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Transplants of Fibroblasts Genetically Modified to Express BDNF Promote Axonal Regeneration from Supraspinal Neurons Following Chronic Spinal Cord Injury

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Transplants of fibroblasts genetically modified to express BDNF (Fb/BDNF) have been shown to promote regeneration of rubrospinal axons and recovery of forelimb function when placed acutely into the injured cervical spinal cord of adult rats. Here we investigated whether Fb/BDNF cells could stimulate supraspinal axon regeneration and recovery after chronic (4 week) injury. Adult female Sprague-Dawley rats received a complete unilateral hemisection injury at the third cervical spinal cord segment (C3). Four–five weeks later the injury site was exposed and rats received transplants of unmodified fibroblasts (Fb/UM) or Fb/BDNF. Four–five weeks after transplantation, locomotor recovery was examined on a test of forelimb usage and regeneration of supraspinal axons was studied following injection of the anterograde tracer biotin dextran amine (BDA). Rubrospinal tract (RST), reticulospinal tract (ReST), and vestibulospinal tract (VST) axons regenerated into transplants of either Fb/UM or Fb/BDNF but the length of axonal growth was significantly different in the two groups. The absolute distance of ReST growth was 1.8-fold greater in Fb/BDNF than in Fb/UM and the absolute distance of growth of RST and VST axons showed a statistically significant 4-fold increase. All three types of regenerated axons occupied a greater proportional length of Fb/BDNF transplants than of Fb/UM transplants. Only VST axons extended into the host spinal cord caudal to the Fb/BDNF grafts, but these axons were sparse. Rats receiving Fb/BDNF used both forelimbs together to explore walls of a cylinder more often than rats receiving Fb/UM, indicating partial recovery of forelimb usage. These results demonstrate that fibroblasts genetically modified to express BDNF promote axon regeneration from supraspinal neurons in the chronically injured spinal cord with accompanying partial recovery of locomotor performance.

Key Words: **spinal cord injury; neurotransplantation; supraspinal; neurotrophin; BDNF; regeneration.**

INTRODUCTION

Little spontaneous regeneration of axons occurs after acute or chronic injury to the adult mammalian spinal cord, but lesioned axons can regenerate if suitable substrates and growth factors are provided. Various approaches have been utilized to promote regeneration and recovery after injury, including grafts of peripheral nerve (8, 33), fetal spinal cord tissue (15–17) and nonneuronal cells (25, 43); supplementary neurotrophic factors (24, 37) and antibodies that block myelin-associated or other growth inhibitors (6, 36). Recently, gene therapy has been used to promote axon regeneration after brain and spinal cord injury (2, 13, 25, 39–41). This therapy is an especially promising approach because a single manipulation can modify the CNS environment and deliver a continuous supply of neurotrophic factors to enhance the intrinsic neuronal growth response. Genetically modified cells producing nerve growth factor grafted to the lesioned septohippocampal projection prevented basal forebrain cholinergic neuronal degeneration and promoted axonal regeneration to the hippocampus (23, 34). When administered at the time of injury, intraspinal transplants of fibroblasts expressing BDNF support axonal growth from rubrospinal tract neurons which correlated with recovery of forelimb function (26) and intraspinal transplants of fibroblasts expressing neurotrophin-3 (NT-3) have been shown to promote regeneration of corticospinal tract axons and recovery of hindlimb function (13).

In the present report we examine whether the same gene therapy strategy can promote axon regeneration

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and locomotor recovery when the injury is chronic. We consider a 1-month delay between injury and transplantation to represent a chronic injury because by this time the abortive regenerative response of CNS neurons has subsided, an astrocytic scar has become established at the injury site, Wallerian degeneration is advanced and spontaneous behavioral recovery has reached a plateau (11, 31, 32, 42). These features of chronic injury make it more challenging to treat than the acute injury. It is, however, essential to determine the extent to which chronic injuries respond to therapies that have been beneficial for the acute because nearly 95% of patients now survive their initial hospitalization following spinal cord injury (10) and these patients far outnumber the ~10,000 patients who suffer an acute spinal cord injury each year in the United States. The aims of the present study were to determine whether transplants of fibroblasts genetically modified to secrete BDNF (Fb/BDNF) could promote regeneration of supraspinal axons after chronic spinal cord injury and whether they could promote recovery of forelimb function. The distance and extent of regrowth by neurons contributing to the rubrospinal tract (RST), reticulospinal tract (ReST), and vestibulospinal tract (VST) were examined because these are major descending spinal pathways associated with forelimb use and they express the trk B receptor necessary for interaction with BDNF (28).

MATERIALS AND METHODS

Adult female Sprague-Dawley (225–250g) rats (N = 24) were studied. All procedures were carried out under the supervision of the Institutional Animal Care and Use Committee and according to the *NIH Guide for the Care and Use of Laboratory Animals*. Animals were housed 3–4 per cage in an environmentally controlled facility with a 12-h light/dark cycle. Food and water were available *ad libitum*.

