Polyethylene Glycol Rapidly Restores Axonal Integrity and Improves the Rate of Motor Behavior Recovery After Sciatic Nerve Crush Injury

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1Department of Psychology, 2Institute for Cellular and Molecular Biology, 3Section of Neurobiology, and 4Institute for Neuroscience, University of Texas at Austin, Austin, Texas; 5Department of Neurosurgery, University of Michigan, Ann Arbor; and 6Department of Neurology, Henry Ford Health Science Center, Detroit, Michigan

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Britt JM, Kane JR, Spaeth CS, Zuzek A, Robinson GL, Gbanaglo MY, Estler CJ, Boydston EA, Schallert T, Bittner GD. Polyethylene glycol rapidly restores axonal integrity and improves the rate of motor behavior recovery after sciatic nerve crush injury. J Neurophysiol 104: 695–703, 2010. First published May 5, 2010; doi:10.1152/jn.01051.2009. The inability to rapidly (within minutes to hours) improve behavioral function after severance of peripheral nervous system axons is an ongoing clinical problem. We have previously reported that polyethylene glycol (PEG) can rapidly restore axonal integrity (PEG-fusion) between proximal and distal segments of cut- and crush-severed rat axons in vitro and in vivo. We now report that PEG-fusion not only reestablishes the integrity of crush-severed rat sciatic axons as measured by the restored conduction of compound action potentials (CAPs) and the intraaxonal diffusion of fluorescent dye across the lesion site, but also produces more rapid recovery of appropriate hindlimb motor behaviors. Improvement in recovery occurred during the first few postoperative weeks for the foot fault (FF) asymmetry test and between week 2 and week 3 for the Sciatic Functional Index (SFI) based on analysis of footprints. That is, the FF test was the more sensitive indicator of early behavioral recovery, showing significant postoperative improvement of motor behavior in PEG-treated animals at 24–48 h. In contrast, the SFI more sensitively measured longer-term postoperative behavioral recovery and deficits at 4–8 wk, perhaps reflecting the development of fine (distal) motor control. These and other data show that PEG-fusion not only rapidly restores physiological and morphological axonal continuity, but also more quickly improves behavioral recovery.

INTRODUCTION

Crush-severance is the most common form of traumatic injury to PNS axons in humans (Bozkurt et al. 2007, 2008). Crush- (or cut-) severance injuries completely disrupt the axolemma at the lesion site and also produce smaller plasmalemmal holes proximal and distal to the severance site (Fig. 1B; Bittner et al. 1986; Lore et al. 1999). Distal segments of severed mammalian PNS and CNS axons undergo Wallerian degeneration within 12–72 h (Ramón y Cajal 1928; Waller 1850). If proximal PNS axonal segments survive axotomy, regeneration by outgrowth can occur at rates of 1–2 mm/day (Hadlock et al. 2005; Ramón y Cajal 1928). Thus acute (within days) recovery in mammals is nonexistent. Chronic behavioral recovery is delayed and often very inadequate or nonexistent because PNS outgrowths take months to years to reach denervated target tissues in larger mammals such as humans and those target tissues are often nonspecifically reinnervated (Bozkurt et al. 2007, 2008; Das and Wallace 1986; Ingoglia and Murray 2001).

In the last several decades, various procedures have improved the number and specificity of PNS axons that reestablish connections following severance, but not the outgrowth rate or time for PNS axons to reestablish those connections. For example, nerve grafts (Jeng and Coggeshall 1986; Lago et al. 2007), connective tissue matrices (Bozkurt et al. 2007; Herbert et al. 1996; Lore et al. 1999), and nerve growth guides (Aebischer et al. 1990; Kalbermatten et al. 2009) have all been reported to improve the extent of regeneration by severed PNS axons, but not the rate or time at which PNS axons reestablish their connections.

We now describe the further use of an unconventional technique to improve the time to reinnervate and specificity of acute and chronic repair of mammalian PNS axons by directly applying polyethylene glycol (PEG) solutions to the lesion site of severed axons (Fig. 1, B–E).

