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Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor

(avulsion/spinal cord/amyotrophic lateral sclerosis)

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ABSTRACT **Glial cell line-derived neurotrophic factor (GDNF) has been shown to rescue developing motoneurons *in vivo* and *in vitro* from both naturally occurring and axotomy-induced cell death. To test whether GDNF has trophic effects on adult motoneurons, we used a mouse model of injury-induced adult motoneuron degeneration. Injuring adult motoneuron axons at the exit point of the nerve from the spinal cord (avulsion) resulted in a 70% loss of motoneurons by 3 weeks following surgery and a complete loss by 6 weeks. Half of the loss was prevented by GDNF treatment. GDNF also induced an increase (hypertrophy) in the size of surviving motoneurons. These data provide strong evidence that the survival of injured adult mammalian motoneurons can be promoted by a known neurotrophic factor, suggesting the potential use of GDNF in therapeutic approaches to adult-onset motoneuron diseases such as amyotrophic lateral sclerosis.**

Factors that promote motoneuron survival have potential as therapeutic agents for the treatment of human neurodegenerative diseases (1). It has been shown that several neurotrophic factors, including brain-derived neurotrophic factor (BDNF) (2–4), insulin-like growth factor (IGF) (5), ciliary neurotrophic factor (6), and glial cell line-derived neurotrophic factor (GDNF) (7–10), can rescue *developing* motoneurons from both naturally occurring and axotomy-induced cell death. However, whether these trophic factors also play a role in *adult* motoneuron survival is not known. Because many motoneuron diseases, such as amyotrophic lateral sclerosis, have a late (i.e., adult) onset, it is important to determine whether neurotrophic factors are effective on injured adult motoneurons.

Interactions between motoneurons and their target muscles have been extensively investigated. For example, it is known that transection of axons of motoneurons or removal of their target during embryonic and early postnatal development results in massive motoneuron cell loss, whereas axotomy of adult peripheral nerve induces little if any neuronal death (11–19). A plausible explanation for this difference is that trophic support derived from mature nonneuronal cells (e.g., Schwann cells) associated with the peripheral nerve maintains the survival of adult motoneurons.

A different type of lesion, ventral root avulsion, which involves pulling the root out of the spinal cord, induces the death of virtually all motoneurons in the adult rat and provides a good model to examine the response of adult motoneurons to trophic factors (20, 21). The expression of nitric oxide synthase (NOS), an enzyme for synthesis of the free radical nitric oxide (NO), can be induced in adult rat motoneurons following both spinal root avulsion (20, 22) and cranial nerve axotomy (23), and it has been suggested that the cell death following these lesions may be induced by oxidative stress and reactive oxygen species such as NO (20–22).

Although the target dependency of motoneuron survival is diminished in adult animals (15, 18), adult rat motoneurons continue to express neurotrophin receptors (24) and they remain biologically responsive to BDNF and neurotrophin 4/5 (25, 26). For example, treatment with BDNF or neurotrophin 4/5 can attenuate the decrease in choline acetyltransferase immunoreactivity in adult motoneurons induced by axotomy and can enhance the lesion-induced reexpression of the low-affinity neurotrophin receptor p75 in adult motoneurons (25, 26). GDNF can prevent the loss of choline acetyltransferase expression in adult rat facial motoneurons following axotomy (10), and GDNF mRNA has been shown to be present in peripheral nerves and cultured Schwann cells (8, 27). Reverse transcription–polymerase chain reaction has been used to demonstrate that GDNF mRNA is present in the striatum, hippocampus, cortex, and spinal cord of adult rat and human (28). Therefore, the expression of GDNF mRNA is widespread in the central and peripheral nervous systems, suggesting that GDNF may serve a neurotrophic role *in vivo* (29). We have examined the time course of motoneuron death and NOS expression following avulsion and have investigated whether treatment with nerve growth factor (NGF), BDNF, IGF-I, or GDNF can prevent avulsion-induced death of adult mouse motoneurons.

