration of a survival-promoting effect of BDNF on developing motoneurons suggests its potential use in the treatment of amyotrophic lateral sclerosis (ALS), a degenerative motoneuron disease (Williams and Windebank, 1991). However, since ALS is a late-onset disease, it is important to determine if BDNF can affect adult motoneurons in an animal model. In the present study, we showed that adult motoneurons express *trk*B receptor mRNA and protein. In addition to possessing BDNF receptors, we demonstrated that adult motoneurons are capable of responding to exogenous BDNF in a dose-dependent manner. We also compared the differences in the efficacy of BDNF administered by different routes.

Materials and Methods

Materials. Recombinant human BDNF was produced in *Escherichia coli* transfected with a BDNF expression vector and purified by the protein chemistry group at Amgen. The biological activities of BDNF were routinely assessed using a chicken DRG explant assay (Lindsay et al., 1985). Mouse anti-rat low-affinity NGF receptor, $p75^{LNGFR}$ antibody (192-IgG) was a gift from Dr. Eugene M. Johnson. Affinity-purified rabbit anti-rat *trk*B peptide (amino acid sequence 23–36, Middlemas et al., 1991) polyclonal antibody was a gift from Drs. Stuart Feinstein and Monte Radeke. Mouse monoclonal antibody against choline acetyltransferase was purchased from Chemicon (Temecula, CA). Biotinylated affinity-purified horse anti-mouse antibodies, goat anti-rabbit antibodies, and ABC kits were bought from Vector Laboratories (Burlingame, CA). ^{33}P-UTP was purchased from Du Pont/New England Nuclear (Boston, MA). Reagents for the synthesis of riboprobes were obtained from Promega (Madison, WI). Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). All other reagents, unless specified, were obtained from Sigma (St. Louis, MO).

In situ *hybridization and immunohistochemical studies of* trk*B mRNA and protein in adult rat spinal cord.* Adult Sprague–Dawley rats were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains and cervical spinal cords were removed, postfixed in the same fixative for 2 hr at 4°C, and cryoprotected in 30% sucrose in PBS overnight at 4°C.

^{33}P-UTP–labeled antisense and sense riboprobes corresponding to the extracellular domain of rat *trk*B receptors were made with the Promega Riboprobe Kit. The *trk* receptor constructs were gifts from Dr. Jeff Milbrandt and have been described previously (Carroll et al., 1992).

For *in situ* hybridization, brains and spinal cords were cut into 8 μm coronal sections with a cryostat microtome and stored at −20°C until use. The *in situ* hybridization was carried out as previously described (Carroll et al., 1992).

For *trk*B immunohistochemistry, brainstems and cervical spinal cords were cut into 80 μm serial sections. Sections were then processed for immunohistochemistry using affinity-purified rabbit anti-trkB (trkB23-36) or normal rabbit IgG as control at 2 μg/ml and followed by 2 μg/ml secondary biotinylated goat anti-rabbit and the ABC method as described previously (Yan and Johnson, 1988).

Animal surgery and BDNF treatments. Adult rats were anesthetized with a cocktail (43 mg/ml of ketamine hydrochloride, 8.6 mg/ml of xylazine, and 1.43 mg/ml of acepromazine) at a dose of 0.7 ml/kg body weight. The right facial nerve was transected near the stylo-mastoid foramen. For local administration, a 5 × 5 × 3 mm^3 piece of Gelfoam soaked in 2.8 mg/ml of BDNF in PBS (n = 10 for ChAT activity assay and n = 7 for immunohistochemistry) or PBS alone (n = 10 for ChAT activity assay and n = 7 for immunohistochemistry) was implanted into the cut site at the time of surgery. For subcutaneous treatment, BDNF in PBS at the doses of 0, 2, 5, 10, and 20 mg/kg body weight were subcutaneously injected daily behind the neck of rats (n = 4 for each dose). For intravenous administration, animals were cannulated through the jugular vein with PE 50 tubing at the time of facial nerve lesion and were treated daily with BDNF in PBS at concentrations of 0, 2, 5, 10, and 20 mg/kg body weight (n = 4 for each dose). For the intracerebral ventricular administration study, a catheter was stereotaxically implanted into the right lateral ventricle of the brain (0.9 mm posterior to the bregma, 1.5 mm lateral to the midline, and 4.0 mm from the surface of the skull) (Paxinos and Watson, 1986) at the time of facial nerve lesion. The catheter was attached to a miniosmotic pump with an infusion rate of 0.1 μl/hr (model 2002) (Alzet, Palo Alto, CA). The miniosmotic pumps were filled with BDNF in PBS at the concentrations of 0, 0.2, 1, and 5 mg/ml (n = 4 for each dose). The miniosmotic pump was implanted subcutaneously according to manufacturer's instructions. Animals from all experiments were killed 7 d after the facial nerve transection.

ChAT activity assay. The effect of BDNF on the ChAT activity of lesioned adult facial motoneurons was assessed in rats receiving local BDNF treatment. Rats were killed by CO_2 inhalation, and their brains were removed. A 1.7-mm-thick slice of the brainstem containing the facial nucleus was cut out with a tissue chopper according to a brain atlas (Paxinos and Watson, 1986) and ipsilateral and contralateral nuclei were dissected out. Tissues were sonicated and then centrifuged at 16,000 × g for 10 min at 4°C. The supernatants were collected and the protein contents of individual samples were determined by the Pierce protein assay (Pierce, Rockford, IL). Samples were normalized for protein content and assayed for ChAT activity in triplicate according to the published protocol (Fonnum, 1975). Since the ChAT activities of nonlesioned nuclei of both PBS (47.13 ± 4.64, pmol/min/mg protein) and BDNF (45.72 ± 4.43, pmol/min/mg protein) treatments were the same, the results were expressed as a ratio of lesioned over control facial nuclei. Data were analyzed statistically with a two-tailed Student's t test.

ChAT and 192-IgG immunohistochemistry. Rats were killed by an overdose of anesthesia and perfused transcardially with PBS followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The brainstems were removed, cryoprotected with 30% sucrose in PBS, and frozen onto a sliding microtome chuck, and 80 μm serial coronal sections were cut through the facial nucleus region and collected into four sets (three to five sections per set). Sections were then processed for immunohistochemistry with either mouse monoclonal antibody against ChAT (ascites, 1:500), mouse monoclonal antibody (192-IgG) against $p75^{LNGFR}$ (2 μg/ml), or a non-sense mouse myeloma IgG followed by 2 μg/ml of secondary biotinylated horse anti-mouse and the ABC method (Yan and Johnson, 1988).

