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Combining Schwann Cell Bridges and Olfactory-Ensheathing Glia Grafts with Chondroitinase Promotes Locomotor Recovery after Complete Transection of the Spinal Cord

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Numerous obstacles to successful regeneration of injured axons in the adult mammalian spinal cord exist. Consequently, a treatment strategy inducing axonal regeneration and significant functional recovery after spinal cord injury has to overcome these obstacles. The current study attempted to address multiple impediments to regeneration by using a combinatory strategy after complete spinal cord transection in adult rats: (1) to reduce inhibitory cues in the glial scar (chondroitinase ABC), (2) to provide a growth-supportive substrate for axonal regeneration [Schwann cells (SCs)], and (3) to enable regenerated axons to exit the bridge to re-enter the spinal cord (olfactory ensheathing glia). The combination of SC bridge, olfactory ensheathing glia, and chondroitinase ABC provided significant benefit compared with grafts only or the untreated group. Significant improvements were observed in the Basso, Beattie, and Bresnahan score and in forelimb/hindlimb coupling. This recovery was accompanied by increased numbers of both myelinated axons in the SC bridge and serotonergic fibers that grew through the bridge and into the caudal spinal cord. Although prominent descending tracts such as the corticospinal and reticulospinal tracts did not successfully regenerate through the bridge, it appeared that other populations of regenerated fibers were the driving force for the observed recovery; there was a significant correlation between numbers of myelinated fibers in the bridge and improved coupling of forelimb and hindlimb as well as open-field locomotion. Our study tests how proven experimental treatments interact in a well-established animal model, thus providing needed direction for the development of future combinatory treatment regimens.

Key words: spinal cord injury; regeneration; locomotion; plasticity; rat; treatment combination

Introduction

Spinal cord injury (SCI) in adult mammals comprises complex pathophysiological processes characterized by extensive cell death, incomplete self-repair, and limited axonal regeneration (for review, see Schwab and Bartholdi, 1996). These processes suggest that a combination of strategies may be required to prevent ongoing tissue damage and promote regrowth and reconnection of axons.

Successful experimental interventions used to promote regeneration of axotomized axons have relied on the following: (1) blockade of inhibitory cues within the injury site involving the glial scar and myelin [e.g., chondroitin sulfate proteoglycans (CSPGs) and NOGO, respectively] (Fawcett and Asher, 1999; Brosamle et al., 2000; Merkler et al., 2001; Bradbury et al., 2002; Li and Strittmatter, 2003; Silver and Miller, 2004); (2) supplementation of growth-promoting cues such as neurotrophic factors (Schnell et al., 1994; Mocchetti and Wrathall, 1995; Kobayashi et al., 1997; Ye and Houle, 1997; Blesch and Tuszynski, 2003), extracellular matrix components, or biopolymers (Novikova et al., 2002, 2003; Woerly et al., 2004); (3) or cellular transplantation (Li et al., 1997; Xu et al., 1997; Ramón-Cueto et al., 1998, 2000; Takami et al., 2002) (for review, see Bunge and Pearse, 2003); and (4) stimulation with agents capable of facilitating or activating the intrinsic capacity of neuronal regeneration programs (for review, see Pearse, 2004), including the elevation of intracellular cAMP (Neumann et al., 2002; Qiu et al., 2002) or manipulation of the Rho pathway (Dergham et al., 2002; Fournier et al., 2003).

Whereas treatments targeting these processes have individually proven efficacious in facilitating regeneration and functional recovery after SCI, the number of regenerating axons is generally small and functional recovery modest, illustrating the need to overcome multiple factors that limit axon growth. Therefore, it is conceivable that a combination of strategies may bring about increased axonal growth and functional recovery. In searching for the optimal combination strategy for SCI repair, a number of

studies have demonstrated the additive potential of two or more therapeutic approaches. Such strategies combine cell grafting with approaches including neuroprotective agents (Pearse et al., 2004b), exogenous or cellular overexpressed growth factors (Xu et al., 1995a; Bregman et al., 1997; Menei et al., 1998; Coumans et al., 2001), elevation of cAMP (Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004a), or scar-reducing enzymes (Chau et al., 2004). Combinatory pharmacological therapies have also been tested (Guth et al., 1994; Mu et al., 2000). With regard to Schwann cell (SC) implants specifically, the addition of methylprednisolone (Chen et al., 1996), neurotrophins (Xu et al., 1995a; Menei et al., 1998; Weidner et al., 1999; Bamber et al., 2001), olfactory ensheathing glia (OEG) (Ramón-Cueto et al., 1998), or elevated cAMP (Pearse et al., 2004a) substantially improved axonal regeneration.

The current study applies a combination of interventions by reducing inhibitory cues of the scar tissue (chondroitinase ABC), providing a growth-supportive substrate for axonal regeneration (SC bridge) and enabling regenerated axons to leave the bridge (OEG) in adult rats with complete spinal cord transection.

Materials and Methods

Cell culture
Schwann cells. SCs were obtained from sciatic nerves of adult female Fischer rats (Harlan, Indianapolis, IN) as described previously (Morrissey et al., 1991). Nerves were cut into small pieces and placed in culture dishes in DMEM/10% heat-inactivated fetal bovine serum (FBS) without mitogens. After 2 weeks, the pieces were transferred to new dishes where they were enzymatically dissociated and then replated in DMEM/10% FBS supplemented with three, rather than two, mitogens: bovine pituitary extract (2 mg/ml; Invitrogen Corporation, Carlsbad, CA), forskolin (0.8 μg/ml), and heregulin (2.5 nM; Genentech, San Francisco, CA) as described previously (Meijs et al., 2004). The purity of the SCs assessed by using the S100 and Hoechst stains was 95–98%.

Olfactory ensheathing glia. Highly purified cultures of OEG were prepared from the nerve fiber layer of the olfactory bulb of adult female Fischer rats (Harlan) using a procedure modified from one described previously (Ramón-Cueto et al., 1998). The pia was removed and care was taken to minimize the inclusion of non-nerve fiber layer bulb tissue. OEG were dissociated and, unlike the method of Ramón-Cueto et al. (1998), were exposed for 5 d to forskolin (0.8 μg/ml) and pituitary extract (2 mg/ml) in DMEM/F-12/10% FBS before purification by p75 immuno-panning as described previously (Takami et al., 2002). The OEG were used after 2 passages (at ~18 d after extraction from the olfactory bulb). A second period in mitogens preceded harvesting at confluency for transplantation. Checking the purity was similar to SCs except that p75, rather than S100, was used with Hoechst nuclear staining. Purity was found to be between 94 and 98%.

Preparation of the cells for grafting. Cultured SCs or OEG were harvested in DMEM/F-12 medium. Before transplantation, OEG were trypsinized to remove them from the dishes, and the cells were counted using a hemocytometer. SCs were resuspended in a 60:40 (v/v) solution of DMEM/F-12:Matrigel (BD Biosciences, San Jose, CA) immediately before grafting. Each animal received a total of 5×10^6 SCs for transplantation. OEG were resuspended in aliquots of 4 μl of DMEM/F-12 medium containing 4×10^5 cells immediately before spinal cord injection.