This PEG-fusion technique and its rationales are as follows: Ca2+ influx through partially constricted axonal ends and nearby small holes produced by the trauma of crush-severance (Fig. 1B) induces vesicles derived from nearby undamaged membranes (Eddleman et al. 1997), lysosomes (Reddy et al. 2003), and/or myelin delaminations (Ballinger et al. 1997) to migrate, accumulate, and pack tightly at the damage site. These membrane-bound structures interact with each other and nearby, undamaged membrane to continuously reduce the influx and efflux of ions and other substances until a complete seal is formed in 10–20 min (Bittner and Fishman 2000). Eventually, the plasmalemma is completely repaired (Fig. 1A) and vesicles are no longer observed 24 h after severance (Lichstein et al. 1999). Vesicle interactions are practically impossible to image in vivo in small-diameter unmyelinated or myelinated mammalian axons, but biochemical and dye exclusion data show that the same proteins and processes are involved with time courses similar to those in invertebrates (Bittner and Fishman 2000; Detrait et al. 2000a,b; Nguyen et al. 2005; Yoo et al. 2003, 2004).

Bathing recently severed invertebrate or mammalian axons in Ca2+-free hypotonic salines containing EGTA opens severed axonal ends, flushes out most previously formed vesicles, and prevents new vesicle formation (Fig. 1, B and C). PEG applied in pure H2O to proximal and distal ends of severed axons removes waters of hydration from membrane proteins so
that plasmalemmal lipids flow together at points where axonal open ends are closely apposed (Fig. 1D; Krause and Bittner 1990; Krause et al. 1991; Lore et al. 1999). That is, two open, largely vesicle free, axonal ends can be more easily fused by PEG than two constricted ends filled with vesicles. For decades, hybrid cell lines have been made by using PEG solutions to remove waters of hydration from membrane-bound proteins, thereby allowing membrane lipids to fuse when cell plasmalemmas are closely apposed (Ahkong et al. 1987.) The subsequent application of Ca$^{2+}$-containing isotonic saline to the lesion site induces vesicles to seal any remaining plasmalemmal holes (Fig. 1E). Crush-severed nerves that are PEG-fused may be mechanically weak at the lesion site because severance (Fig. 1B) disrupts the extracellular matrix (ECM) that normally prevents intact axons (Fig. 1A) from tearing when stretched or stressed by joint or muscle movements.

In the present study, we confirm that PEG applied directly to the lesion site rapidly restores morphological and physiological continuity to crush-severed PNS axons. We report for the first time that this PEG-fusion technique rapidly (within 24–48 h) improves behavioral function in rats with crush-severed sciatic axons as measured by a modified foot fault (FF) test (Schallert et al. 2002; Yang et al. 2006). Only behavioral measures in this or any other study reported to date indicate whether PEG-fusion or any other technique to enhance regeneration has reconnected proximal and distal axonal ends of individual mammalian axons with sufficient specificity to enable the restoration of some or all of their original functions.

**METHODS**

**Subjects**

All experimental procedures were approved by the University of Texas at Austin’s Institutional Animal Care and Use Committee.

Our experimental group was composed of crush-severed axons that received a PEG treatment. Our control groups and their rationales were composed of crush [-severed] controls treated with distilled water to examine whether our vehicle affects any of our three measures of nerve repair. Sham controls examined whether the injuries to skin and adjacent muscles impair any measure of nerve repair. Cut [-severed] controls examined whether any measure of repair occurs naturally when a rat sciatic nerve receives a very severe and less common injury, in which the ends of proximal and distal sciatic nerve axons separate by about 2 mm and are not surgically reapposed.

One set of experimental and control groups totaling 40 adult male Sprague–Dawley rats (250–350 g) received in vivo compound action potential (CAP) measurements to both sciatic nerves (80 nerves total: 18 PEG-crush; 21 crush; 4 distilled water crush; 6 cut; 31 sham-operated) to assess axonal continuity immediately followed by in vitro intraaxonal dye diffusion assessments of axonal continuity (Table 1).