Quantification of immunohistochemical sections. A Quantimet 520 image analyzer (Leica, Inc., Deerfield, IL) coupled to a Nikon Optiphot-FXA microscope was used to quantify the relative intensity of ChAT and 192-IgG staining. A 510 nm narrow-band pass filter (Oriel Corp., Stratford, CT) was used with a Nikon-Plan Apochromatic 2× objective lens to produce high-contrast images of the facial nucleus region in histology sections. The relative intensity of ChAT immunoreactivity was determined by obtaining the mean gray scale intensity for each outlined nucleus minus the background staining of adjacent ChAT negative gray matter. The relative intensity of 192-IgG staining was determined by obtaining the mean gray scale intensity for each outlined nucleus without the subtraction of the adjacent non-facial-nucleus gray matter, since the nonlesioned facial nucleus was 192-IgG negative and had the same optical intensity as that of adjacent gray matter. Between two and five sections of the facial nucleus per animal (average of 3.2 sections per animal) were used for the quantification. Since BDNF treatments via local, systemic, and intracerebroventricular routes did not affect the ChAT and 192-IgG immunostaining of the nonlesioned facial nucleus, the data were expressed as the ratio of the relative optical density of lesioned over nonlesioned facial nuclei of same sections. The data were statistically analyzed by ANOVA followed by the Dunnett t test for a given route of administration or by a two-tailed Student's t

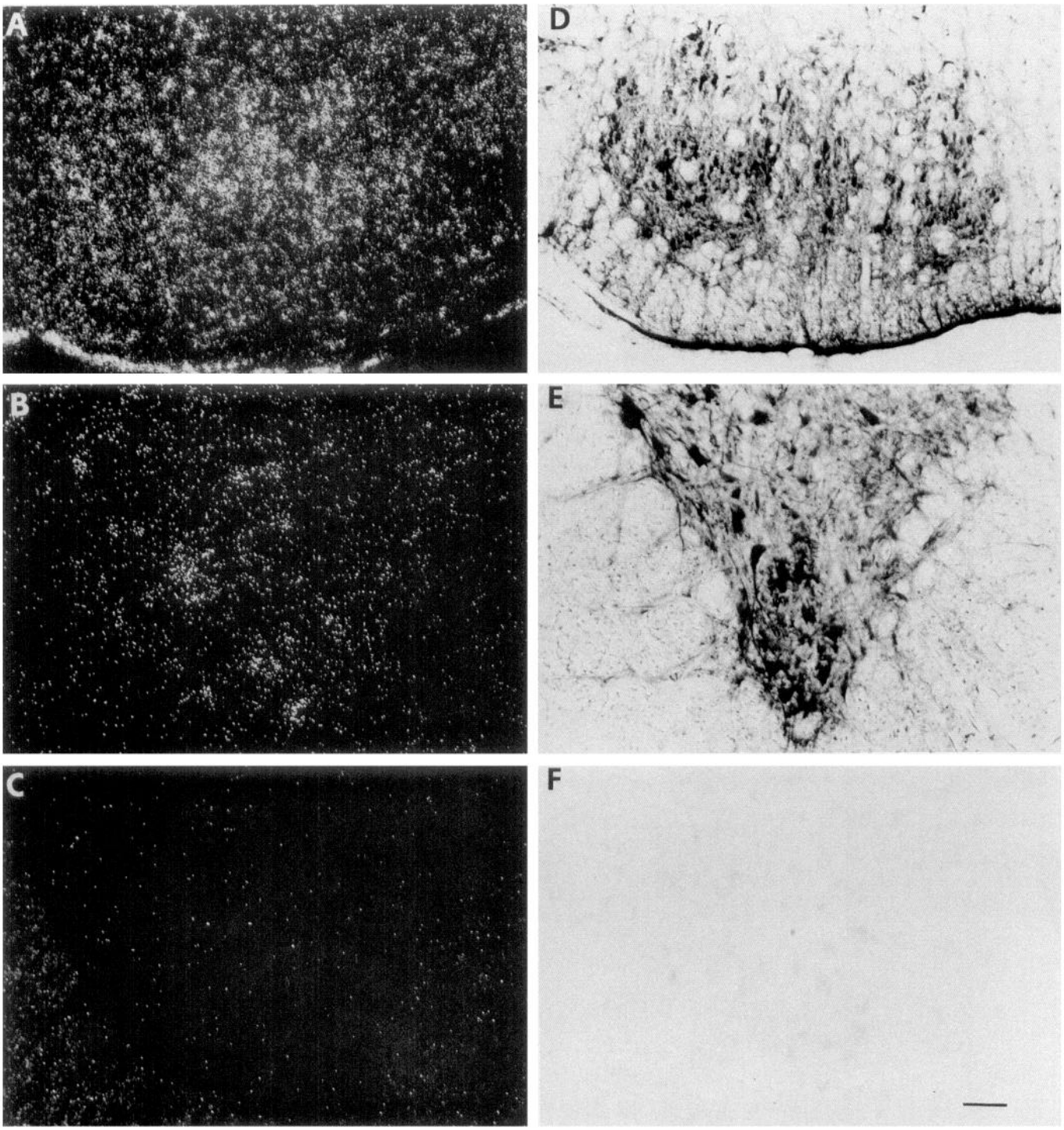


Figure 1. Expression of *trk*B in adult motoneurons. The expression of *trk*B by motoneurons was studied by *in situ* hybridization (*A–C*, dark-field photographs) and immunohistochemical (*D–F*, bright-field photographs) techniques. Motoneurons in the facial nucleus (*A*) and spinal cord (*B*) were positively labeled by an antisense riboprobe to *trk*B. There was no labeling of motoneurons by a corresponding sense riboprobe (*C*). Motoneurons in the facial nucleus (*D*) and spinal cord (*E*) were also positively stained by affinity-purified rabbit anti-rat *trk*B polyclonal antibodies. The staining was localized to the motoneuron soma and processes. Replacement of the primary antibodies with a control rabbit IgG resulted in no staining of the motoneurons of the spinal cord (*F*). Scale bar (in *F*): 150 μm for *A* and *D*, 375 μm for *B, C, E,* and *F*.

test for the same dose of BDNF by different route of administration (subcutaneous vs intravenous).