Surgery and Cell Transplantation

Rats were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg). A laminectomy of the second and third cervical vertebrae was performed to expose the dorsal surface of the spinal cord. The underlying meningeal membranes were cut and a complete hemisection lesion of the third cervical spinal segment (C3) was prepared by gentle aspiration of spinal cord tissue on the right side. The cavity was rinsed with saline, and a Silastic sheet was placed on the dorsal cord surface to cover the cavity. The dura mater, overlying muscles and skin, were closed with sutures and animals received penicillin G (3000 units, sc) and buprenorphine (0.1 mg/kg) as an analgesic. Cyclosporin A (CsA) injection solution (Sandoz, East Hanover, NJ) was administered subcutaneously at a dose of 1 mg/100 g body weight. The daily CsA injections started 2–3 days before the transplantation procedures and continued for 2 weeks after transplantation. After this, oral CsA solution (Sandoz) was administered via the drinking water (50 μg/ml) throughout the remaining postoperative period. Animals were divided into two groups: one group (N = 12) received transplants of unmodified fibroblasts (Fb/UM), the other group (N = 12) received transplants of fibroblasts genetically modified to secrete BDNF (Fb/BDNF). Four–five weeks after the hemisection lesion, the cavity was exposed and cellular debris was removed. Scar tissue lining the cavity had a gray, fibrotic appearance that was easily distinguished from the well-vascularized, healthy spinal cord tissue adjacent to the lesion. Obvious scar was aspirated from both the rostral and the caudal aspects of the lesion cavity until normal appearing spinal cord tissue was observed (less than 0.5 mm was removed from either end). The dura mater was apposed with loose sutures prior to filling the cavity with a slurry of Fb/UM or Fb/BDNF. This technical approach provided a reservoir for transplanted cells such that most remained in the cavity as the meninges were more tightly closed. It has been established that BDNF is secreted from these cells in culture at a rate of 12.8 ng/10^6 cells/24 h (26) and this property of Fb/BDNF is monitored routinely. No BDNF was detected in the medium of Fb/UM cells. Approximately 10 μl of cell suspension (2–3 $\times$ 10^6 cells) was injected into the cavity through a glass micropipette and the dura was closed with 10-O sutures and muscles and skin were closed in layers. One week before sacrifice, four animals from each transplant group received a unilateral injection of 10% biotin dextran amine (BDA, 10,000 MW, Molecular Probes) into the left red nucleus (N = 8, stereotaxic coordinates: bregma, −5.8 mm; lateral, 0.75 mm; depth, 7.0 mm), the right lateral vestibular nucleus (N = 8, bregma, −11.0 mm; lateral, 2.2 mm; depth, 6.2 mm) or the right gigantocellular reticular nucleus (N = 8, bregma, −11.6 mm; lateral, 1.0 mm; depth, 8.5 mm). Animals were euthanized 1 week after BDA injection (5–6 weeks posttransplantation) with an overdose of sodium pentobarbital and perfused with 4% paraformaldehyde. Spinal cord segments were post fixed with paraformaldehyde overnight at 4°C, and then placed into 20% sucrose. Cryostat sections (25 μm) cut in a horizontal plane through the graft site were collected and kept in 0.1 M Sorenson's phosphate buffer (PBS) for staining of BDA-labeled axons. BDA labeled axons were detected by processing with the BDA-Neuronal Tracer kit (Molecular Probes). After three washes in 0.1 M PBS sections were incubated in avidin-horseradish peroxidase (0.5 μg/ml) for 3 h at room temperature. Sections were rinsed three times in PBS and reacted with DAB and hydrogen peroxide, followed by three 10-min rinses in PBS. Sections were mounted on glass slides, dried, and coverslipped. Sev-

eral sections from each animal were counterstained with cresyl violet to identify more clearly the graft-host interface.

Preparation of Cells for Transplantation

Fb/UM and Fb/BDNF cells were cultured as described previously (26). In brief, for surgery or stock culture, the cells were grown on 75-mm uncoated tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% fetal bovine serum and split weekly at a 1:10 ratio into fresh medium. Twenty-four hours before transplantation, cells were labeled with the nuclear dye bis-benzimide (Hoechst 33342, Sigma) by adding 100 μl of a 1 mg/ml solution to 10 ml of culture medium used to feed the cells. On the day of surgery, confluent cultures of cells were washed with Hanks balanced salt solution (HBSS, GIBCO BRL, Grand Island, NY), trypsinized, gently triturated, counted, washed, pelleted (900 rpm for 5 min), and resuspended in serum-free HBSS at a concentration of 2×10^5 cell/μl. The cells were pelleted by centrifugation and maintained on ice during preparation of animals for transplantation.

Image Analysis and Statistics

Images were captured using a CoolSnap color CCD camera (Roper Scientific, Tucson, AZ) attached to a Zeiss Axioskop microscope and analyzed by Metaview imaging software (Universal Imaging Corporation, West Chester, PA). For all animals, 5 tissue sections displaying the greatest number of BDA-labeled axons at the graft-spinal cord interface were chosen from each animal for analysis. Axons with terminal end bulbs within the transplant were identified and the length of axon extension into the graft was measured and compared to the total length of the graft. The mean distance and the proportion of transplant length occupied by regenerated axons in Fb/UM and Fb/BDNF groups were compared for the three descending tracts using one-way ANOVA followed by Bonferroni post hoc analysis.

Behavioral Testing

Forelimb use during spontaneous vertical exploration was examined once in all rats 5–6 weeks after transplantation and before BDA injection. Animals were placed in a clear Plexiglas cylinder (20 cm in diameter and 30 cm high) for 5 min as described (26). The cylinder encourages use of the forelimb for vertical exploration. A mirror was placed behind the cylinder so that both forelimbs could be viewed at all times. The following behaviors were scored: independent use of the left (unimpaired) or right (impaired) forelimb for contacting the wall of the cylinder during a full rear, to initiate a weight-shifting movement, or to regain center of gravity while moving laterally in a vertical posture along the wall; and simultaneous or near-simultaneous use of both the left and the right forelimb to contact the wall of the cylinder during a full rear and for lateral movements along the wall. Each behavior was expressed in terms of (1) percentage use of unimpaired forelimb relative to the total number of impaired, unimpaired, and both limb use; (2) percentage use of impaired forelimb relative to the total number of impaired, unimpaired, and both limb use; (3) percentage use of both forelimbs relative to the total number of impaired, unimpaired, and both limb use. One-way ANOVA was used to test for differences between groups followed by Bonferroni post hoc analysis.

RESULTS

Transplant Survival and Transgene Expression

Using an established E8 chicken DRG bioassay to determine that BDNF secreted by Fb/BDNF cells was biologically active the Fb/UM and Fb/BDNF cells were tested in Philadelphia by Western blot analysis to verify that Fb/BDNF cells secreted BDNF, and by X-gal histochemistry to monitor transgene expression (26) before they were shipped to the Houle laboratory for preparation for transplantation. Approximately 80% of Fb/BDNF population was positively stained by X-gal histochemistry at the time of shipment, whereas no labeled Fb/UM cells were detected.