EXPERIMENTAL PROCEDURES

Animals and Surgical Techniques. The seventh cervical segment (C7) spinal roots of adult BALB/cByJ mice (16–25 g) and Sprague–Dawley rats (200–300 g) were avulsed by an extravertebral extraction procedure (20). Animals were anesthetized with intramuscular injections of ketamine (80 mg/kg) and xylazine (8 mg/kg). They were placed on the surgical table in a supine position and the right brachial plexus was exposed by separating the scalene muscles with fine forceps. The C7 nerve was identified and isolated from the surrounding adipose/connective tissue to the point where it exits the vertebral foramen. With microhemostat forceps, an extravertebral avulsion was carried out by a steady moderate traction applied away from the intervertebral foramen. The avulsed ventral and dorsal roots as well as dorsal root ganglia (DRG) were cut away from peripheral nerve and examined to confirm the success of the avulsion (the avulsed DRG, ventral rootlets, and dorsal root can be easily observed with a dissecting microscope). In another series of experiments, axotomy was performed by cutting the right C7 nerve with microscissors very close to the vertebral column (about 3 mm distal to the spinal cord and a

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Abbreviations: NOS, nitric oxide synthase; C*n*, *n*th cervical segment; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; IGF, insulin-like growth factor; NGF, nerve growth factor; CNTF, ciliary neurotrophic factor; DRG, dorsal root ganglion (ganglia).

3-mm segment of nerve was removed to avoid reinnervation). The wounds were suture closed and animals were examined at various times following surgery for motoneuron survival and compared with those which had avulsion. The rats were also lesioned by avulsion and examined in parallel as positive controls for NOS expression in lesioned cervical motoneurons (20).

Following avulsion of the right C7 roots in the mouse, a small piece (2 mm^3) of Gelfoam (Upjohn) presoaked in 10-μl solutions of either saline (0.9% NaCl, pH 7.4) or various human recombinant neurotrophic factors (NGF, BDNF, IGF-I, and GDNF), at 1 $\mu g/\mu l$ was placed in contact with the lesioned spinal cord. The wounds were closed with 3-0 sutures. The location above where the Gelfoam was implanted was marked by a skin suture. All mice which had Gelfoam implantation also received one injection (10 μl) of either saline or various trophic factors at the lesion site 1 week after the surgery. By knowing the depth and angle of the lesion/implantation site relative to the overlying skin suture it was possible to place the tip of the needle in close proximity to the site of avulsion. The majority of mice were sacrificed 3 weeks following avulsion. Spinal cords were dissected, embedded in paraffin, and processed for motoneuron cell counting.

NOS (NADPH-Diaphorase) Enzyme Histochemistry. At the end of the appropriate survival time, animals were perfused with phosphate-buffered saline (PBS, containing 137 mM NaCl, 2.7 mM KH_2PO_4, 8 mM Na_2HPO_4) followed by a fixative solution containing 4% (wt/vol) paraformaldehyde and 0.2% (wt/vol) picric acid in 0.1 M phosphate buffer (pH 7.4). The cervical spinal cord was dissected out, and the C7 segment was easily identified by the absence of both ventral and dorsal roots on the side of the lesion. The boundaries of the C7 spinal segment were marked by small notches made in the white matter. The region of spinal cord spanning C6–C8 was fixed by immersion in the same fixative as above for 2–4 hr and then placed in phosphate-buffered 30% (wt/vol) sucrose overnight. On the following day, 40-μm-thick serial sections were cut with an Oxford vibrating microtome. The sections were then collected in 0.1 M PBS and processed for NADPH-diaphorase histochemistry. Although NADPH-diaphorase staining has been reported to detect other NADPH-mediated oxidative reactions besides the NOS reaction, we have previously shown that the NADPH-diaphorase method used here identifies the same population of spinal neurons as detected with specific NOS antibodies (30). Colocalization studies of other investigators have also confirmed that immunoreactive neuronal NOS and NADPH-diaphorase staining are found in the same cells (31–33). Therefore, NADPH-diaphorase histochemistry can be used as a specific and reliable method for detecting neurons expressing NOS. In brief, sections cut with a vibrating microtome were incubated at 37°C for 1 hr in 10 ml of 0.1 M Tris·HCl (pH 8.0) containing 10 mg of NADPH (Sigma) and 2.5 mg of nitro blue tetrazolium, washed with PBS three times, air dried, and then counterstained with neutral red. Sections were dehydrated and mounted in Permount.