Results

Adult motoneurons express BDNF receptor, trk*B*

Developing and adult spinal motoneurons have been shown to express *trk*B mRNA (Ernfors et al., 1992; Merlio et al., 1992; Yan et al., 1993). In an extension of these findings, high levels of *trk*B mRNA were detected in other populations of adult motoneurons including facial (Fig. 1*A*) and hypoglossal motoneurons (not shown). Comparable high levels of expression were seen in adult spinal cord motoneurons (Fig. 1*B*). Consistent with these *in situ* hybridization results, facial and spinal motoneurons of adult rats were positively stained with affinity-purified rabbit

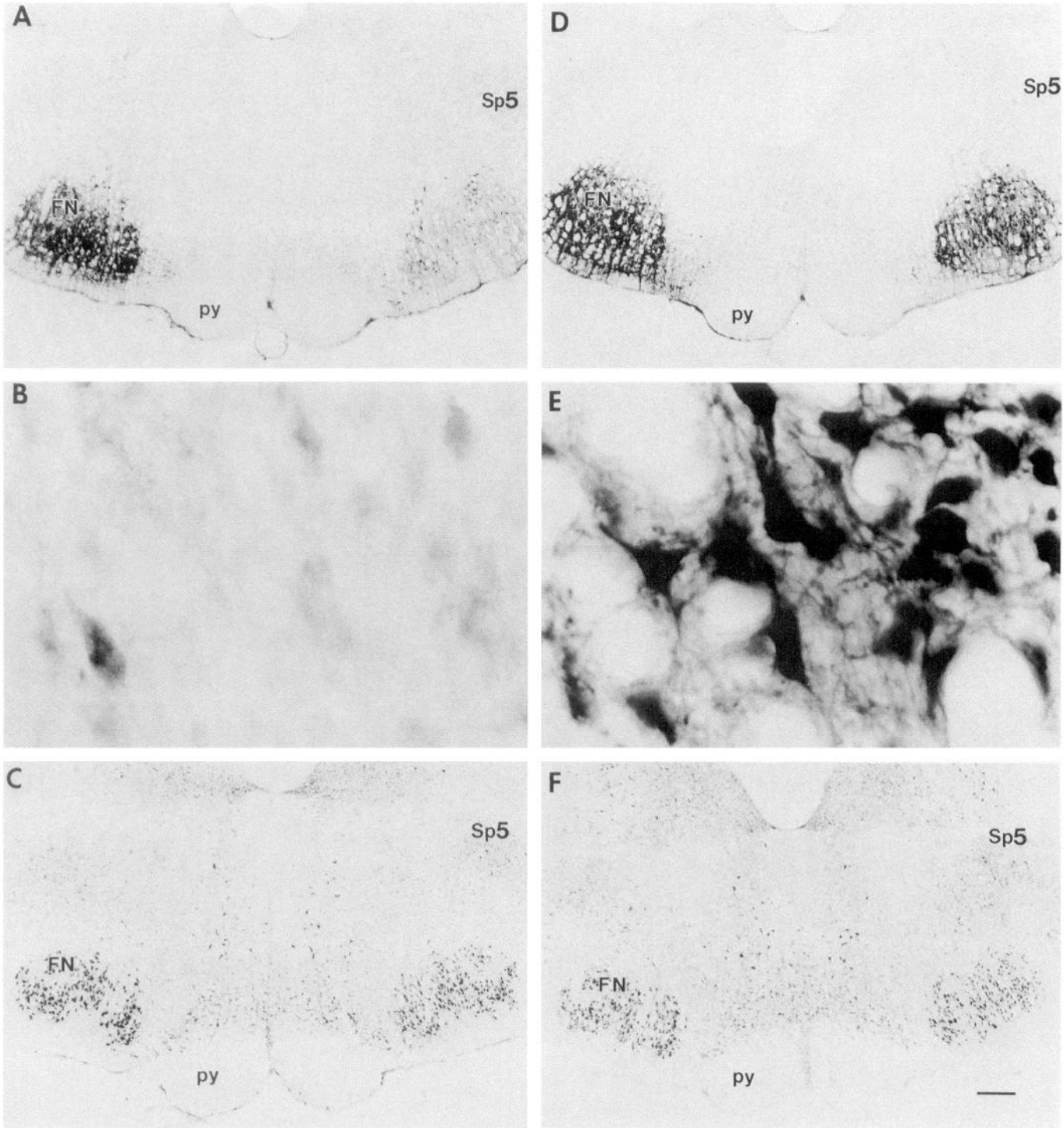


Figure 2. ChAT immunohistochemistry and Nissl staining of axotomized facial motoneurons. The right facial nerve was transected and animals were locally treated with either PBS (*A–C*) or BDNF (*D–F*) for 7 d. In the PBS-treated rats, axotomy resulted in a large drop in ChAT immunoreactivity in the lesioned facial nucleus (*A*). BDNF treatment largely attenuated the lesion-induced drop of ChAT immunoreactivity (*D*). The staining on individual motoneurons after the BDNF treatment (*E*) was much higher than those receiving PBS treatment (*B*). Nissl staining of adjacent sections of either PBS- (*C*) or BDNF- (*F*) treated rats indicated that the change in ChAT immunoreactivity was not due to the loss of neuronal cell bodies after axotomy. *FN*, facial nucleus; *py*, pyramidal tract; *Sp5*, nucleus of spinal tract of trigeminal. Scale bar (in *F*): 500 μm for *A, C, D,* and *F*; 50 μm for *B* and *E*.

polyclonal anti-trkB peptide antibodies (Fig. 1*D,E*). Staining was localized to motoneuron soma as well as dendrites. In addition to the motoneurons, there were many other cells expressing *trk*B in the brainstem and the spinal cord of adult rats. However, motoneurons were among the highest *trk*B mRNA–expressing and immunoreactive cells in the adult CNS.