Transplants of Fb/UM and Fb/BDNF grew to fill the lesion cavity with no cystic spaces at either the rostral or caudal interface with host spinal cord tissue (Fig. 1A). Densely packed fibroblasts in both groups appeared homogeneous throughout the transplant and most cells in the graft area exhibited nuclear staining with bis-benzimide (not shown) indicating that transplanted cells remained in the graft area.

In general, BDA-labeled axons within the Fb/UM and Fb/BDNF transplants appeared to have grown in a relatively longitudinal plane, with individual axons traceable for hundreds of microns in length in the same focal plane (Fig. 1B). There was no obvious difference in the morphological appearance of axons within the different types of transplants and there was little branching of axons. Within Fb/BDNF transplants most axons remained oriented in a rostral to caudal direction and formed small fascicles of fibers (Figs. 2B, 3C, and 5) whereas the orientation of axons in Fb/UM transplants was less regular (Fig. 2A).

BDA-Labeled ReST Axons in the Transplant

The gigantocellular reticular nucleus was selected for BDA injection because previous studies had shown that most of the neurons in the reticular formation that regenerated into a peripheral nerve graft after chronic

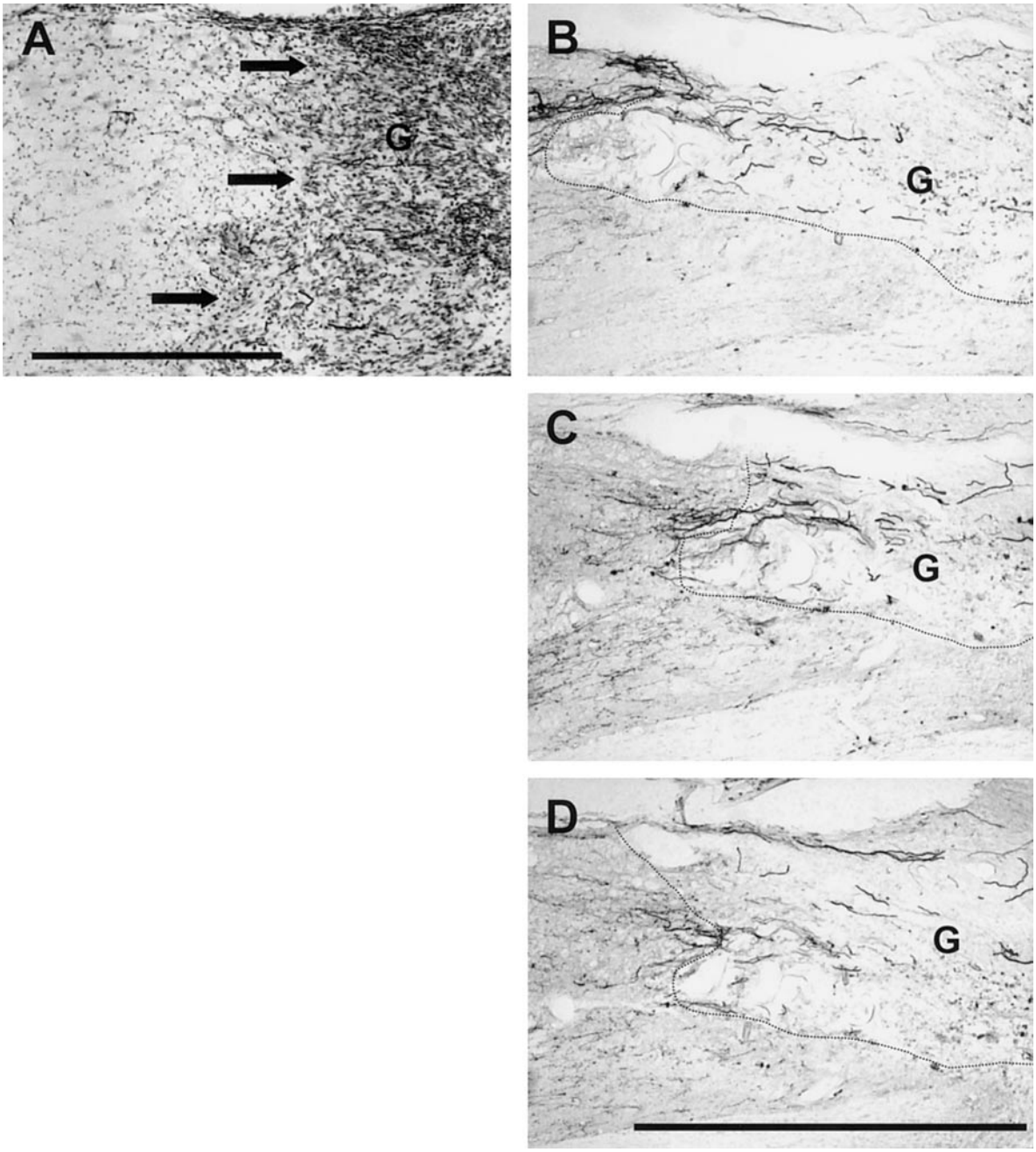

FIG. 1. Horizontal sections through the rostral host-graft interface. In (A) the close apposition of a fibroblast graft (G) with the host spinal cord can be seen. The marked increase in cell density within the graft provides a readily detectable marker for distinguishing the interface (arrows). Bar = 500 μm. Nonserial sections (B, C, D) through a Fb/BDNF transplant demonstrate the variability in morphology of the host-graft (G) interface (dashed lines). BDA-labeled axons are found crossing the interface in each section. Bar = 500 μm.