**TABLE 1. Number of rats and nerves assayed for CAP confirmation of axonal continuity for each experimental and control treatment group followed by dye diffusion or behavioral assessment**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Number of Rats, CAP Confirmation Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Dye diffusion groups</strong></td>
<td></td>
</tr>
<tr>
<td>PEG-crush</td>
<td>9, 18</td>
</tr>
<tr>
<td>Crush</td>
<td>11, 21</td>
</tr>
<tr>
<td>Distilled water crush</td>
<td>2, 4</td>
</tr>
<tr>
<td>Cut</td>
<td>3, 6</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>15, 31</td>
</tr>
<tr>
<td>Total</td>
<td>40 rats, 80 CAP confirmation assays</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>B. Behavioral groups</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-crush</td>
<td>14, 13</td>
</tr>
<tr>
<td>Crush</td>
<td>9, 9</td>
</tr>
<tr>
<td>Distilled water crush</td>
<td>9, 9</td>
</tr>
<tr>
<td>Cut</td>
<td>4, 4</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>4, 4</td>
</tr>
<tr>
<td>Total</td>
<td>40 rats, 39 CAP confirmation assays</td>
</tr>
</tbody>
</table>

For sham-operated and PEG-crush groups, CAP confirmation was the conduction of CAPs through the lesion site. For crush, distilled water crush, and cut groups, CAP confirmation was the absence of CAPs conducted through the lesion site. Dye diffusion data were collected more quickly than behavioral data and both sciatic nerves could be used. Thus more rats were assayed for CAPs and dye diffusion than for CAPs and behavior. Experimental groups (PEG-crush) and control groups (sham-operated, crush) were analyzed in several subgroups to ensure that CAPs did not vary over time due to changes in surgical or assay conditions. That is, there was no significant difference in CAP amplitude between the first and last subgroups of animals tested. Fewer measurements were needed in the cut or distilled water control groups than originally expected to obtain statistically significant differences because there was no variation in CAP amplitude for any control group with cut or crush injuries (CAPs were never detected, i.e., were 0 mV). The smaller number of rats in cut and distilled water crush treatment groups conducted later in this study reflects this observation. CAP confirmation for PEG-crush animals required the conduction of CAPs through the lesion site and 31 PEG-fused sciatic nerves met this criterion. One PEG-crush sciatic nerve in the behavioral PEG-crush treatment group did not meet this criterion and that animal was removed from the study. That is, PEG-fusion was successful in 31 (97%) of 32 attempts.
Another set of experimental and control groups totaling 39 adult male Sprague–Dawley rats (250–350 g) received in vivo CAP measurements to assess axonal continuity of one (left) sciatic nerve (39 nerves: 13 PEG-crush; 9 crush; 9 distilled water crush; 4 cut; 4 sham-operated) and were examined postoperatively for 8 wk using two behavioral tests (Table 1). All animals were housed in groups of three in polycarbonate cages with sawdust bedding, maintained on a 12:12-h dark:light cycle and given unrestricted access to food and water.

**Surgical procedures**

Rats were anesthetized with intraperitoneal injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). An incision about 1.5 cm long was made in the hindlimb posterior-thigh muscles to expose the sciatic nerve. Exposed sciatic nerves were bathed with hypotonic Ca\(^{2+}\)-free Kreb’s physiological saline containing 0.5 mM EGTA (Ca\(^{2+}\)-free saline, in mM: 99 NaCl, 5 KCl, 1.2 KH\(_2\)PO\(_4\), 1.3 MgSO\(_4\), 26 NaHCO\(_3\), 10 Na ascorbate, 10 dextrose, pH 7.35, 295 milliosmolar) and cleaned of connective tissue. Animals in the sham-operated group received no nerve injury following exposure of the sciatic nerve via incision. The sciatic nerve was bathed with isotonic Ca\(^{2+}\)-containing saline (in mM: 124 NaCl, 5 KCl, 1.2 KH\(_2\)PO\(_4\), 1.3 MgSO\(_4\), 26 NaHCO\(_3\), 110 Na ascorbate, 10 dextrose, 2 CaCl\(_2\), pH 7.35, 345 milliosmolar) before closing the incision.

Sciatic crush-severance injuries were made with Dumont #5 forceps. Experimenters carrying out the crush were blind to the assignment of postcrush treatment to eliminate possibility of bias in the force used to make the crush in the PEG versus non-PEG treatments. Following crush injury, the severed ends of the crushed sciatic axons remained closely apposed within their endo-, peri-, and epineurial sheaths. The epineurial sheath of the sciatic nerve was nicked with microscissors to allow better access of PEG or other solutions to axonal tissues.