Effects of locally administered BDNF on axotomized facial motoneurons

Axotomy in adult rats causes a rapid and reproducible decrease of ChAT immunoreactivity in motoneurons (Lams et al., 1988; Armstrong et al., 1991). In addition, axotomy induces the re-

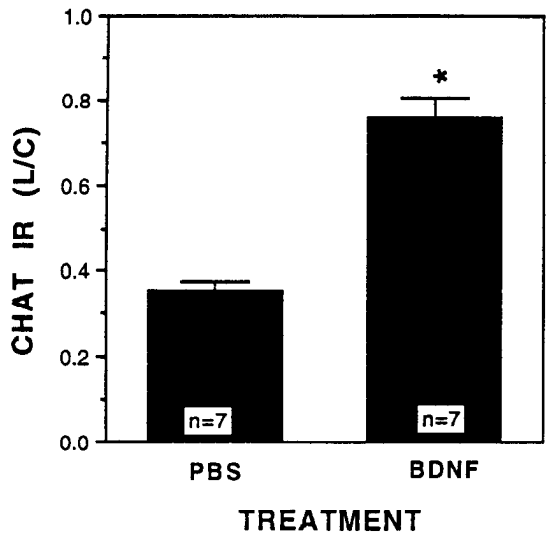


Figure 3. Quantification of ChAT immunoreactivity of the facial nucleus after local administration of PBS or BDNF for 7 d. Values are means ± SEM ($n = 7$). BDNF significantly attenuated the axotomy-induced decrease of ChAT immunoreactivity in the lesioned facial nucleus (*, $p < 0.0001$, two-tailed Student t test).

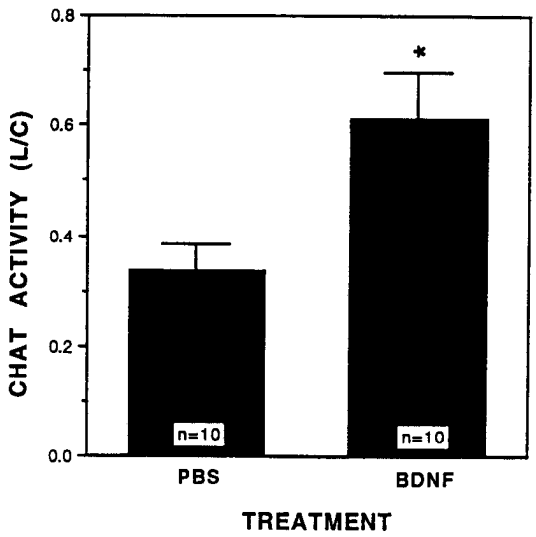


Figure 4. Effect of locally administered BDNF (7 d) on ChAT activity of axotomized facial nucleus. Values are means ± SEM ($n = 10$). BDNF significantly attenuated the axotomy-induced decrease of ChAT activity in the lesioned facial nucleus (*, $p < 0.0001$, two-tailed Student t test).

expression of $p75^{LNGFR}$ in adult motoneurons (Ernfors et al., 1989; Wood et al., 1990; Armstrong et al., 1991; Koliatsos et al., 1991), which is normally expressed only transiently by developing motoneurons (Yan and Johnson, 1988). To evaluate the effects of BDNF on adult motoneurons, we used a facial nerve transection paradigm to study its influence on the expression of ChAT and $p75^{LNGFR}$.

Seven days after right facial nerve transection, ChAT immunoreactivity largely disappeared from the lesioned facial nucleus receiving PBS treatment (Fig. 2*A*). Staining on a few remaining ChAT-positive facial motoneurons was quite diffuse (Fig. 2*B*). BDNF locally applied in a Gelfoam implant to the proximal stump of the cut facial nerve largely attenuated the lesion-induced decrease of ChAT immunoreactivity (Fig. 2*D*). The ChAT immunoreactivity on individual axotomized motoneurons after BDNF treatment (Fig. 2*E*) was much higher than those receiving PBS treatment (Fig. 2*B*) and approached the level of contralateral, nonlesioned motoneurons. Nissl staining of adjacent sections revealed that the disappearance of ChAT-positive cells in the lesioned facial nucleus was not due to the loss of neurons (Fig. 2*C*,*F*, PBS and BDNF treated, respectively), confirming previous reports (Lams et al., 1988; Armstrong et al., 1991).