spinal cord injury were located within this nucleus (45). BDA-labeled ReST axons grew into both Fb/UM and Fb/BDNF transplants (Figs. 2A, and B). In the Fb/UM group, the mean length of ReST axons in the transplant was 877 $\pm$ 75 μm (Fig. 6), which covered just over 40% of the total length of the transplant (Fig. 7). The mean length of ReST axon growth into Fb/BDNF transplants was 1568 $\pm$ 494 μm, which was greater than 76% of the total transplant length. Both of these measures are significantly greater than those observed in Fb/UM transplants. Regenerating ReST axons in Fb/UM transplants showed terminal end bulbs a minimum of several hundred microns beyond the rostral host graft interface. This equaled approximately 20% of the total transplant length. In contrast, ReST axons in Fb/BDNF transplants extended for at

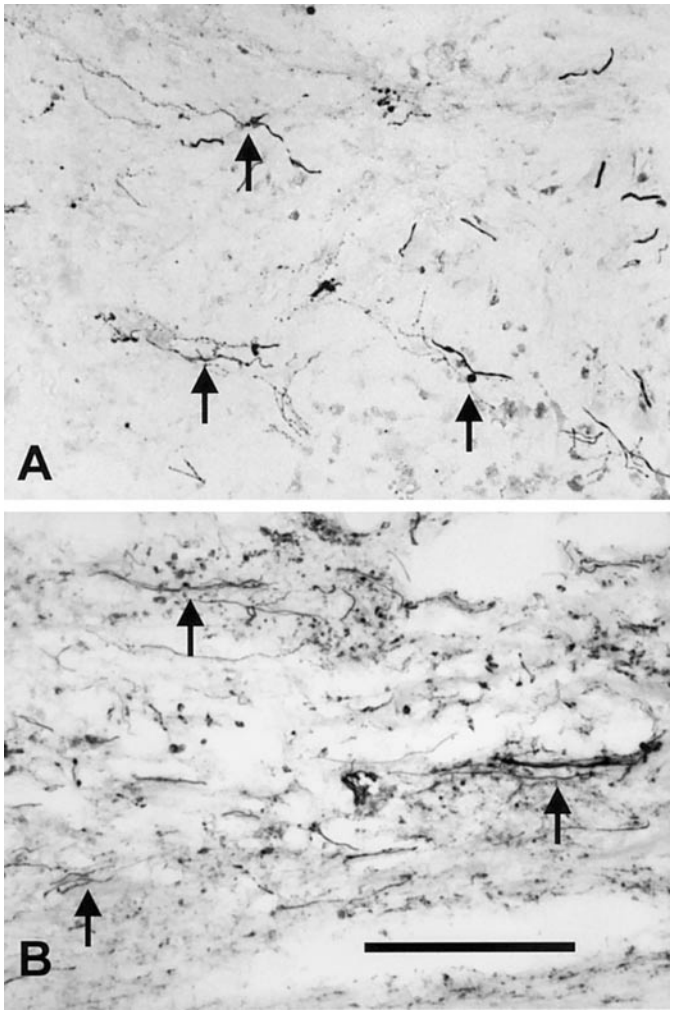

FIG. 2. BDA-labeled reticulospinal tract axons within fibroblast transplants. (A) ReST axons (arrows) in the rostral portion of Fb/UM transplants were few in number and appeared to grow in all directions. (B) Fb/BDNF transplants supported long-distance growth of numerous axons (arrows) in a relatively straight and orderly plane. Several small bundles of axons are evident in this central portion of the transplant. Sections are in a horizontal plane. Bar = 100 μm.

least 850 μm, covering nearly 60% of the transplant length. While there were isolated examples of more extensive growth into Fb/UM transplants, growth into Fb/BDNF transplants was consistently longer. Some ReST axons in the Fb/BDNF group grew close to the caudal edge of the host spinal cord (within 200 μm), but there was no evidence of growth across the graft-host interface.

BDA-Labeled RST Axons in the Transplant

To visualize the regrowth of RST axons into Fb transplants, BDA was injected into the magnocellular portion of the red nucleus contralateral to the side of the spinal cord lesion. In the Fb/UM group, many BDA-labeled axons extended up to the host-graft interface