Motoneuron Counting and Morphometric Analysis. Animals were perfused intracardially with 4% paraformaldehyde 1–6 weeks following the lesion, and spinal cords were dissected out from the vertebral column and postfixed in Bouin's fixative overnight. After fixation, spinal cords were dehydrated with increasing concentrations of ethanol and embedded in paraffin. Serial transverse sections (12 μm) were cut through the C6–C8 spinal region. For motoneuron counts, the C7 segment was defined as the region beginning halfway between the end of C6 DRG and the beginning of C7 DRG (contralateral side) and ending in the region halfway between the end of C7 DRG and the beginning of C8 DRG. Motoneurons in C7 were counted blindly in every fifth section on both sides of the spinal cord, as described previously (19, 34, 35). Recently, we compared our counting method with a different method that involves the use of a new correction factor (36) and we found no statistically significant difference between the two methods (19). We conclude that our counting method is as accurate and reliable as those that use correction factors or stereology (37).

Following neuronal cell counts, two sections (five sections apart) were selected from the middle of the C7 segment of the spinal cord from each animal. From these, the outline of motoneurons was traced by using a camera lucida attached to the microscope. Only those motoneurons that met the same criteria as for cell counting (see ref. 19) were included in this analysis. The perimeters of motoneuron somata were digitized with a Summagraphics bit pad interfaced to a microcomputer, and the areas were calculated with Sigmascan software (Jandel, Corte Madera, CA) (19).

RESULTS

NOS Expression in Adult Mouse and Rat Motoneurons Following Avulsion. Avulsion in the mouse did not induce NOS expression in motoneurons either 2 weeks (Fig. 1*A*) or 4 weeks after injury, when 80% of the motoneurons had already degenerated (Fig. 2). In contrast, in the rat, NOS-positive motoneurons appeared in the lesioned segment 1 week after avulsion, and about 50% of surviving motoneurons were NOS positive by 2 weeks (Fig. 1*B*), reaching a maximum of 80% by 4 weeks after the lesion (ref. 20). It is possible that the absence of NOS in mouse motoneurons following avulsion may be attributed to the inability of the histochemical method to detect murine NOS. Because other neurons in the spinal cord of normal and avulsed mice that express NOS can be detected with this method (Fig. 1*A Inset*), however, we can exclude this possibility. It is also possible that NOS expression in mouse motoneurons occurs at a different time than in rat following avulsion. This also seems unlikely, since NOS-positive mouse motoneurons were not seen at 1, 3, 5, and 7 days or 3, 6, or 8 weeks following avulsion (data not shown).

Time Course of Adult Mouse Motoneuron Loss Following Avulsion. The spinal root avulsion lesion in cervical segments induces a massive motoneuron death in the adult mouse (Fig.