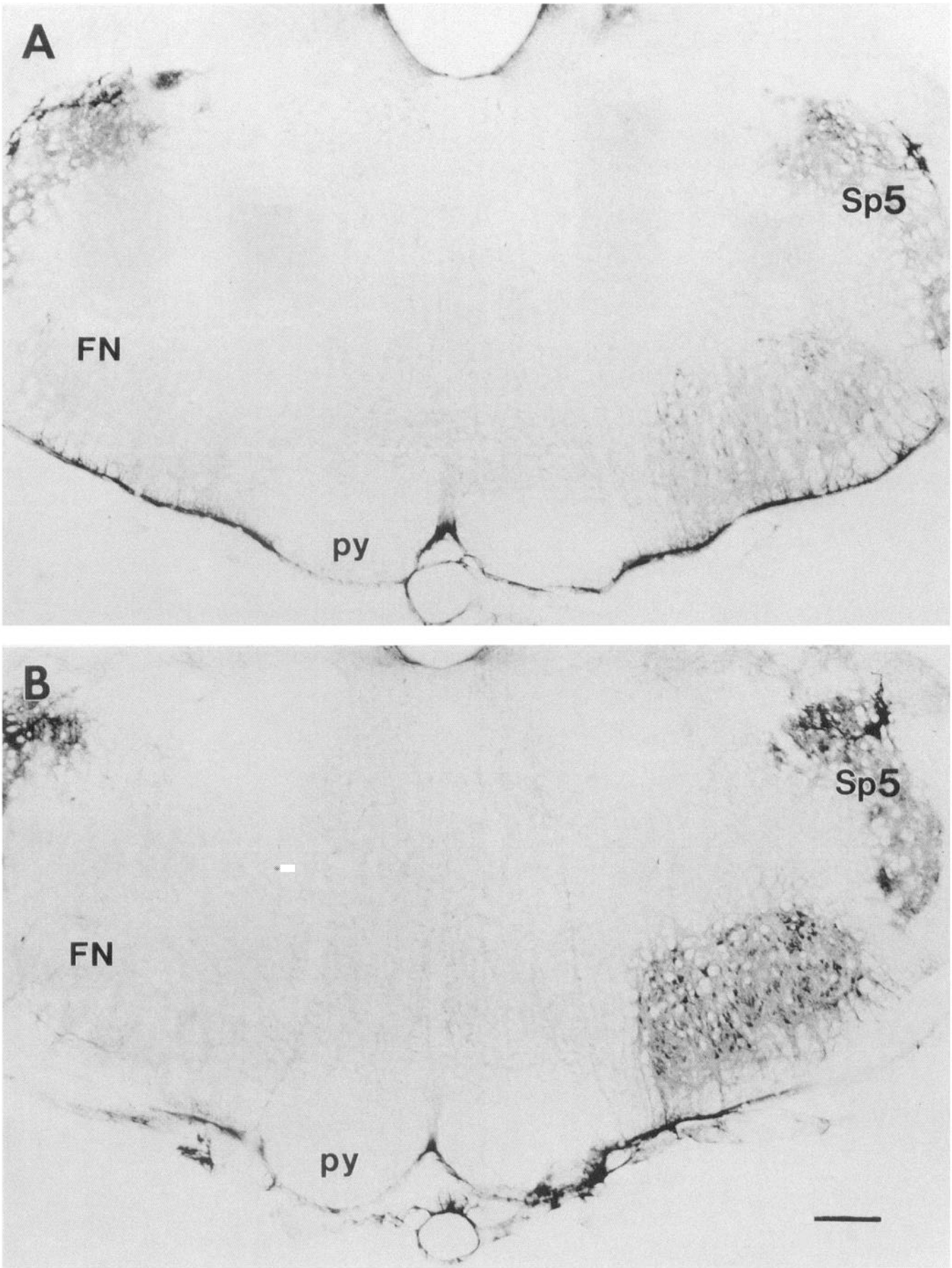
To quantify this phenomenon, the average optical densities of both lesioned and nonlesioned facial nuclei of ChAT-immunostained sections were measured. Since locally applied BDNF did not influence the ChAT immunoreactivity of nonlesioned motoneurons (Fig. 2*D*), the result was expressed as the ratio of the gray scale value of lesion over control. Only 35% of the ChAT immunoreactivity remained in lesioned facial nuclei in PBS-treated animals. Locally applied BDNF resulted in the retention of 76% of the ChAT immunoreactivity in the lesioned facial nucleus, a twofold improvement compared to PBS-treated controls (Fig. 3).

To verify the protective effect of BDNF on ChAT immunoreactivity of lesioned motoneurons, the ChAT activities of dissected lesioned and nonlesioned facial nuclei from animals treated with locally applied PBS or BDNF were measured. The lesion caused a large decrease of ChAT activity (34% of nonlesion) and BDNF treatment could attenuate this decrease (62% of nonlesion) in the facial nucleus (Fig. 4). As found in our ChAT immunohistochemistry studies, BDNF treatment markedly attenuated the decrease of ChAT activity but did not stimulate its level over that of nonlesioned motoneurons.

In the adult facial nucleus, axotomy induced an upregulation of $p75^{LNGFR}$ immunoreactivity from a nondetectable to a detectable level (Fig. 5*A*). BDNF locally applied to the proximal stump of the cut facial nerve for 7 d further potentiated the lesion-induced reexpression of $p75^{LNGFR}$ immunoreactivity (Fig. 5*B*). The quantification of $p75^{LNGFR}$ immunoreactivity by measuring the average optical densities of facial nuclei is shown in Figure 6. Lesion plus PBS treatment resulted in a slight increase of $p75^{LNGFR}$ immunoreactivity (119% of nonlesion, not statistically significant) and BDNF treatment significantly augmented this reexpression of $p75^{LNGFR}$ immunoreactivity (207% of nonlesion).

Comparison of the effectiveness of different routes of BDNF administration

The results presented above demonstrate a significant effect of BDNF when administered locally at the site of axotomy on adult motoneurons. We next determined whether systemic or intracerebroventricular administration of BDNF could influence axotomized motoneurons in a dose-dependent fashion. Since ChAT activity measurements were less sensitive and more variable than the measurements of the average optical densities of ChAT immunoreactivity of stained sections, the latter method was used to quantify the effects of axotomy and BDNF treatment. The ChAT immunoreactivities of the left, nonlesioned facial nuclei of animals treated with PBS or BDNF at different doses and via different routes did not differ significantly (relative optical densities of PBS treatment vs BDNF treatments of all doses pooled: 99.0 vs 95.6 for subcutaneous; 100.3 vs 101.7 for intravenous, and 97.0 vs 94.2 for intracerebroventricular). The left facial nucleus was therefore used as a internal control, and the data were expressed as the ratio of lesion over control.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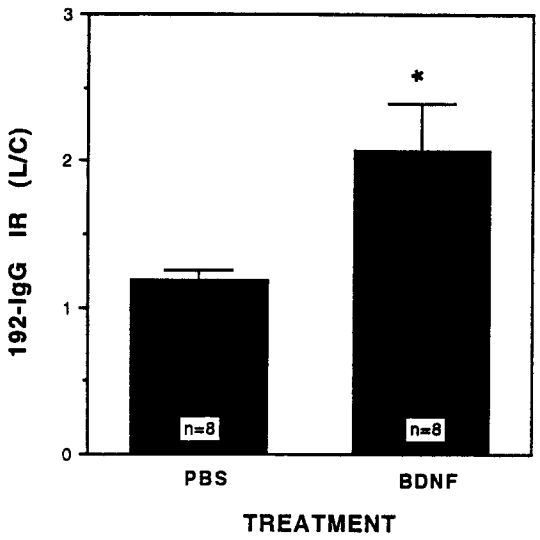

Figure 6. Quantification of 192-IgG immunoreactivity of the facial nucleus in rats that received local administered PBS or BDNF for 7 d. Values are means ± SEM (n = 8). BDNF significantly enhanced the axotomy-induced increase of 192-IgG immunoreactivity in the lesioned facial nucleus (*, $p < 0.016$, two-tailed Student t test).