Surgical procedures
The experiments were performed using adult female Fischer rats (165–180 g; Charles River Laboratories, Wilmington, MA). All rats were kept at a 12 h light/dark cycle and received water and food *ad libitum*. A complete injury model ensured that regeneration of axons could be identified definitively.

The experiment was approved by the animal welfare committee at the University of Alberta. After arrival, the rats were handled for 2 weeks and
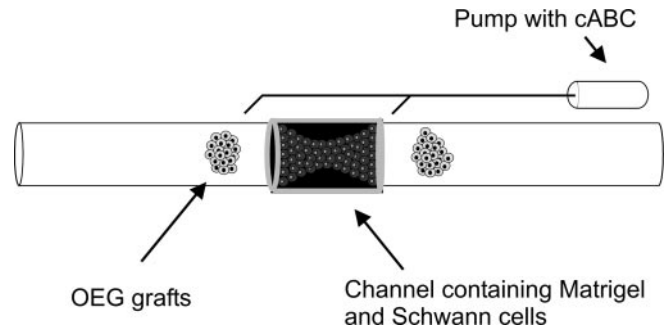

Figure 1. Experimental design for the cell grafting and cABC treatment after the removal of the thoracic 8 segment of the spinal cord.

trained to drink Ensure (complete meal replacement, Ross products division; Abbott Laboratories, Irving, TX) from a syringe.

For the different treatment combinations, the rats were divided into three experimental groups: (1) control group with transection and Matrigel-only bridge ($n = 8$); (2) transection with SCs and Matrigel bridge, rostrocaudal OEG grafts, and pump delivery of galactosidase and mouse IgG (referred to as graft-only group; $n = 7$); and (3) transection with SC:Matrigel bridge, rostrocaudal OEG grafts, and pump delivery of chondroitinase ABC and mouse IgG (referred to as cABC group; $n = 5$). The treatments are illustrated in Fig. 1.

The IgG was accommodated into the experiment as a control for an additional treatment group, which is not reported here.

Spinal cord transection and Schwann cell grafting
Animals were anesthetized with a subcutaneous injection of Hypnorm (120 μl per 200 g body weight; Janssen Pharmaceutics, Beerse, Belgium) and Midazolam (0.75 mg in 150 μl/200 g body weight; 750 μl total volume diluted with H$_2$O; Sabex, Boucherville, Quebec, Canada). Eye lubricant (Tears Naturale; Alcon, Mississauga, Ontario, Canada) was applied to protect the eyes from dehydration.

After a laminectomy at the T 7.5–9 vertebral level, the meningeal membranes were severed along with the dura mater, the spinal cord was completely transected, and a 4 mm region of spinal cord encompassing T8 was removed. The rostral and caudal stumps were lifted after removal of the spinal cord segment to ensure complete discontinuity. All spinal roots visible in the injury gap were removed, and bleeding was stopped using Gelfoam (Upjohn, Kalamazoo, MI). Thereafter, ~2×10^5 OEG were drawn into a glass pipette with a tip diameter of 150–200 μm mounted onto a 5 μl Hamilton syringe (Hamilton, Reno, NV) attached to a micromanipulator. The cells were deposited into two injection sites at the rostral as well as the caudal stump, ~2 mm from the lesion, at 300 μm lateral to the midline, and to a depth of 800 μm. A volume of 1 μl containing ~10^5 OEG in DMEM/F-12 was grafted into each site. Next, the rostral cord stump was inserted 2 mm into a 6 mm-long hollow, semipermeable 60:40 polyacrylonitrile/polyvinylchloride copolymer guidance channel (2.6 mm inner diameter, 3.0 mm outer diameter; provided by T. Hazlett, CytoTherapeutics, Providence, RI), previously cleaned and sterilized (Aebischer et al., 1988). The channel was then drawn back 1 mm to permit the insertion of the caudal stump 1 mm into the channel. Opening two small windows that had been crafted into the dorsal surface of the channel before implantation allowed 30 μl of SCs suspended in a 60:40 (v/v) solution of DMEM/F-12:Matrigel to slowly fill the channel. The windows were then closed and sealed with Matrigel, and surgery was halted for 5 min to allow semisolidification of the Matrigel-SC cable at 37°C.

In addition to the drug delivery using pumps (see below), immediately adjacent to the lesion, the wound was rinsed with chondroitinase or vehicle. The surgeon was blinded for both kinds of treatments.

cABC delivery
A double catheter was constructed by sealing two fine catheters (ReCathCo, Allison Park, PA) into a connecting tube from the minipumps (model 2002; Alzet, Cupertino, CA). The ends were cut to provide deliv-

ery at the rostral and caudal spinal cord outside the guidance channel, thus ensuring the delivery of the chondroitinase (cABC; Seikagaku America, East Falmouth, MA) to the sites of scarring at the SC bridge/host spinal cord interfaces. The tubing was fixed with sutures to the caudal musculature. The connected minipump contained sterile 0.9% NaCl to keep the catheters clear during the intervals between cABC injections (2 μl of 10 μg/ml cABC). Injections were performed every other day for 4 weeks under short isoflurane anesthesia. Pumps were placed into skin pouches on the back of the rats and kept in position with sutures. Finally, the overlying muscles were sutured in layers, and the skin was closed with wound clips.

Antibody delivery

A fine (32 gauge) intrathecal catheter (ReCathCo) was inserted subdurally from the lumbar level to its delivery site at the caudal SC bridge/host spinal cord interface. The catheter supplied the antibody (model 2002; Alzet) to the injury site for 4 weeks. Pumps contained antibody (IgG) at a concentration of 3 mg/ml in PBS, and the delivery rate of the pumps was 0.6 μl/h (=1.8 μg/h).

After surgery, the animals were kept on a thermostatically regulated heating pad until completely awake. The analgesic Buprenex (buprenorphine; Reckitt and Colman, Richmond, VA) was administered subcutaneously (0.03 mg/kg) immediately after operation and every 8 h for 74 h. Ringer's solution (4 ml) was given subcutaneously daily for the first week and at later stages if animals showed signs of dehydration. The antibiotic, cefazolin (5 mg/kg, s.c.; Novopharm, Toronto, Ontario, Canada) was administered once each day for 4 d starting at 1 d before surgery.

Bladders were expressed three times per day until the voiding reflex of the bladder was reestablished. For 2 weeks after injury, up to 1 ml of Ensure (chocolate or vanilla) was fed to each rat twice each day. After 2 weeks, pumps were exchanged under isoflurane anesthesia, and at 4 weeks, pumps and catheters were removed.