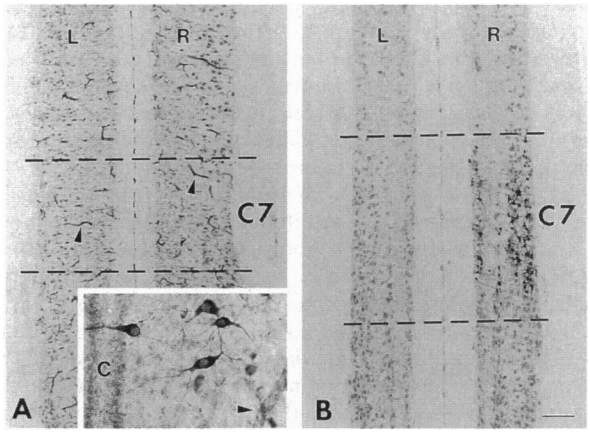

FIG. 1. Photomicrographs of horizontal sections through the ventral horn of mouse (*A*) and rat (*B*) C6–C8 spinal cord stained by NADPH-diaphorase (NOS) histochemistry and counterstained with neutral red 2 weeks following avulsion of the right C7 spinal roots. (*A*) In the mouse, no NOS-positive motoneurons can be observed on the lesion side of the C7 segment; arrowheads indicate NOS-positive blood vessels. (*B*) In contrast, NOS expression can be seen in rat motoneurons ipsilateral to the lesion; note the lack of NOS activity in motoneurons in upper (C6) and lower (C8) nonoperated segments in the rat. (Bar = 200 μm.) However, NOS staining is prominent in other mouse spinal neurons, including cells in the dorsal horn, surrounding the central canal and in the intermediolateral column. The NOS-positive cells in the *Inset* in *A* are around the central canal (C) of an avulsed mouse in which no NOS-positive motoneurons were detected. L and R are left and right sides, respectively.

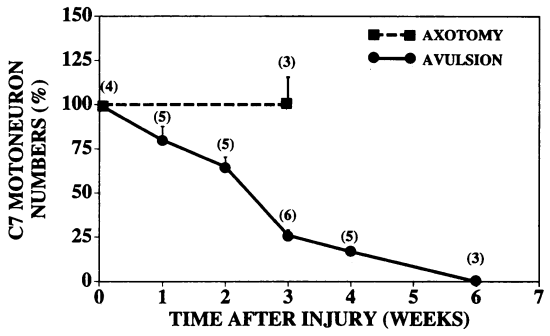

FIG. 2. Time course of motoneuron loss following C7 root avulsion or axotomy in adult mice. Data are expressed as percent (mean ± SEM) of contralateral (control) cell numbers, which represent 100%. Numbers in parentheses above the bars indicate the number of mice examined in each group.

2), similar to previous reports in the adult rat (20) and in lumbar segments in the adult rat (21). No apparent cell loss was detected 1, 3, or 5 days after lesion. One week after injury, there was a 20% cell loss on the lesioned side of the spinal cord (Fig. 2). The cell loss increased steadily and by 3 weeks, there was a 70% loss of motoneurons. By 6 weeks, all motoneurons on the lesioned side had degenerated (Fig. 2). In contrast, a different kind of injury, axotomy of the C7 nerve at a point 3 mm distal to the spinal cord, did not cause any significant motoneuron loss 3 weeks after axotomy (Fig. 2). Similar results have been obtained following axotomy in the adult rat (20).

Effects of Trophic Factors on Lesioned Adult Mouse Motoneurons. Treatment of avulsion-lesioned adult mice with GDNF rescued a significant number (50%) of injured motoneurons from cell death and induced a cellular hypertrophy in surviving cells at 3 weeks after injury, whereas NGF, BDNF, and IGF-I all failed to significantly enhance survival or cell size (Table 1; Figs. 3 and 4). Because GDNF was administered at only two time points following the lesion, it seems likely that a different treatment strategy (e.g., see ref. 6) could rescue even more motoneurons. Although we cannot exclude the possibility that GDNF affects motoneurons in this situation by a systemic route, we consider this unlikely. Both the Gelfoam implant and the later injection were placed at the site of the avulsion. Additionally, if GDNF were acting systemically, then one would expect hypertrophy of motoneurons on the side contralateral to the lesion, but this did not occur (data not shown).

DISCUSSION

Recently, it has been reported that NOS can be induced in spinal motoneurons by root avulsion lesion in adult rats, and the induction of NOS coincides with the death of the lesioned

Table 1. Motoneuron soma size (mean ± SEM) in C7 spinal cord of adult mice 3 weeks after avulsion and treatment with either saline or neurotrophic factors

Motoneurons	Treatment	Size, μm^2	n*
Contralateral	—	489 ± 21	5
Ipsilateral	Saline	491 ± 21	5
	NGF	456 ± 17	3
	BDNF	473 ± 18	5
	IGF-I	489 ± 14	5
	GDNF	558 ± 22†	5

*Number of animals in each group. For each animal, the value is from a pooled population of neurons (7–18 cells).
†$P < 0.03$ vs. all other groups, including contralateral (control), as judged by one-way analysis of variance followed by Newman–Keuls test.