Experimental animals in the PEG-crush group received a topical application of a 50% solution (w/w) of 2 kDa PEG dissolved in distilled water following crush injury. PEG was applied from a micropipette positioned so that the PEG-containing solution flowed in a narrow stream (about 1 mm wide) over the crushed axons at the lesion site and allowed to bathe the injured nerve for about 1.5 min. Control animals in the crush injury group received no further treatment. Control animals in the distilled water crush group received a vehicle treatment of distilled water applied as previously described for the PEG-treated group. Control animals in the cut group received a sciatic nerve transection with microdissection scissors so that the proximal and distal ends retracted for 1–2 mm and were not reapposed or further treated, which produced a complete severance of all axons and their epineural sheaths. After assessing CAP conduction (see following text), the skin incision was closed with staples in all rats that later received behavioral tests.

All experimental and control animals used for behavioral testing received a 5 mg/kg subcutaneous injection of ketoprofen after surgery. Pharmacokinetic studies show that ketoprofen is almost completely excreted within 24 h (Kantor 1986). Our first behavioral analysis was performed 14 h after performing a CAP confirmation assay, we excised a 3- to 4-cm length of the sciatic nerve (including the lesion site) from each animal as described in Lore et al. (1999). We removed most of the epineurial sheath and placed the nerve in a watertight well made of Vaseline ejected from a 20 mL syringe on a 60 × 15 mm petri dish. For all sham operations and crush-severed nerves (PEG-treated, distilled water treated, and no treatment), the proximal end of the sciatic nerve was placed within the Vaseline well containing Ca\(^{2+}\)-free saline and 20 μL of hydrophilic dye (Texas Red dextran; Molecular Probes). The remainder of the nerve, including the crush site, was bathed in Ca\(^{2+}\)-free saline. For cut nerves, two Vaseline wells were made; one well contained the proximal end of the nerve bathed in Ca\(^{2+}\)-free saline and 20 μL of 15% Texas Red dextran (Molecular Probes), whereas the distal segment of the nerve was anchored within the second well such that the transected segments were 1–2 mm apart. The petri dishes containing nerves in Vaseline wells were refrigerated for 14 h at 4°C and usually examined for intraaxonal diffusion of fluorescent dye beyond the crush or transection site using a Zeiss ICM-405 inverted fluorescence microscope. Some nerves were imaged using a Leica DM IRBE with a ×20 objective outfitted with a Leica DFC350 FX fluorescence camera.

**Behavioral tests**

Behavioral assessments were performed by experienced testers blind to the treatment condition during the dark portion of each animal’s daily light cycle in which rats are more active. Animals were handled daily for 7 days prior to the start of behavioral testing. After receiving a CAP continuity assay of their left sciatic nerves, experimental and control groups of rats were behaviorally evaluated at 14, 24, 48, and 72 h after surgery, and then at weekly postoperative intervals for 8 wk. Animals were first tested at 24 h postoperatively to allow animals to recover from anesthesia.

**Foot-fault test**

Animals were allowed to roam freely on a wire mesh grid (45 × 30 cm, with 2.5 × 2.5 cm openings) elevated 1.5 cm above a solid base floor. Trials for each animal were recorded for 50 total steps per hindlimb. A foot fault was scored when a misstep resulted in the
hindlimb falling through an opening in the grid. If the hindlimb
misstepped, but was pulled back before touching the floor beneath
the grid, the movement was scored as a partial fault and given a fault
score of one. A full fault occurred when the animal’s hindlimb
touched the floor beneath the grid for support. Full faults were given
a fault score of two. A composite fault score was calculated (see
the following equations) for each of the injured and uninjured hindlimbs
every animal at each postoperative time. The composite fault score
was further divided by 50 (total number of steps/limb) to obtain a fault
percentage for each hindlimb. The percentage of faults by the injured
hindlimb was subtracted from the percentage of faults by the unin-
jured hindlimb, yielding an asymmetry score for each animal at a
given postoperative time according to the following three equations

\[
\text{Composite FF Score} = (# \text{ Partial faults} \times 1) + (# \text{ Full faults} \times 2)
\]
\[ (1) \]

\[
\% \text{FF} = \text{Composite FF Score/50 (total number of steps)} \times 100\%
\]
\[ (2) \]

\[
\text{FF Asymmetry Score} = \% \text{FF (uninjured limb)} - \% \text{FF (injured limb)}
\]
\[ (3) \]

FF asymmetry tests were conducted two times at 1–5 days prior to
surgery and their scores averaged to obtain preoperative baseline
values plotted at 0 postoperative days.