Evaluating chondroitin sulfate digestions

To demonstrate that cABC degraded chondroitin sulfate at the host cord/SC graft interface, three monoclonal antibodies that recognize either intact or digested chondroitin sulfate were used for immunochemical staining on alternative spinal cord sections of 2 week cABC-treated ($n = 2$) or galactosidase-treated grafted animals ($n = 3$). One antibody, 2B6 (1:150; Seikagaku America), recognizes an epitope created after cABC degradation of chondroitin-4 sulfate and does not identify the intact form (Moon et al., 2001). The other antibodies used were CS-56 (1:500; Sigma-Aldrich, St. Louis, MO), which recognizes an epitope located on various intact chondroitin sulfate glycosaminoglycan chains, and 1D1 (1:200; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), which recognizes the CSPG core protein of neurocan (Moon et al., 2002). An increase of 2B6 and a reduction in the amount of either CS-56 or 1D1 immunoreactivity at the interface would indicate cABC efficacy. To measure changes in immunoreactivity intensity of 2B6, CS-56, and 1D1 at the caudal host cord/SC graft interface, 40 μm sagittal sections at every 200 μm (10–12 sections/animal) were evaluated using computer-assisted microscopy and MetaMorph software (Universal Imaging Corporation, West Chester, PA). In each section, images encompassing an 800 × 800 μm area equally overlapping both SC and the contiguous host cord region of the caudal interface and symmetrical to a line drawn through the center of the cord were evaluated. Immunoreactivity intensity (arbitrary units; higher value indicating greater staining) for each antibody at the caudal interface was determined per animal by averaging measured intensity values across all sections examined.

Testing locomotor recovery

Recovery of hindlimb stepping movements was evaluated using several locomotor tests to determine whether recovery was attributable to increased excitability of spinal networks or to partial reestablishment of descending control.

Basso, Beattie, and Bresnahan score. The open-field locomotor score (Basso et al., 1995) was determined by two blinded observers. Before testing, bladders were expressed, because spontaneous bladder contraction often accompanies hindlimb activity. The rats were placed in a trans-

parent Plexiglas box (30 × 90 × 120 cm) with a smooth floor and were observed for 4 min. Hindlimb movements immediately after contact with experimenters were disregarded. During the open-field activity, the animals were also video-monitored with a digital camera using a shutter speed of 1/250 s for additional analysis of forelimb/hindlimb coupling.

Forelimb/hindlimb coupling. This analysis adds valuable information to the Basso, Beattie, and Bresnahan (BBB) score, because both the frequency of hindlimb activity and the coordination with the forelimb during walking sequences are taken into account. To quantify forelimb/hindlimb coupling, the order of steps made by an ipsilateral forelimb/hindlimb pair was recorded. Percentage scores of forelimb/hindlimb coupling were determined by dividing the number of correct couplings (in which a forelimb step was followed by a hindlimb step, or vice versa) by the total number of couplings in the sequence (the number of steps in the sequence minus one). For this analysis, five video sequences per animal, with a minimum of six forelimb steps, were chosen.

Role of sensory feedback on hindlimb movement. A major issue when testing locomotor recovery is to resolve whether hindlimb movements are triggered by volitional drive or by sensory feedback from the hindlimbs passively sliding over the ground. To investigate this question, we tested open-field locomotion on different surfaces. In addition to the smooth Plexiglas surface in the open field described above, we used a slippery surface and a rough surface. These tests are new and have not been described in the literature. To test locomotion on a slippery surface, we used a Plexiglas runway (80 × 10 cm, with 10 cm high walls) with its walking surface covered by a thin layer of paraffin oil. On this surface, forward propulsion was reduced to "stationary walking" when rats performed stepping movements with their forelimbs. Therefore, the amount of sensory feedback to the hindlimbs from passive sliding was limited. Events where forelimb and hindlimb movements occurred in a stepping sequence were scored. The test was performed once at 9 weeks after transection and only in animals with BBB scores of 6 and higher.

To test the influence of increased sensory feedback, the rats were placed on a rough carpet, and the forelimb/hindlimb couplings were analyzed as described during open-field locomotion. This test was also performed at 9 weeks after transection; all animals were tested on the carpet.

Sensory testing

Each rat was wrapped in a towel and held such that its covered head could move freely, and the hindlimbs remained exposed. Because the rats were used to handling and restraining, they remained calm, and five different locations on dermatomes that lost their ascending projections (i.e., base of the tail, skin above the femur on both hindlegs, and the dorsal surfaces of both hindpaws) were stimulated with a set of von Frey hairs (Stoelting, Wood Dale, IL). The von Frey hair was placed on the skin and pressure applied (target force, 0.04–1 g), so that it would slightly bend and then be directly removed. The stimulation started with a low intensity (small hair diameter; 0.04 g target force) and was repeated three times. If no twitch response was observed, the next thicker hair was used. The lowest stimulation intensity to produce a response was repeated three times before the sensation threshold was recorded. Repeated stimulations of any given spot were separated by at least 2 min to avoid desensitization.

Tracing and histology

At 10 weeks after injury, the corticospinal tract (CST) and reticulospinal tract (RtST) were unilaterally traced under Hypnorm/Midazolam anesthesia (as described above). For the CST tracing, 2 μl of biotinylated dextran amine (BDA) [molecular weight (MW), 10,000; D-1956, 10% in 0.01 M PBS; Molecular Probes, Eugene, OR) were injected into four sites in the sensorimotor cortex using a Hamilton syringe as described previously (Fouad et al., 2001). For tracing of the RtST, a long-necked glass microelectrode mounted onto a Hamilton syringe was stereotaxically guided into the giant nucleus of the reticular formation (coordinates from Paxinos and Watson, 1986). A volume of 0.35 μl 5% Micro Ruby (dextran, tetramethylrhodamine, and biotin; 3000 MW; Molecular Probes) was slowly injected over a period of 5 min.

Two weeks after the tracing, the animals were killed with pentobarbital (Euthanyl, Bimeda-MTC; 70 mg/100 mg body weight) and transcardially

perfused with phosphate-buffered 4% paraformaldehyde (0.1 M, pH 7.4) (Takami et al., 2002). The spinal cord and brain were removed and postfixed overnight in the same fixative at 4°C. The spinal cords were examined under a surgical microscope for continuity of the bridge and the spinal cord and photographed. A 1-mm-thick transverse slice was taken from the middle of the SC bridge and placed in 2% glutaraldehyde with 100 mM sucrose in phosphate buffer (PB; 0.05 M, pH 7.4) for at least 24 h at 4°C. This tissue was then further fixed in 1% buffered osmium tetroxide for 2–16 h, dehydrated, embedded in Epon-Araldite (Electron Microscopy Sciences, Fort Washington, PA), and cut transversely in semithin sections (1 μm). These sections were stained with a 1% toluidine blue–1% methylene blue–1% sodium borate solution to analyze SC bridge histology and to determine the number of myelinated axons and blood vessels therein. The remaining SC bridge and adjacent rostral and caudal spinal cord and brain were cryoprotected in 30% sucrose in PB (0.1 M, pH 7.4) for at least 24 h, frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and cut into 30-μm-thick sagittal sections. Efficacy of the tracing was evaluated in cervical cord sections.