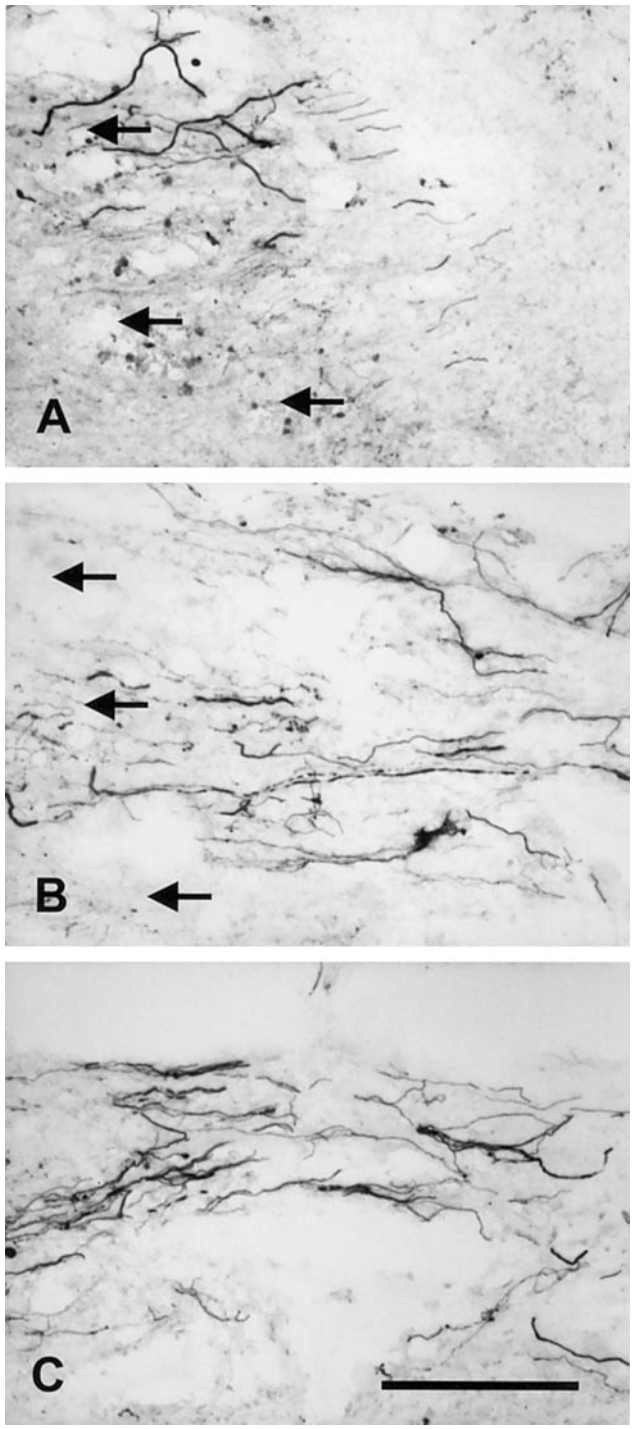

FIG. 3. BDA-labeled rubrospinal tract axons within fibroblast transplants. RST axons crossing the host-transplant interface are found in Fb/UM (A) and Fb/BDNF (B) transplants. Host spinal cord tissue is to the left of the arrows, which are aligned along the interface. Length and the number of axons growing into Fb/BDNF transplants (B) are greater than into Fb/UM (A) transplants. (C) BDA-labeled RST axons are prominent in the central portion of this Fb/BDNF transplant. Sections are in a horizontal plane, with the lateral spinal cord edge at the top of each figure. Bar = 100 μm.

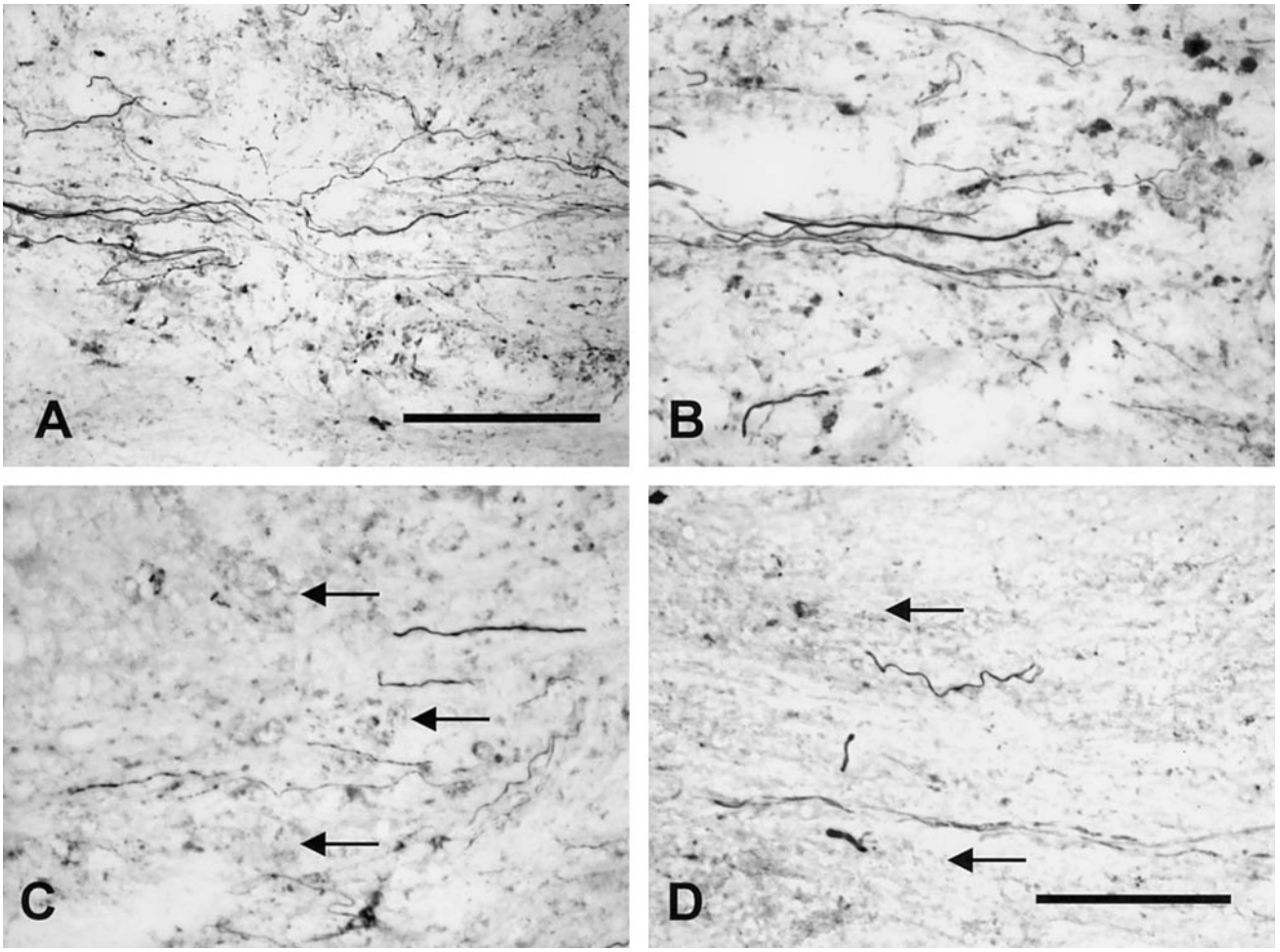

FIG. 4. BDA-labeled vestibulospinal tract axons within fibroblast transplants. (A, B) VST axons exhibit a relatively straight course of growth through the central portion of Fb/BDNF transplants. (C, D) A few VST axons extend through the entire length of Fb/BDNF transplants, up to and across the caudal transplant-host spinal cord interface (indicated by arrows). The host spinal cord caudal to the injury site is to the left of the arrows. Sections are in a horizontal plane. Bar for (A) = 100 μm and for (B, C, D) = 50 μm.