The results of daily systemic administration for 7 d by either subcutaneous or intravenous routes along with intracerebroventricular infusion of BDNF for 7 d on ChAT immunoreactivity of axotomized facial nuclei are summarized in Figure 7. Both subcutaneously and intravenously administered BDNF reduced the lesion-induced decrease of ChAT immunoreactivity (Fig. 7*A*) in a dose-dependent fashion. At a dose of 2 mg/kg body weight, intravenously administered BDNF was significantly better than subcutaneously administered BDNF in attenuating the lesion-induced decrease of ChAT immunoreactivity ($p < 0.001$, two-tailed Student t test). At higher doses, these two routes of systemic administration resulted in similar ChAT staining levels and reached the efficacy of locally applied BDNF (Fig. 3). BDNF infused intracerebroventricularly also attenuated the lesion-induced decrease of ChAT immunoreactivity in the facial nucleus in a dose-dependent fashion. Much lower daily doses of BDNF administered by intracerebroventricular infusion were needed versus systemic administration. The maximum protective effect of BDNF by intracerebroventricular administration was achieved at a dose of 60 μg/d (Fig. 7*B*) and was similar to the results of locally applied BDNF (Fig. 3) and systemically applied BDNF at the high doses (Fig. 7*A*).

Effect of BDNF on animal weight gain after different routes of administration

The potential side effects of BDNF treatment on animals by different routes of administration were evaluated by monitoring animals' weight gain during these experiments (Table 1). Animals receiving BDNF subcutaneous treatment at all the dosages did not show significantly different weight gain compared to

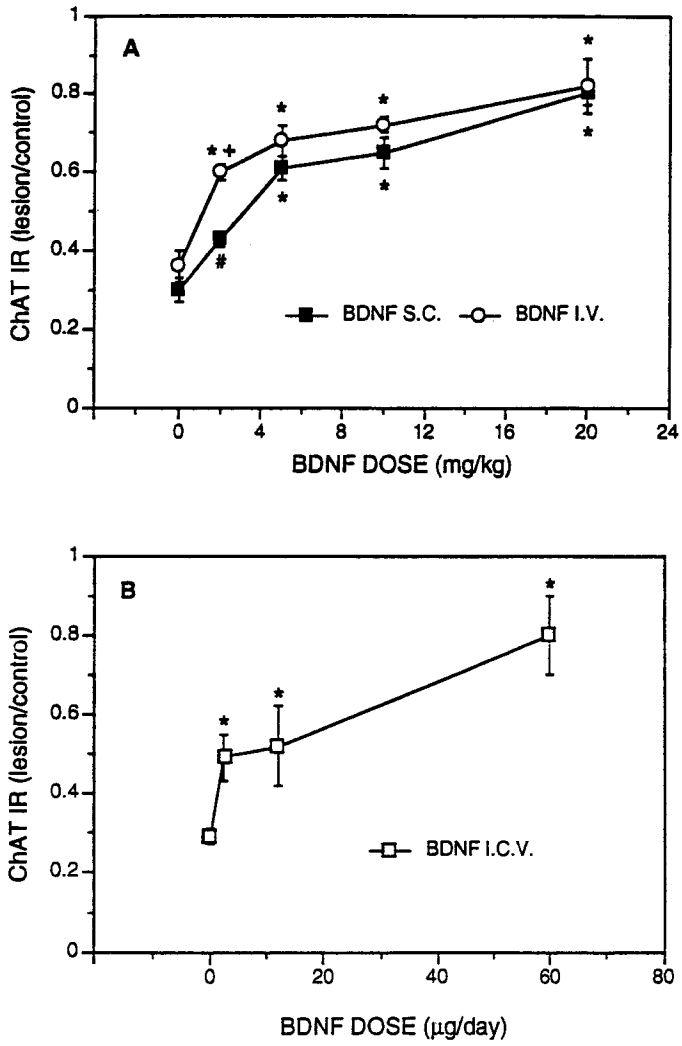

Figure 7. Dose–response curve of systemicly (*A*) or intracerebroventricularly (*B*) administered BDNF on ChAT immunoreactivity of the lesioned facial nucleus. *A*, Animals received daily injections of BDNF either subcutaneously (*S.C.*, ■) or intravenously (*I.V.*, ○) at the doses indicated for 7 d. Systemically administered BDNF by both routes resulted in attenuation of the lesion-induced decrease of ChAT immunoreactivity in a dose-dependent fashion. Values are means ± SEM (n = 4). Data were analyzed by ANOVA followed by Dunnett t test (*, $p < 0.01$; #, $p < 0.05$, BDNF vs control). At the dose of 2 mg/kg, BDNF administered intravenously was more effective than BDNF administered subcutaneously (+, $p < 0.001$, two-tailed Student t test). There were no significant differences in ChAT immunoreactivity at higher doses of BDNF when we compared these two different routes of administration. *B*, Animals received BDNF continuously infused into the lateral ventricle (*I.C.V.*) through miniosmotic pumps at the doses indicated for 7 d. BDNF administered intracerebroventricularly resulted in attenuation of the lesion-induced decrease of ChAT immunoreactivity in a dose-dependent fashion. Values are means ± SEM (n = 4). Data were analyzed by ANOVA followed by Dunnett t test (*, $p < 0.01$, BDNF vs control).

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Figure 5. 192-IgG immunohistochemistry of the axotomized facial motoneurons. The right facial nerve was transected and animals were locally treated with either PBS (*A*) or BDNF (*B*) for 7 d. In the PBS-treated rats, axotomy resulted in a detectable increase of 192-IgG staining in the lesioned facial nucleus. The treatment of BDNF augmented the lesion-induced upregulation of 192-IgG immunoreactivity. There was no detectable staining in the left, nonlesioned facial nucleus. 192-IgG also stained the nucleus of spinal tract of trigeminal (*Sp5*), which is known to be $p75^{LNGFR}$ positive (Yan and Johnson, 1988). *FN*, facial nucleus; *py*, pyramidal tract; *Sp5*, nucleus of spinal tract of trigeminal. Scale bar, 500 μm.