Total numbers of myelinated axons and blood vessels were counted as described previously (Xu et al., 1995b). Briefly, in stained, 1-μm-thick transverse plastic sections (three sections from a 1 mm slice from the center of the SC bridge; $n = 6$ per group), the bridge area was outlined and then scanned using a fractionator grid at 630× magnification (optical fractionator method; West and Collins, 1989). Every blood vessel and every myelinated axon present within each grid square (surface area, 0.028 mm^2) was counted.

Immunohistochemistry
The sections chosen for evaluation were postfixed a second time on the slides with 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.1% picric acid for 20 min and then washed in 0.1 M PB three times. A quenching step was added by leaving the sections for 10 min in 50% EtOH plus 0.3% H$_2$O$_2$, followed by two washing cycles in PB for 10 min. Blocking was performed with 0.4% NaBH$_4$ for 10 min. After washing the slides in PB three times for 20 min, they were incubated with 0.1 M citrate buffer, pH 4.5, overnight at 4°C.

The next day, the slides were warmed to room temperature and microwaved for 1 min at 600 W, followed by two cycles of 30 s at 600 Watts in a separate glass container in 200 ml of H$_2$O. Slides were left to cool for 30 min and were subsequently blocked in 1% Top Block gelatin (Juro Supply, Lucerne, Switzerland) for 20 min [200 mg of Top Block in 0.3% Tris-buffered saline with Triton X-100 (TBS-TX)]. The 5-HT antibody (DiaSorin, Stillwater, MN) was added in a dilution of 1:8000 containing 4% normal goat serum (NGS) and left on the slides overnight at 4°C. Slides were washed twice in PB for 10 min before the second biotinylated goat anti rabbit antibody (1:300 in 0.3% TBS-TX plus 2% NGS) was added and left for 45 min at room temperature. Slides were then rinsed twice in PBS for 10 min, and the ABC elite complex was added for 45 min. After three washing steps of 10 min each in PBS, they were preincubated with 0.05 M Tris, pH 8.0, for 5 min. Slides were then reacted in 50 ml of 0.05 M Tris plus 25 mg of DAB plus 25 μl of 30% H$_2$O$_2$ for ~1–2 min. The reaction was stopped in ice-cold PBS, followed by two washes in PB for 10 min and one rinse in water. Slides were dried overnight and coverslipped.

5-HT fiber counts
The number of 5-HT-positive fibers caudal to the lesion site was analyzed quantitatively using a light microscope with bright-field illumination at a final magnification of 400×. Because alternating 25 μm sagittal sections were cut to visualize CST and RtST fibers and for spare sections, the sections chosen for the analysis of 5-HT-positive fibers were separated by 75 μm intervals. This minimized the possibility of counting the same fiber twice. On each of these remaining sections (an average of ~11 sections per animal), all fibers in the caudal spinal cord with a length of >100 μm were counted.

Corticospinal and reticulospinal tract staining
Sections were first observed under a fluorescent microscope to detect labeling with Micro Ruby. In all animals, fibers were detected rostral to the lesions and, in some cases, in the bridge.

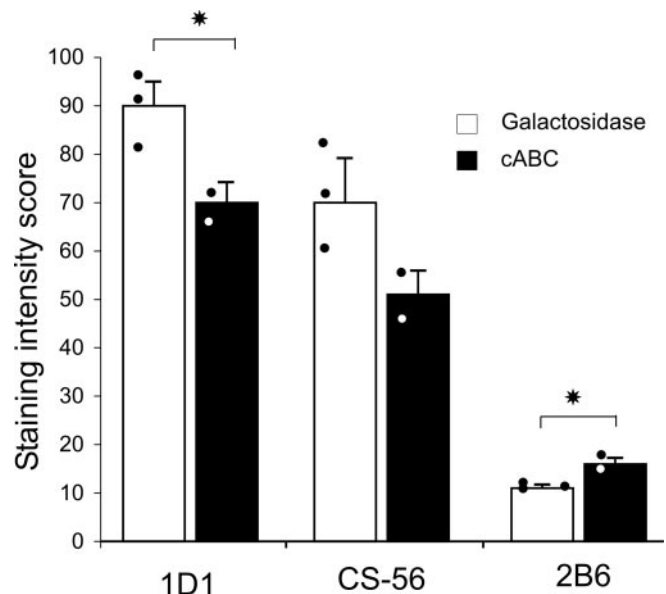Sections were then processed with avidin-HRP (ABC Elite; Vector

Figure 2. Effectiveness of cABC application as demonstrated by the use of antibodies against either intact (1D1, CS-56) or digested (2B6) chondroitin sulfate. *$p < 0.05$.

Laboratories, Burlingame, CA) followed by a nickel-enhanced diaminobenzidine HRP reaction for the visualization of the BDA tracer, in a semifree-floating technique as described previously (Herzog and Brosamle, 1997). Because both CST and RtST fibers were absent at the caudal end of the bridge or in the caudal stump of the spinal cord, no additional analysis was performed.

Statistics
Before statistical analysis, exclusion criteria were defined in an attempt to reduce the variability in the groups: animals in which a spinal cord stump (rostral or caudal) had pulled out of the channel, or the injection of the OEG created inflammation and subsequently a lesion that was larger than 50% of the spinal cord.

Statistical comparisons were performed using GraphPad Prism. Comparisons between the treatment groups were made using the Mann–Whitney U test. Differences of $p < 0.05$ were considered significant; $p < 0.01$ was considered highly significant. Errors are given as SEM.

Results
cABC degraded chondroitin sulfate in injured spinal cord
Two weeks after spinal cord transection, tissue from three animals with grafts and two that also received cABC was examined to determine whether cABC had degraded chondroitin sulfate. Three monoclonal antibodies that recognize either digested or intact chondroitin sulfate were used; an increase of 2B6 and reduced CS-56 and 1D1 immunoreactivity at the caudal SC/spinal cord interface indicated cABC efficacy. The average values of staining intensity were as follows: for Neurocan (1D1), 89.6 ± 5.1 (SEM) and 70.3 ± 4.4; for chondroitin sulfate proteoglycans (CS56), 69.8 ± 8.6 and 50.7 ± 5.3; for degraded chondroitin sulfate proteoglycans, 10 ± 0.6 and 16.2 ± 1.1 in the controls and the cABC-treated rats, respectively (Fig. 2).