(Fig. 2A) but most failed to extend into the graft for long distances (Fig. 6). Those few axons that grew well beyond the host-graft interface reached a mean length of 443 ± 202 μm, which represented 22% of the total length of the transplant (Fig. 7). The terminal end bulbs of RST axons reached at least 125 μm in Fb/UM transplants, with a maximum distance reached of 1410 μm from the rostral end. The maximum length of ingrowth by RST axons represented just 42% of the total transplant length.

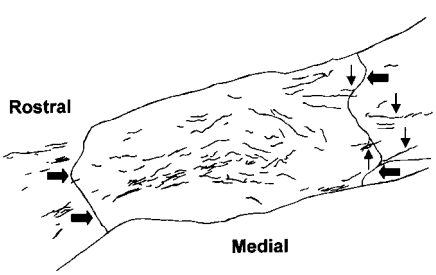

FIG. 5. Camera lucida drawing of BDA-labeled VST axon growth into and through a Fb/BDNF transplant. Only the transplant side of the spinal cord is shown, with uninjured spinal cord tissue present medial to the transplant. Large arrows indicate the rostral and caudal boundaries of the transplanted cells. Small arrows indicate VST axons that have reached the caudal end of the transplant, with some extending back into the host spinal cord. The transplant length between the large arrows is approximately 2.1 mm.

In comparison, the growth of RST axons into Fb/BDNF transplants was significantly greater in terms of mean length of growth and the percentage of transplant length occupied by regenerating axons (Figs. 5 and 7). In the presence of BDNF-secreting cells, the mean length of RST axon growth was 1772 ± 506 μm, which represented over 68% of the total length of the transplants. This was a fourfold increase over the length of growth observed in Fb/UM transplants. At least 37% of the total transplant length was occupied by RST axons, reaching a peak of 88% in some specimens. While the mean length of axon growth was enhanced in Fb/BDNF transplants (Figs. 3B and C), no

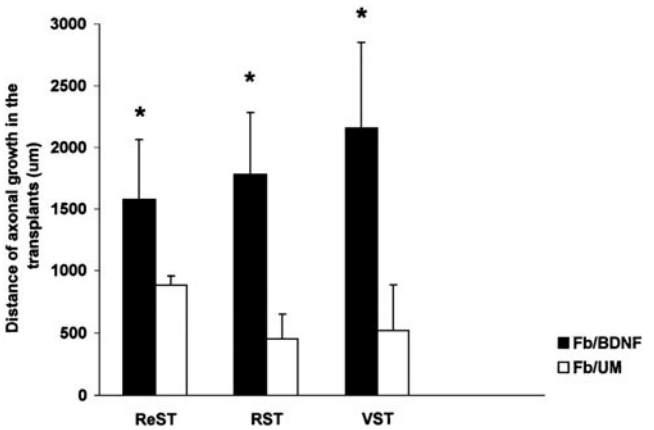

FIG. 6. Distance of BDA-labeled axon growth into transplants. The mean distance of axon growth into Fb/BDNF transplants was significantly greater for all tracts compared to the distance of growth into Fb/UM transplants. The mean length of growth of ReST, RST, or VST axons into Fb/UM transplants was not significantly different, nor was there any difference in the extent of axon growth from these tracts into Fb/BDNF transplants. Values are mean ± S.D., $*P < 0.05$.

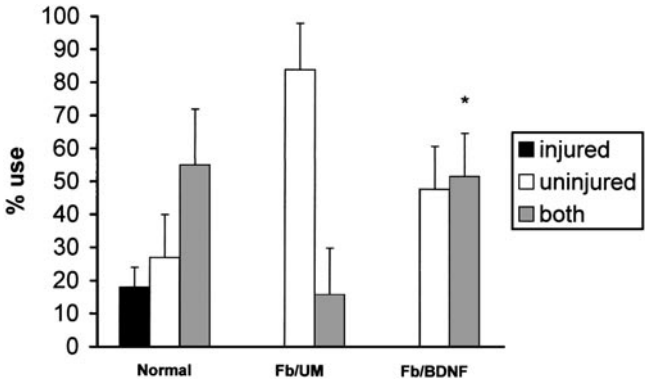

FIG. 8. Behavioral analysis of forelimb use during vertical exploration. Five to six weeks after transplantation, animals with Fb-only transplants (control) relied upon their left forelimb (uninjured side) for support during vertical exploration, with the right forelimb (injured side) being added less than 20% of the time. The Fb/BDNF transplant animals showed significant use of both left and right forelimbs for exploration compared to the control. Values are mean ± SD, $*P < 0.05$.

axons were found growing into the caudal stump of the host spinal cord.

BDA-Labeled VST Axons in the Transplant

The lateral vestibular nucleus was chosen as the site for BDA injection to label VST axons that had regenerated into Fb transplants in the chronically injured spinal cord. Both Fb/UM and Fb/BDNF transplants (Figs. 4A and B) supported VST axons but the extent of growth among BDNF-secreting cells was significantly greater than in Fb/UM transplants. In the Fb/UM group, the mean length of VST axons was 508 ± 369 μm (Fig. 6). This represented growth over 32% of the total transplant length (Fig. 7), but in all samples there was a large range (6–76%) of total transplant length reached by axons, with some VST axons extending just 150 μm into Fb/UM transplants. With Fb/BDNF transplants there was a significant fourfold increase in the mean axon length (2146 ± 697 μm) compared to Fb/UM transplants. Over 80% of the total Fb/BDNF transplant length was occupied by VST axons and in one animal axons grew across the caudal graft-host interface (Figs. 4C and D and Fig. 5) reaching a length up to 500 μm within the host spinal cord.