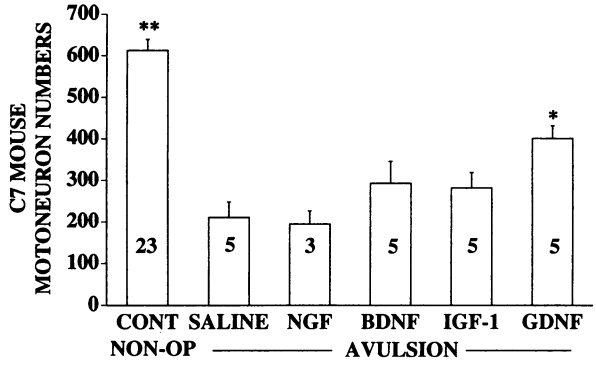

FIG. 3. Effects of neurotrophic factors on adult mouse motoneuron survival 3 weeks following C7 root avulsion. Data are expressed as mean ± SEM. Numbers in bars represent numbers of animals examined in each group. *$P < 0.04$ vs. saline-, NGF-, BDNF-, and IGF-I-treated groups; **$P < 0.001$ vs. all other groups. Statistical comparison was done by one way analysis of variance followed by Newman–Keuls test.

motoneurons. From this it was suggested that avulsion-induced cell death may result from oxidative stress and the production of reactive oxygen species such as NO (20–22). In contrast to rats, however, no NOS expression was detected in motoneu-

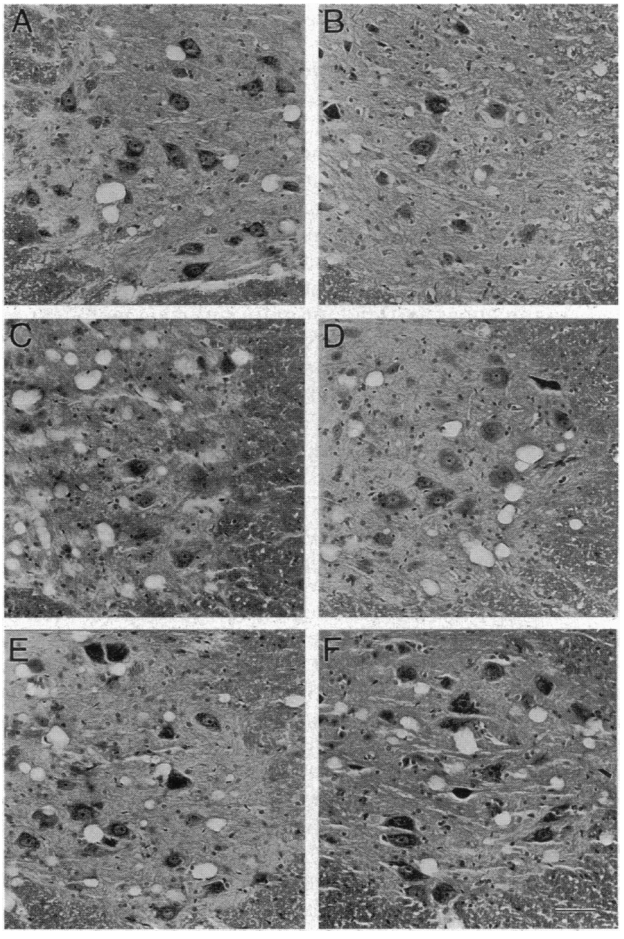

FIG. 4. Photomicrographs of hematoxylin- and eosin-stained transverse sections of the C7 spinal cord of adult mice 3 weeks following avulsion and after treatment with either saline or a total of 20 μg of neurotrophic factor as indicated. (*A*) Nonoperated (contralateral) side. (*B*) Lesioned (ipsilateral) side treated with saline. (*C*) Lesioned side treated with NGF. (*D*) Lesioned side treated with BDNF. (*E*) Lesioned side treated with IGF-I. (*F*) Lesioned side treated with GDNF. (Bar = 50 μm for *A–F*).

rons of avulsed mice at any time period examined (1 day to 8 weeks) following the lesion (Fig. 1). However, motoneuron loss following avulsion in the mouse (Fig. 2) is more dramatic than that in the rat (figure 1 in ref. 20). The reason for the difference in avulsion-induced NOS expression in rat and mouse motoneurons is not clear. Accordingly, although the role of NOS in motoneuron death is still not completely understood, it is clear from the present results that the expression of NOS (and NO) is not necessary for avulsion-induced motoneuron death in the adult mouse.