Partial locomotor recovery followed combined treatment
Initially, after the spinal cord transection, all animals exhibited complete, flaccid paralysis of the hindlimbs. Slight recovery, represented by spontaneous movements of up to two joints of the hindlegs, was found over the next 5 weeks after injury (Fig. 3C). Rhythmic limb movements were not observed before 6 weeks after lesioning. Because of the extensive laminectomy, many animals suffered from severe lordosis, manifested by a hunched
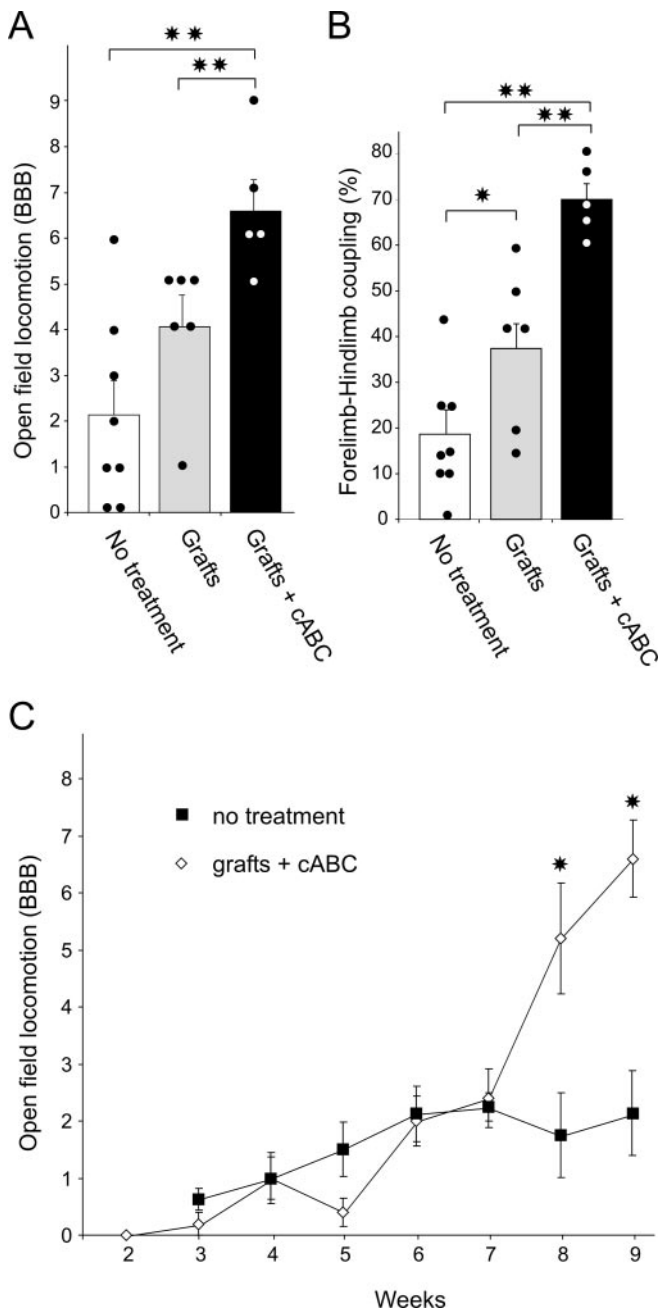
Figure 3. Comparison of the open-field locomotion scores between the different treatment groups quantified with the BBB score (*A*) and by using the percentage of successful forelimb/hindlimb coupling (*B*). *C*, The time course of recovery shows treatment-induced benefits start to become apparent at 8 weeks after surgery. The dots represent the scores of single animals. *$p < 0.05$, **$p < 0.01$.

posture rostral to the laminectomy site. Some rats also displayed signs of spasticity, including asymmetric muscle tone resulting in asymmetric body posture. When spasticity was quantified using a subjective ranking score (ranging from 0, no sign of spasticity, to 3, extensive spasticity), no difference was found between groups (data not shown). Both lordosis and spasticity impacted locomotor ability and consequently limited the animal's functional recovery independently of the ablation of descending axons.

One unexpected finding was that some treated rats exhibited rhythmic and alternating movements of the hindlegs during phases when the animals were standing and the forelimbs did not

step. This could be interpreted as spontaneous activity of the pattern generating networks in the lumbar spinal cord. These sequences were excluded from the evaluation of open-field locomotion using the BBB rating scale.

In none of the animals were the spinal cords pulled out of the channels, but in one animal (grafts only), the rostral injection of OEG resulted in strong inflammation and tissue destruction, creating a second lesion rostral to the transection spanning ~80% of the spinal cord. Because this fulfilled the exclusion criteria, this animal was removed from the study.

Open-field locomotor score
Behavioral testing was performed until 9 weeks postlesion (Fig. 3*A*). The average score in the control group ($n = 8$) was 2.1 ± 0.7 (SEM), in the group with transplants was 4 ± 0.6 ($n = 6$), and in the cABC group was 6.6 ± 0.7 ($n = 5$). The average BBB score of the cABC group was significantly higher than that of the other groups ($p < 0.01$). The group with the transplants did not exhibit any significant improvements in the BBB score when compared with the control group.

No significant difference between the cABC and control groups was seen until 8 weeks after injury (Fig. 3*C*). During the 7 weeks after injury, the BBB scores ranged between 0 and 4 points. The first signs of recovery in locomotor functions were observed and were statistically significant between the cABC group and the rats with Matrigel only, at 8 weeks. The results indicate that the recovery did not plateau at 9 weeks after the injury, the time point at which testing was discontinued. A representative walking sequence of a rat from the cABC group with forelimb and hindlimb activity is shown in Figure 4.

Forelimb/hindlimb coupling
This analysis was added to include information on the tendency of forelimb and hindlimb movements to alternate. Forelimb/hindlimb coupling is considered in the BBB score only in animals that are able to perform weight-supported plantar stepping. However, in animals with lower locomotor capabilities, this is also an important outcome measure. A measure of the frequency of movements and a relation to forelimb stepping is especially needed after complete SCI. Therefore, the percentage of hindlimb steps after forelimb steps was analyzed.

For assessment, one video recording for each animal at 9 weeks after injury was used. The results are summarized in Figure 3*B*. As in the open-field locomotor score, the untreated rats scored the lowest percentage of forelimb/hindlimb coupling with $18.5 \pm 0.5\%$. This was statistically different from the group with grafts only ($38.3 \pm 0.7\%$). The best coupling was evident in the group with grafts and cABC ($70 \pm 3.4\%$). This improvement was highly significant compared with both the untreated and the grafted groups.

Role of sensory feedback in hindlimb movement
To examine whether hindlimb movements are triggered by sensory feedback resulting from the sliding of hindlegs during forelimb stepping or rather by descending commands from reestablished motor tracts, we examined locomotion on three different surfaces. The first was a smooth Plexiglas surface used for all open-field testing, the second was a Plexiglas surface covered with a thin layer of oil (referred to as a slippery surface), and the third was a carpet (rough surface).

None of the groups on the rough surface were able to verify the prediction that increased cutaneous feedback to the hindlimbs from sliding over the surface would enhance the hindlimb activity. Because the carpet increased friction, the forelimbs were able

to increase propulsion, and this resulted in a nonquantified, but obvious, increase in walking speed. The forelimb/hindlimb coupling score was either reduced ($p < 0.05$ in four animals) or statistically unchanged. Walking of the fore limbs on the slippery runway (and if necessary, a slight tilt of the surface) resulted in stationary walking. The rats produced a rhythmic stepping pattern with the forelimbs without moving the rest of the body relative to the surface, thus, strongly reducing sensory feedback to the hindlimbs. Under these conditions, it was hard to motivate the rats to walk, and only very few stepping sequences were recorded. We focused on sequences in which forelimb locomotion was tied to hindlimb activation, because this would support the idea that forelimbs and hindlimbs are coupled and direct descending control is involved in triggering locomotion. Alternating activity of both forelimbs and hindlimbs during stationary walking was found in only two of five rats in the cABC group and in no animals in the remaining groups. This result supports the idea that in animals with alternating forelimb/hindlimb activity, a reconnection of the caudal with the rostral spinal cord has occurred.