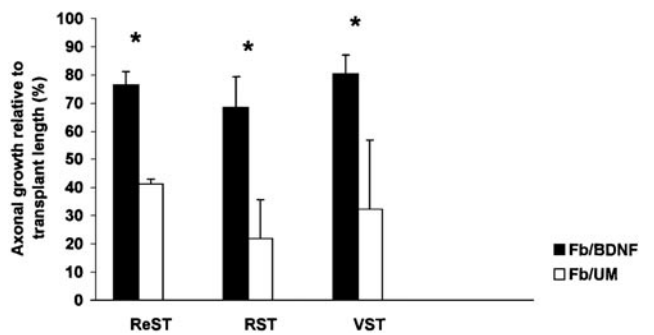

FIG. 7. Percentage of transplant length occupied by BDA-labeled supraspinal axons. The mean length of growth into Fb/BDNF transplants relative to the length of the transplant was significantly greater for all tracts compared to the proportion of Fb/UM transplant length occupied by growing axons. The percent of Fb/UM transplant length containing ReST, RST, or VST axons was not significantly different, nor was there any difference in the percent of Fb/BDNF transplant length supporting ReST, RST, or VST axons. Values are mean ± SD., $*P < 0.05$.

Behavioral Analysis

A clear Plexiglas cylinder was used to test for selective forelimb use during spontaneous vertical exploration. Normal rats used a single forelimb (right or left) alone (approximately 50% of the time) or both forelimbs together (approximately 50 % of the time). We analyzed the percentage of wall exploration behavior that was initiated by right (impaired) or left (unimpaired) forelimb alone or both forelimbs together (Fig. 8). Hemisection at the upper cervical level induced asymmetry in forelimb use so that rats with either unmodified or modified fibroblast transplants did not use the impaired limb alone. Rats in the Fb/UM group infrequently used both forelimbs (16 % of the time) to explore the wall, whereas Fb/BDNF-treated rats used both forelimbs together as often (approximately 50% of the time) as they used the unimpaired limb alone. Transplantation of Fb/BDNF cells significantly increased the usage of both forelimbs compared to the Fb/UM group ($P < 0.05$), indicating partial recovery of a specific locomotor task.

DISCUSSION

Of the transplantation techniques developed to promote structural repair and functional improvement after acute spinal cord injury, only a few have been applied to a chronic injury. The results of the present study confirm previous observations of the behavior of chronically injured supraspinal neurons, i.e., that axonal regrowth depends upon the availability of a suitable substratum (transplanted cells or tissues) *in combination with* the application of specific neurotrophic factors that are necessary to maintain the survival and initiate the regenerative effort of neurons treated long after their initial injury. Here we show that transplants of fibroblasts genetically modified to produce BDNF support the regrowth of three major descending spinal pathways even when the intervention is delayed for 4 weeks after injury. Although most regrowing axons remained within the transplant and only a few extended across the caudal graft-host interface, the long-term release of BDNF from modified cells was sufficient to improve the use of the affected forelimb during vertical exploration. In addition to the resiliency of chronically injured supraspinal neurons, these results show that the recovery of certain locomotor behaviors does not depend upon the reestablishment of synaptic contact between axons of specific descending pathways and their targets distal to the site of spinal cord injury.

We found that rubrospinal, reticulospinal, and vestibulospinal tract axons regenerated into fibroblast transplants and that cells genetically modified to produce BDNF supported significantly greater growth than unmodified fibroblasts. This is consistent with earlier studies where treatment of a chronic lesion cavity with BDNF significantly increased the number of axons from these same chronically injured supraspinal neurons that regenerated into a peripheral nerve graft (45) and that transplants of Fb/BDNF cells enhanced regeneration of serotonergic axons (22). The present results provide additional support for the observation that regeneration is more efficient when both transplants and trophic factor support are provided than when either is provided alone. Here we show that transplantation of Fb/BDNF cells led to a 4-fold increase in the distance of growth of RST and VST axons and to a 1.8-fold increase in length of ReST axons compared to the extent of growth observed into Fb/UM transplants. It is not surprising that RST, VST, and ReST neurons demonstrated similar levels of regeneration, because their response to Fb/BDNF transplants likely is mediated through the trkB receptor, which is expressed by all of these types of neurons (28).

The present results support a recent study examining the regrowth of serotonergic axons following delayed transplantation of fetal spinal cord tissue with BDNF or NT-3 provided to the transplant surface through an osmotic minipump (7). The total length of serotonergic axons and the density of serotonergic axon clusters were enhanced by the combined therapies, particularly when there was a 2- or 4-week delay from the time of injury to the beginning of treatment. It is important to note that successful axonal growth into the caudal spinal cord demonstrated by Coumans *et al.* (7) may be due to the nearly 1000-fold greater concentration of neurotrophic factor provided posttransplantation, compared to the amount estimated to be produced by the Fb/BDNF transplants of the present study (see 26). Together these studies clarify some of the conditions necessary to promote regeneration of chronically injured neurons, although there is reason to be concerned that the neuronal response may weaken if an intervention is delayed for too long after the initial injury. The extent of axonal regrowth was greater when the intervention began at 2 weeks than at 4 weeks (7) and Tobias *et al.* (38) detected greatly reduced regrowth of RST axons with a 6-week delay between injury and transplantation of Fb/BDNF compared to our present observations after a 4-week delay. Fu and Gordon (12) have shown that regeneration by spinal motoneurons becomes less effective as the interval between peripheral nerve injury and apposition to a distal nerve stump increased. One possibility is that the prolonged absence of target-derived neurotrophin support is responsible for a diminished regenerative response. An appropriate growth environment formed by the resident nonneuronal cells also appears to be critical for long-term regeneration by peripheral axons, possibly because of the production of numerous growth and trophic factors by these cells (reviewed in 12). Retrograde neuron death may also contribute to a limited regenerative response. A second injury resulting from removal of scar tissue prior to transplantation, as was performed in the present study, is known to exacerbate neuron cell death (19). As the interval between the first and second injury increased, the extent of cell death also increased. When the second injury occurred 4 or 8 weeks after the first, the loss of neurons could be lessened by treatment of the lesion site with BDNF or CNTF (19).