Table 1. The effect of different routes of BDNF administration on animal body weight

Route	Dose	Day 0 (mean, gm)	Day 7 (mean, gm)	% gain (or loss)
s.c.	0.0	200.75 ± 7.11	218.75 ± 10.29	8.88 ± 1.97
(mg/kg)	2.0	200.25 ± 6.30	213.50 ± 7.50	6.61 ± 1.49
	5.0	210.00 ± 3.56	221.25 ± 3.07	5.38 ± 0.91
	10.0	192.25 ± 1.38	204.50 ± 3.84	6.35 ± 1.29
	20.0	217.25 ± 8.39	228.75 ± 6.61	5.41 ± 1.06
i.v.	0.0	197.67 ± 2.03	216.00 ± 2.31	9.27 ± 0.10
(mg/kg)	2.0	194.00 ± 3.37	207.50 ± 6.06	6.92 ± 1.99
	5.0	208.50 ± 8.70	216.25 ± 9.42	3.94 ± 4.05
	10.0	201.00 ± 5.34	204.00 ± 5.80	1.48 ± 0.44
	20.0	207.50 ± 7.03	195.50 ± 14.79	(−5.69 ± 6.65)*
i.c.v.	0.0	264.25 ± 7.50	270.00 ± 6.10	2.23 ± 0.80
(μg/day)	2.4	284.50 ± 9.00	254.75 ± 11.37	(−10.35 ± 3.73)**
	12.0	251.00 ± 10.35	207.25 ± 8.44	(−17.37 ± 1.56)**
	60.0	268.00 ± 10.66	216.00 ± 14.91	(−19.52 ± 3.88)**

Body weights of each individual rat ($n = 4$ for all groups) were measured daily, beginning on day 0 (prior to surgery) and ending on day 7 (prior to perfusion); only those weights of day 0 and of day 7 are shown. *, $p < 0.05$; **, $p < 0.01$ compared with the corresponding values of vehicle treated animals (one-way ANOVA followed by Dunnett t test).

animals receiving PBS treatment. In contrast, animals receiving BDNF intravenously showed a dose-dependent reduction in weight gain. At 5 mg/kg and 10 mg/kg, animals showed a tendency to gain less weight (not statistically significant due to the small number of animals used) while those receiving 20 mg/kg significantly lost weight ($p < 0.05$). Animals receiving BDNF intracerebroventricular treatment at all doses had a significant weight loss in a dose-dependent fashion.

Discussion

In an extension of our previous finding that BDNF functions as a survival-promoting factor for developing motoneurons (Oppenheim et al., 1992; Yan et al., 1992), we found in this study that adult motoneurons remain biologically responsive to BDNF. Specifically, BDNF treatment attenuated the decrease of ChAT immunoreactivity and activity in adult facial motoneurons induced by axotomy. Furthermore, we found that exogenous BDNF augmented the lesion-induced reexpression of $p75^{LNGFR}$ in adult facial motoneurons.

We have shown here that adult brainstem and spinal motoneurons express *trk*B mRNA and protein. These findings are in agreement with those of previous *trk*B *in situ* hybridization results (Ernfors et al., 1992; Merlio et al., 1992; Yan et al., 1993). Spinal motoneurons express *trk*B mRNA as early as E13 and continue into adulthood (Yan et al., 1993). This continued expression of the BDNF receptor by motoneurons suggests a capacity to respond to BDNF from early development through to adulthood. The *trk*B gene encodes at least three cell surface receptors, including a full-length form and two truncated forms (Klein et al., 1990; Middlemas et al., 1991). The full-length form with the intracellular tyrosine kinase catalytic domain apparently mediates the biological activity of BDNF. In the present study, a riboprobe corresponding to the extracellular domain of *trk*B was used for *in situ* hybridization. The antibody used in the immunohistochemistry was also directed against *trk*B extracellular domain and would detect both truncated and full-length *trk*B receptors. Thus, it could not be determined whether motoneurons express only one form or both full-length and truncated forms. The demonstration of *trk*B immunoreactivity is consistent with the fact that motoneurons are capable of retrogradely transporting BDNF in a receptor-mediated fashion (DiStefano et al., 1992; Yan et al., 1992, 1993). The presence of *trk*B protein on motoneuron dendrites suggests that motoneurons may receive BDNF from within the brainstem or spinal cord in addition to their muscle target (Henderson et al., 1993; Koliatsos et al., 1993) and Schwann cells (Acheson et al., 1991; Meyer et al., 1992). Since the level of BDNF mRNA present in the adult spinal cord and brainstem is relatively low (Maisonpierre et al., 1990), the major CNS source of BDNF for motoneurons may be derived from the monosynaptic afferent fibers of a subset of sensory neurons that express high levels of BDNF mRNA (Ernfors and Persson, 1991; Schecterson and Bothwell, 1992).

The dependence of motoneurons on their targets and neurotrophic factors decreases as animals mature. Unlike neonatal rats (Schmalbruch, 1984; Snider et al., 1992; Yan et al., 1992), axotomy of motor nerves in the adult does not cause motoneuron cell death (Lams et al., 1988). In order to determine whether BDNF can influence adult motoneurons, we examined whether BDNF could prevent the axotomy-induced decrease of ChAT immunoreactivity and activity (Lams et al., 1988; Armstrong et al., 1991), and prevent the axotomy-induced increase of $p75^{LNGFR}$ immunoreactivity (Ernfors et al., 1989; Wood et al., 1990; Koliatsos et al., 1991) in facial motoneurons. We found that exogenous BDNF could markedly attenuate the axotomy-induced decrease of ChAT immunoreactivity and activity and enhance the reexpression of $p75^{LNGFR}$.

It is interesting that although adult injured motoneurons readily responded to BDNF, BDNF did not stimulate the ChAT activity or immunoreactivity over the level of nonlesion motoneurons. In addition, systemic and intracerebroventricular administration of BDNF did not influence the ChAT levels of the nonlesioned motoneurons. Previously, we found that in neonatal rats, BDNF had no protective effect on the ChAT activity of lesioned spinal motoneurons, although it was effective in protecting neonatal motoneurons from axotomy-induced cell death (Yan et al., 1993). It is likely that, in neonatal rats, BDNF provides a replacement of target-derived neurotrophic factor(s) for the ax-

otomized motoneurons for their survival but BDNF is not sufficient for the maintenance of the expression of ChAT. In contrast, axotomized adult motoneurons do not depend on trophic factor for survival but they apparently need trophic factor, such as BDNF, for the maintenance of the expression of ChAT. The dissociation of a protective effect and a neurotransmitter enzyme stimulation effect of BDNF on motoneurons is markedly different from the effects of NGF on its responsive neurons. NGF has been shown to promote neuronal survival and stimulate neurotransmitter enzyme synthesis in normal as well as lesioned sympathetic (Thoenen et al., 1971; Hendry and Campbell, 1976; Kessler and Black, 1980) and medial septal cholinergic neurons (Hefti et al., 1984; Hagg et al., 1989; Higgins et al., 1989). The difference in neurotrophic effects of BDNF and NGF on their responsive neurons could arise from differences in the signal transduction pathways or intrinsic differences in their responsive neurons. It is interesting to note that BDNF stimulates the ChAT activity of cultured motoneurons over controls (Henderson et al., 1993; Wong et al., 1993). Possible explanations for the discrepancy between our data and the *in vitro* studies include (1) the effect of BDNF on ChAT activity of cultured motoneurons may partially result from its survival-promoting effect on cultured motoneurons (more neurons and therefore more ChAT) and partially result from its effect to prevent the decrease of ChAT activity of cultured motoneurons that are axotomized during the preparation, (2) the differences in the ages of animals studied (embryonic vs postnatal), (3) differences between the *in vitro* and *in vivo* environment.