Figure 4. Video sequence from a rat treated with OEG and Schwann cell grafts as well as with cABC. Note the range of leg motion as well as the alternation between the forelimb and hindlimb movements. The panels are organized in rows from left to right.

Cell grafts increased response to tactile stimuli

Spinal cord injury is often accompanied by increased responses to mechanical stimuli of cutaneous receptors (allodynia) below the lesion (Siddall et al., 1995; Hutchinson et al., 2004). To test whether the experimental treatments influenced these responses, we tested the reaction to mechanical stimulation with calibrated von Frey hairs at five different locations below the lesion. A general finding was that in all rats, the base of the tail was the most sensitive and most reliably excitable location. Because the relation of excitability at the different locations was consistent between animals, only data gathered from stimulation at the base of the tail are presented. The lowest sensitivity (highest target force to trigger a response) was found in untreated rats (Fig. 5); the average target force at the tip of the von Frey hairs was 0.63 ± 0.1 g. This was significantly higher than in rats with grafts only (0.31 ± 0.07 g) and with grafts and cABC (0.33 ± 0.08 g).

Grafts and cABC enabled serotonergic fiber regeneration

In this study, we focused on descending axons from three groups of neurons: the hindlimb area of the motor cortex, the medial reticular formation, and the raphe nuclei, which project serotonergic (5-HT-positive) fibers.

Serotonergic fibers were detected within the graft and caudal to it. Even more noteworthy was the abundance of highly branched serotonergic fibers in the caudal spinal cord (Fig. 6A). The quantitative analysis of these fibers caudal to the bridge revealed a high variability within the groups. Because of the large SD, the increase caused by grafting was not significant (70.1 ± 12.2, SEM in the rats with grafts only). This increase was further enhanced by the application of cABC (94 ± 14.5) and was statistically significant when compared with the untreated animals (15.5 ± 2.2) (Fig. 6B). Although serotonin in the lumbar spinal cord of injured rats has powerful effects on locomotor function (Ribotta et al., 1998, 2000), no correlation between the number of serotonergic fibers found caudal to the bridge and locomotor recovery was found in our experiment (Fig. 6C).

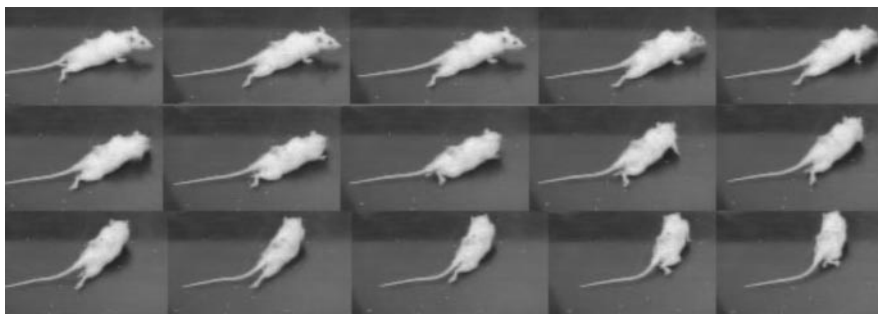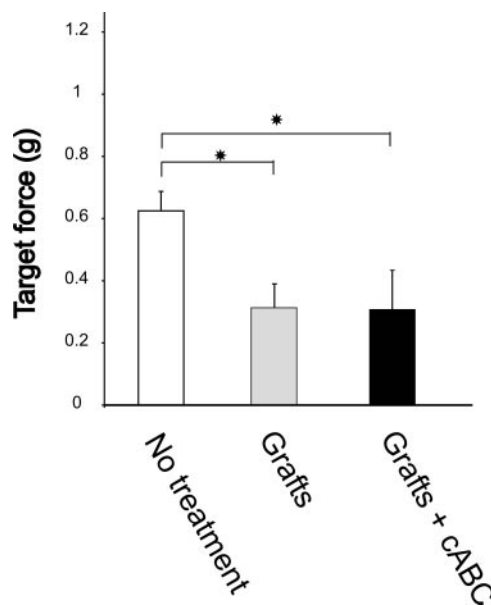Detectable tracing of axons of two descending motor tracts

Figure 5. Sensory testing using von Frey hairs at the base of the tail shows increased responsiveness in all of the treated groups. *$p < 0.05$.

(CST and RtST) was confirmed rostral to the lesion. Only a few of these traced axons were able to enter the bridge, and none were observed to enter the caudal part of the spinal cord (data not shown).

Myelinated axons and blood vessels in the bridge

Quantification of myelinated axons and blood vessels was performed in transverse 1 μm sections obtained from the center of the bridge. These counts likely include both descending and ascending fibers that cannot be distinguished by this approach (Fig. 7). The lowest count was found in the control group with an average of 1009 ± 237 (SEM) myelinated axons. In the group with grafts, the average number was (1949 ± 486), which was not statistically different. The cABC group displayed significantly higher numbers of myelinated axons when compared with the control group (3058 ± 600) (Fig. 8A).

When the individual axon counts were compared with the functional outcomes (open-field locomotion and forelimb/hindlimb coupling), a significant correlation ($r = 0.63$) was found (Fig. 8C), indicating that axonal regeneration through the bridge is involved in the observed functional recovery.