The acute application of neurotrophic factors to hemisected or completely transected adult rat spinal cord has been shown to prevent atrophy of rubrospinal neurons (5) and to promote supraspinal axonal growth into fetal spinal cord transplants (4). Exogenous administration of BDNF adjacent to neuron perikarya in the RN (24) or into the lesion cavity (45) increased the number of RN neurons whose axons regenerated into a peripheral nerve graft. Additional reports indicate that a combination of exogenously administered BDNF and NT-3 induced vestibulospinal axons to regrow into Schwann cell transplants placed acutely into a thoracic spinal cord injury site (44), whereas no VST axons entered Schwann cell transplants alone (43). Little

information is available concerning the regeneration of ReST axons in either acute or chronic spinal cord injuries, although an earlier report from our laboratory showed that treatment with exogenously administered growth and neurotrophic factors leads to an increase in the total number of chronically injured ReST neurons that extend axons into a peripheral nerve graft (18, 45). Some of the difficulty obtaining long-term release of growth promoting factors into the CNS has been overcome by fashioning genetically modified cells which can synthesize and secrete a neurotrophin after transplantation into the CNS. Schwann cells genetically modified to secrete BDNF promote growth of 5-HT axons along the transplant, indicating that the extent of regeneration depended upon the presence of both Schwann cells and higher than normal levels of BDNF (27).

Regrowing axons approached the caudal end of Fb/BDNF transplants, but very few traversed the graft-host interface despite the continued release of BDNF from modified fibroblasts. This is a discrepancy with results reported with acute Fb/BDNF transplants where long distance growth into and through transplants was described (26). It is likely that changes occur in extracellular matrix components distant to a lesion, which may become more prominent with a second injury of the spinal cord. The response of nonneuronal cells adjacent to a site of spinal cord reinjury is ill-defined, yet glial scarring likely reforms and remains inhibitory to extensive axonal growth across the interface (14). Even axons that enter the transplant almost immediately after reinjury will take several days to grow to the distal interface, which may be sufficient time to reestablish scar tissue at the graft-host interface. The transplants in this study were derived from the same source of Fb/BDNF cells that elicited elongation of RST axons through the transplant into the caudal host spinal cord when used to treat an acute spinal cord injury (26). It is possible that diffusion of BDNF beyond the transplant boundaries occurs more readily after an acute lesion compared to the chronic condition, thereby accounting for some of the more long-distance growth observed with acute transplants (26) While unmodified fibroblast transplants readily supported short-distance axonal ingrowth in the present study, it is obvious that regeneration by chronically injured neurons is not sustained or enhanced without additional neurotrophic factor support (7, 19, 46). Davies *et al.* (9) have shown that axonal growth from transplanted neurons ceases at a spinal cord injury site where there is a local accumulation of chondroitin sulfate proteoglycan (CSPG). Plant *et al.* (30) have shown that higher concentrations of CSPG occur at the caudal interface between a Schwann cell transplant and host spinal cord, than at the rostral interface, supporting the present observation that it is more difficult for regrowing axons to extend beyond a transplant than initially to grow into a transplant. Enzymatic degradation of chondroitin sulfate has been shown to provide an effective strategy for improving regeneration of injured nigrostriatal axons (29) and of crushed corticospinal tract axons (3). In this latter study, the extent of anatomical regeneration was limited even though there was evidence of recovery of locomotor and proprioceptive behavior, correlating with the results of the present study.

Our behavioral results based on observations of explorative activity along the walls of a transparent cylinder showed that delayed transplants of BDNF-secreting fibroblasts significantly reduced the asymmetry in forelimb usage caused by the unilateral C3/4 hemisection This limb-use asymmetry test has been shown to be a reliable and sensitive measure for demonstrating impairment and recovery in unilateral models of stroke and Parkinson's disease as well as spinal cord injury (reviewed in 35). Normal rats use either the right or the left forelimb independently or both forelimbs together to contact the wall of the cylinder when they rear or shift their weight laterally in a vertical position. We found that right-sided C3/4 hemisection abolished the independent use of the impaired (right) forelimb and that independent use of this forelimb did not recover in rats that received delayed grafts of either Fb/BDNF or Fb/UM. Rats that received Fb/BDNF, however, used both forelimbs together far more often (~50% of the time) than those that received Fb/UM (~16% of the time) and they used both limbs in combination as frequently as they used the unimpaired (left) forelimb alone. Because we found that supraspinal axons regenerated only modestly into the delayed Fb/BDNF transplants and virtually not at all into caudal host spinal cord, the recovery of forelimb function is likely due to an effect of BDNF on local spinal cord circuits rostral and/or caudal to the transplants. Several reports have previously demonstrated that BDNF enhanced locomotor recovery through an effect on local circuitry independent of regenerating axons (1, 20, 21). Here the observed functional recovery was attained in the absence of significant axonal regeneration beyond the site of transection injury, suggesting that BDNF was able to affect these hind limb movements by enhancing local synaptic activity or by promoting similar plastic changes in local circuitry. This enhancement of local circuitry might allow greater use of the impaired forelimb in response to movements initiated by the unimpaired forelimb that result in shifts in the center of gravity. Another possibility is that the BDNF-expressing grafts contributed to reorganization of local circuits sufficient to permit simultaneous use of both forelimbs but insufficient for independent use of the impaired forelimb. It is possible that Fb/BDNF transplants serve as a center for the interaction of descending supraspinal axons with dendritic processes of caudal spinal cord interneurons that have grown into the

transplant. In this case, additional experiments designed to study the effects of inactivating the unimpaired forelimb or relesioning the spinal cord rostral to the grafts might clarify the mechanisms responsible for recovery when the transplants are delayed.

In summary, we demonstrate that BDNF secreted by genetically modified fibroblasts promotes axonal regeneration of chronically injured rubrospinal, reticulospinal, and vestibulospinal axons. Local delivery of BDNF from transplants at least partially improves the recovery of forelimb usage after a long-term injury, despite the near absence of axonal regrowth across the caudal graft-host interface.

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