It seems likely that the difference between axotomy and avulsion may reflect the deprivation of trophic support from nonneuronal cells associated with peripheral nerve after avulsion (e.g., Schwann cells). This source of trophic support would remain following axotomy but would be absent following avulsion. Consistent with this proposal is the finding that implantation of a peripheral nerve graft at the site of avulsion in the adult rat prevents motoneuron degeneration (22). A detailed examination of the peripheral stump following axotomy could perhaps provide some clues regarding the putative role of nonneuronal cells in the peripheral nerve in promoting neuron survival following axotomy. Following avulsion, motoneurons appear to die in two phases, an early rapid phase (1–3 weeks), in which 70% of motoneurons die, and a later phase (3–6 weeks) which is more protracted and slower, during which the remaining 30% of the motoneurons are lost. This difference may reflect the differential response of subclasses of motoneurons with distinct target muscles or functions (e.g., slow vs. fast motoneurons).

GDNF has been shown to promote the survival of developing motoneurons *in vivo* and *in vitro* (8–10). Our present data indicate that GDNF can also promote the survival of adult mouse spinal motoneurons after injury *in vivo*. GDNF can also rescue adult rat motoneurons from degeneration following avulsion (38). Although BDNF and IGF-I can rescue developing motoneurons from axotomy-induced cell death (2–5, 24), these agents were ineffective in our adult mouse avulsion model. However, although not statistically significant, there was an apparent trend for more surviving motoneurons following treatment with BDNF and IGF-I. Perhaps larger sample sizes or more frequent treatments (or larger doses) could enhance the likelihood of preventing cell loss with these agents. In fact, BDNF was recently reported to promote motoneuron survival in the adult rat after avulsion (38).

There appear to be differences in the response to trophic factors between neonatal and adult motoneurons (39). This could be due to differences in uptake, transport, or metabolism of trophic factors in developing vs. adult motoneurons. It is also possible that motoneurons change their trophic dependencies as they mature. Although target muscle-derived trophic factors play a major role in embryonic and postnatal motoneuron survival, different factors derived from afferent inputs or from central and peripheral glial cells may become increasingly important in the adult. Some trophic factors are known to be upregulated in the peripheral nerve following axotomy in adult mammals (40–46). The survival of adult motoneurons following axotomy and their death following avulsion may reflect the availability of local sources of GDNF in the axotomized peripheral nerve (22, 27–29). It is also possible that receptors for neurotrophic factors such as GDNF are upregulated following avulsion or that GDNF stimulates the glia surrounding damaged motoneurons to produce other neurotrophic factors that promote motoneuron survival. GDNF appears to be unique among the trophic factors examined so far in inducing hypertrophy of surviving motoneurons (refs. 8 and 9; present results). Although the mechanism for this hypertrophy is not known, the prototypical trophic agent, NGF, has long been known to induce hypertrophy of sympathetic and sensory neurons (47).

Our demonstration that GDNF can rescue injured adult mammalian motoneurons from cell death *in vivo* indicates that GDNF is a major candidate trophic molecule that could be used in the therapeutic prevention of cell loss following spinal cord injury and in the treatment of adult onset motoneuron disease. In fact, clinical trials of other neurotrophic factors [ciliary neurotrophic factor (CNTF), BDNF, and IGF] are currently being conducted on patients with amyotrophic lateral sclerosis. A major unresolved issue in the use of neurotrophic factors as therapeutic agents is whether they will be effective in preventing death or atrophy for months or years. In neonatal animals, trophic agents appear to have only transient survival-promoting effects despite chronic long-term treatment (48).

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