The number of blood vessels in the bridge was not statistically increased when comparing either of the groups (Fig. 8B). In-
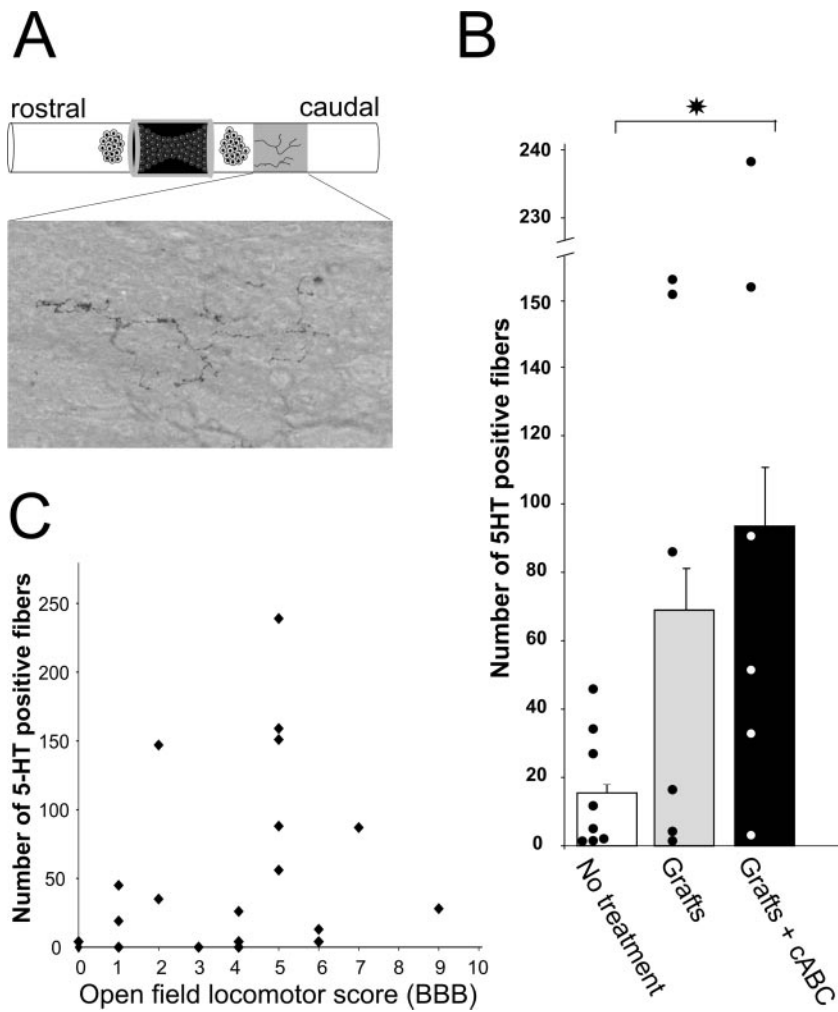
Figure 6. *A*, 5-HT-positive fibers were found to grow into the caudal spinal cord. The quantification of these fibers in the caudal spinal cord is illustrated in *B*. As documented in *C*, there was no correlation between individual functional recovery and the number of 5-HT-positive fibers in the caudal spinal cord. *$p < 0.05$.
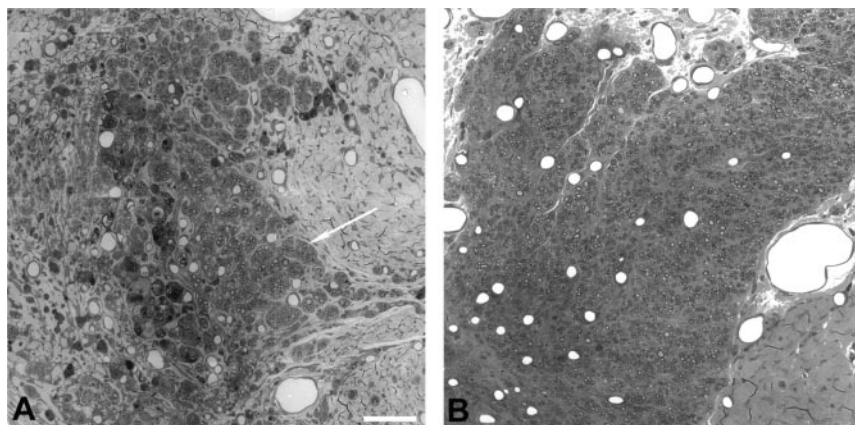

Figure 7. Significantly greater numbers of peripheral-myelinated axons (arrow) are present within the Schwann cell bridge after digestion of CSPGs with chondroitinase ABC. Animals with grafts only (*A*) and animals with grafts and cABC (*B*) are shown. Scale bar, 150 μm.

and grafts/cABC were 264 ± 43 (SEM), 357 ± 43, and 414 ± 50, respectively.

Discussion

Testing the interaction of established experimental treatments is an important step in developing additive, more efficacious treatments for SCI. Our study addresses this need and provides direction for the refinement of future treatments. When compared with untreated rats, only the group of animals subjected to cell grafts and cABC treatment showed statistically significant improvements in locomotor recovery quantified by both the BBB score and forelimb/hindlimb coupling measurements. Recovery occurred only near the endpoint of the study, at 8 weeks after the injury, a time that would theoretically have been sufficient for axonal regeneration through the graft and into the caudal stump (Xu et al., 1995b, 1997; Steward et al., 2003).

It is very likely that the functional benefits of the current treatments are actually much greater than shown for at least two reasons. First, the time course illustrates that recovery did not plateau at the end of the allotted testing period. A longer experimental time course would have been beneficial in determining the long-term benefits of the combinatory treatment but was not chosen because of small animal numbers in the groups, and the possible loss of animals before fiber tracing had been completed. Second, the laminectomy of three vertebral segments without artificial stabilization resulted in strong lordosis, further hampering locomotor recovery. The hunched body posture would have hindered plantar and weight-supported stepping, even if the nervous system had recovered sufficient motor control. Nevertheless, the results clearly demonstrate that the grafts consisting of SCs and OEG and the application of cABC have additive effects. Our results are the first to demonstrate the beneficial effects of cABC on axonal regeneration after complete spinal cord transection and support previous reports in which cABC application also has shown salutary effects after brain injury (Moon et al., 2001) and incomplete lesions of the spinal cord (Bradbury et al., 2002; Yick et al., 2003). The application of cABC appears especially suited when applied in conjunction with grafted tissue, because scar tissue at the graft-host interface represents a barrier for axons to grow into and through the graft (Chau et al., 2004).

It is noteworthy that one control animal reached a BBB score of 6 and its recovery progressed faster than in the treated group. This recovery was unexpected, because the surgery eliminated

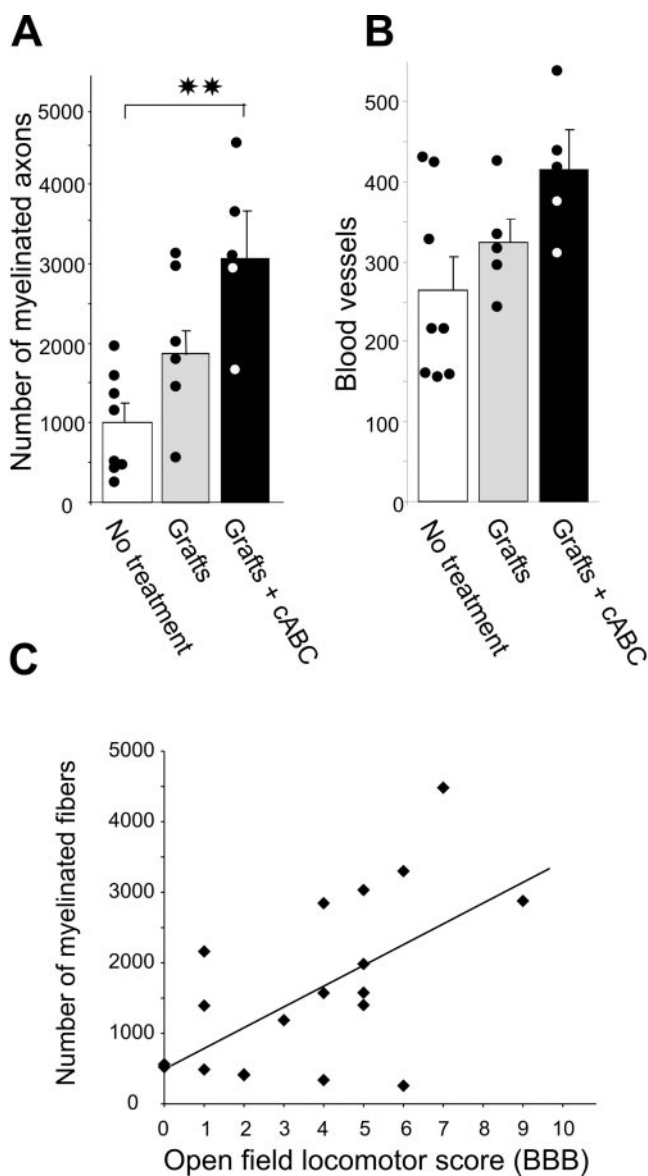creased vascularization of the graft would be desired to enhance the viability of the grafted cells within the bridge and to improve support of axonal regeneration. The average numbers of blood vessels in the graft for the groups with no treatment, grafts only,