$p75^{LNGFR}$ is expressed by motoneurons only during development and has largely disappeared by postnatal day 10 in rats (Yan and Johnson, 1988; Ernfors et al., 1989). In adult rats, lesion causes reexpression of $p75^{LNGFR}$ in motoneurons (Ernfors et al., 1989; Wood et al., 1990; Armstrong et al., 1991; Koliatsos et al., 1991). Here we show that in addition to its protective effect on ChAT, BDNF enhances the reexpression of $p75^{LNGFR}$ in axotomized motoneurons. This enhancement effect of BDNF on the $p75^{LNGFR}$ expression is unexpected since we argued that supplying exogenous BDNF would move the motoneurons back toward their normal, resting, intact state. This is not the case. BDNF attenuates the decrease in ChAT immunoreactivity (normal state), but augments the increase in $p75^{LNGFR}$ (abnormal state). This indicates that the transduction mechanisms that act on these two parameters in motoneurons are effected very differently by BDNF. The biological role of $p75^{LNGFR}$ is not clear at present. The upregulation of its expression following injury by motoneurons and other cells may contribute to the process of regeneration (Johnson et al., 1988). $p75^{LNGFR}$ may also mediate effects of BDNF, since it is a BDNF binding protein. The potential role of $p75^{LNGFR}$ in mediating the effects of BDNF on normal and lesioned motoneurons may be evaluated in the recently developed $p75^{LNGFR}$ knock-out mice (Lee et al., 1992).

In an effort to find a route of administration of BDNF that will be most efficient in affecting motoneurons, we compared subcutaneous, intravenous, and intracerebroventricular administration. All three routes of administration attenuated the decrease of ChAT in lesioned facial motoneurons in a dose-dependent fashion. The highest doses of BDNF administered by subcutaneous or intravenous routes resulted in similar maximal effects on lesioned motoneurons. At lower doses, intravenous administration was more efficient. The difference between subcutaneous and intravenous delivery at the lower doses likely resulted from the lower bioavailability of BDNF from a subcutaneous bolus injection, and this was apparently compensated for at higher doses of BDNF. Due to the technical difficulties associated with intrathecal administration, in which drug solutions are introduced into the subarachnoid space, we chose to deliver BDNF via intracerebroventricular administration. Since very little BDNF injected intracerebroventricularly can penetrate brain parenchyma (Yan et al., 1994), the bulk of BDNF should be carried by cerebral spinal fluid through the cerebral ventricular system into the subarachnoid space. Therefore, for application of BDNF to brainstem motoneurons, intracerebroventricular administration should resemble intrathecal administration. Since BDNF administered intracerebroventricularly was able to attenuate the lesion-induced decrease of ChAT immunoreactivity in the facial nucleus in a dose-dependent fashion, this study suggests that intrathecal administration of BDNF will also be effective in affecting spinal and brainstem motoneurons. Axotomized motoneurons can be protected by BDNF via a peripheral mechanism (e.g., mediated by BDNF receptors on nerve terminals and on motor axons), since a small amount of BDNF administered locally is effective. Although it is likely that systemically administered BDNF acts on lesioned motoneurons through such a peripheral mechanism, due to the large quantity of BDNF used for the systemic administration, one cannot completely rule out the possibility that a small amount of circulating BDNF could leak into the brain and protect motoneurons directly. When administered intracerebroventricularly, BDNF may affect the motoneurons by bathing the motor nerves within the subarachnoid space (similar to the peripheral mechanism) or by penetrating into the brain parenchyma from the surface of the brainstem and directly acting on motoneuron cell bodies. It is unlikely that BDNF injected intracerebroventricularly could affect motoneurons systemically because a similar dose of BDNF has no effect if administered systemically.

We found BDNF injected intracerebroventricularly caused animals to lose weight in a dose-dependent manner as shown previously for NGF (Williams, 1991). The weight loss probably results from reduced food intake (Lapchak and Hefti, 1992; Pelleymounter and Cullen, 1993). Although the underlying mechanism of weight loss by BDNF and NGF injected intracerebroventricularly is not known, it is speculated that direct or indirect activation of the hypothalamus by high dose of BDNF and NGF injected intracerebroventricularly may affect animals' eating behavior (Williams, 1991; Pelleymounter and Cullen, 1993). If the effect of BDNF injected intracerebroventricularly on body weight is mediated by the hypothalamus or other structures adjacent to the ventricles, it may be avoided or reduced by intrathecal administration of BDNF into the lumber or cerebellomedullary cistern. Because of the flow of CSF, the intrathecally administered BDNF would not gain access to brain structures along the ventricles. High doses of BDNF administered intravenously also had a clear negative effect on weight gain. It is possible that peripherally injected BDNF can penetrate into brain areas that lack a blood–brain barrier such as the circumventricular organs (Weindl and Sofroniew, 1978). Whether cells in circumventricular organs express *trk*B and whether the weight loss caused by intravenous administration of BDNF is mediated by CNS mechanisms remain to be studied.

In summary, we found that adult brainstem and spinal motoneurons express *trk*B receptor mRNA and protein. Local, systemic, and intracerebroventricular administration of BDNF effectively attenuated the axotomy-induced decrease of ChAT and enhanced the axotomy-induced reexpression of $p75^{LNGFR}$ in adult

motoneurons. This and other studies provide a strong rationale for the potential clinical use of BDNF for the treatment of motoneuron degenerative diseases, such as ALS.

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