A

B

C

Figure 8. Counts of myelinated axons in the middle of the bridge showed a significant ($p <$ 0.01) increase (A) in the group with grafts and cABC. B, The number of blood vessels in the bridge was not different between the groups. C, There was a significant correlation ($r^2 = 0.63$) between individual functional recovery and the number of myelinated axons.

enter the matrix when the collagen is implanted in solid gel form (Joosten et al., 1995). This study also suggested that when the implant is in fluid form, it may better conform to the surface of the cord stump.

A major question after experimental treatments to repair the injured spinal cord is whether observed functional recovery is caused by regenerated fibers reconnecting to appropriate targets below the lesion or changes in existing circuitries above and below the lesion (for review, see Fouad and Pearson, 2004). Whereas some studies have reported axonal regeneration and functional recovery after complete SCI because of treatment (Cheng et al., 1996; Ramón-Cueto et al., 2000), very few have provided evidence of reconnection. One link between regeneration and functional recovery is histological evidence of fibers growing through the graft and into the caudal stump of the spinal cord. Traced fibers of the CST and the RtST were found to enter the SC bridge, but none were found to grow through and out of the bridge into the contiguous caudal cord.

In contrast, in rats with grafted cells in our study, 5-HT fibers that grew through the bridge and projected into the caudal stump were found in abundance. This confirms previous experiments in which SC bridges combined with OEG grafts led to the presence of 5-HT fibers caudal to the bridge occupying a complete transection gap (Ramón-Cueto et al., 1998). Because the application of serotonin or the grafting of serotonin-secreting cells to the caudal spinal cord is capable of promoting significant locomotor recovery (Ribotta et al., 2000; Antri et al., 2002), it seems counterintuitive that no correlation between an increased number of serotonergic fibers and locomotor recovery was found. Possible explanations could be that even greater numbers of regenerating serotonergic fibers are necessary to promote locomotor recovery, they did not grow far enough into the lumbar cord, or the fibers were not functional.

The significant correlation that we found between functional recovery and the number of myelinated axons in the center of the bridge suggests that fibers other than CST, RtST, or serotonergic axons were involved in the observed recovery. Plausible candidates are axons of propriospinal interneurons that are known to grow through SC bridges (Xu et al., 1995b, 1997); the interneurons can be used as relays for lesioned axons sprouting rostral to a lesion (Fouad et al., 2001; Raineteau and Schwab, 2001; Raineteau et al., 2001; Bareyre et al., 2004). Propriospinal interneurons were not traced in the present study and should be monitored using retrograde tracers in future work. An alternative approach to confirm the relationship between regeneration and functional recovery, a relesion of the spinal cord, was performed only in one rat, because it would have interfered with the anterograde tracing used in our study.

A finding supporting a reestablished connection via the lesion site comes from behavioral testing on a slippery surface. During "stationary" forelimb stepping, sensory feedback to the hindlimbs was reduced to a minimum, because no sliding over the surface occurred. Under these circumstances, the hindlimbs of a few rats with high locomotor scores were activated and alternating with the forelimbs, indicating that both forelimb and hindlimb stepping was triggered by descending input. Promoting axonal regeneration in the injured spinal cord does not only involve descending motor axons. Sprouting and growth of sensory fibers will also be enhanced. Although our study did not monitor sprouting of sensory fibers, we assessed responses to tactile cutaneous stimuli in dermatomes caudal to the lesion. The finding that treated rats presented significantly increased sensitivity to tactile stimuli impacts the clinical value of regeneration-

any chance of axonal sparing. Even a relesion 9 weeks later could not reduce hindlimb activity. This phenomenon illustrates the plastic capabilities of the spinal networks controlling locomotion (for review, see Pearson, 2000; Edgerton et al., 2001; Rossignol et al., 2002).

Another unexpected result was the considerable number of myelinated axons and serotonergic axons observed in Matrigel-only grafts; these fibers were not found in Matrigel-only bridges in previous studies (Xu et al., 1997). This and the finding that in none of the animals was the spinal cord pulled out of the channel may be attributable to the new technique for implanting an SC bridge used in this study. Inserting the channel first, then adding the SCs in fluid Matrigel through windows on the dorsal surface of the channel, may enable better apposition between the SCs and the spinal cord stumps. It has been reported that HRP-labeled CST axons enter a graft of collagen when the collagen is introduced in fluid form, which self-assembles *in situ* but does not

promoting treatments, because increased pain after a treatment would be undesirable. However, our results do not necessarily have to be interpreted as increased pain or allodynia. Paralyzed rats showed signs comparable with spasticity in human patients, and thus the increased responses to tactile stimuli could simply be a sign of increased spasticity triggered by disinhibition of spinal pathways (Calancie et al., 1993) or changes in neuronal properties (Bennett et al., 2004; Li et al., 2004). Increased sensory feedback has been suggested to accentuate locomotor recovery after SCI (Pearson, 2001). Changes in sensitivity as reported above would predict enhanced stepping movements when walking on a rough surface because of greater levels of cutaneous feedback. However, this was not the case. Rather, it appears that changes in ground texture during a walking sequence initiate reflex-like stepping.

The implications of the current study form an important stepping stone toward the development of a combined treatment for SCI. Additional treatment strategies that involve the neutralization of myelin-associated inhibitors by blocking Nogo A, Nogo 66, MAG, and oligodendrocyte–myelin glycoprotein (Merkler et al., 2001; GrandPre et al., 2002; Liu et al., 2002), the application of growth and survival-promoting neurotrophic factors (Schnell et al., 1994; Mocchetti and Wrathall, 1995; Xu et al., 1995a; Kobayashi et al., 1997; Ye and Houle, 1997; Menei et al., 1998; Bamber et al., 2001; Blesch and Tuszynski, 2003), and the stimulation of the intrinsic capacity of neurons to grow (Dergham et al., 2002; Neumann et al., 2002; Qiu et al., 2002; Fournier et al., 2003; Lu et al., 2004; Nikulina et al., 2004; Pearse et al., 2004a) should be integrated and tested for their compatibility after complete transection of the spinal cord.

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