**Sciatic Functional Index**

Footprints have been used previously to measure gait quality in rat
models of Parkinson’s disease (Schallert et al. 1978) and sciatic nerve
severance by the Sciatic Functional Index (SFI; de Medinaceli et al. 1982). Rats were trained to traverse a wooden beam ending in their
home cage. After a few habituation trials, during which rats frequently
stopped and paused en route to their home cage, rats traversed the
beam to the home cage without hesitation. For each trial run, a white
strip of paper was secured to the wooden beam to collect footprints
two trials per rat at a given postoperative time. Animals had their
injured and uninjured hind paws inked with black and red ink,
respectively, and were placed near the end of the wooden beam
farthest from the home cage. Three consecutive footprints from each
limb (for a total of six consecutive prints) were used to measure (in
millimeters) the following: NPL, normal footprint length; EPL, ex-
perimental footprint length; NTS, normal toe spread between toes one
and five; ETS, experimental toe spread; NIT, normal intermediary
toe spread; EIT, experimental intermediary toe spread (Carlton and Goldberg 1986). SFI scores were then com-
puted for each animal at a given postoperative time using the follow-
ing formula (Carlton and Goldberg 1986)

\[
\text{SFI} = \left( \frac{\text{NPL} - \text{EPL} + \text{ETS} - \text{NTS} + \text{EIT} - \text{NIT}}{\text{NPL} + \text{EPL} + \text{ETS} + \text{NTS} + \text{EIT} + \text{NIT}} \right) \times 73
\]

SFI scores of about −100 indicate complete impairment of behav-
iors mediated by the sciatic nerve and scores of about 0 indicate
normal use/complete recovery of behaviors mediated by the sciatic
nerve (de Medinaceli et al. 1982; Mackinnon et al. 1989). SFI tests
were conducted three times at 1–5 days prior to surgery and their
scores averaged to obtain preoperative baseline values plotted at 0
postoperative days.

**Video recordings**

We recorded foot fault (FF) tests using a Canon XL1 with a shutter
speed of 1/420 s (all videos are available in online Supplemental
data). Foot fault tests were recorded during the light phase of the
animals’ reverse dark/light cycle in the presence of experimenters
scoring the animals’ behavior (videos SV1–SV4). Open field obser-
vations were recorded in the dark using the camera’s night vision
setting, sacrificing some resolution in the videos for increased loco-
motor activity (videos SV5–SV6). The video recordings show qual-
tative differences in behavior between experimental groups.

**Statistical analyses**

Student’s t-test was used to assess differences (P < 0.05) in
preoperative CAP amplitudes versus postoperative CAP amplitudes.
ANOVA was used to assess differences in SFI and FF asymmetry
scores and Tukey’s test was used for post hoc analysis to adjust for
multiple comparisons. Hindlimb motor behaviors were subjected to a
linear regression t-test to determine whether the regression line slopes
of two treatment groups differed significantly (P < 0.05) over the
8-wk observational period following surgery.

**RESULTS**

**CAP assessments of axonal continuity**

To evaluate the ability of PEG-fused axons to conduct action
potentials across the lesion site in vivo, we measured pre- and
postinjury CAP amplitudes for all treatment groups. Peak CAP
amplitudes of ≥0.5 mV were easily detectable with hook
electrodes. For dye diffusion experimental and control groups (n = 40
rats, 80 nerves), we recorded CAPs in vivo (Fig. 2) from both

![CAP amplitudes](http://jn.physiology.org/)

**FIG. 2.** Compound action potential (CAP) amplitudes of rat
sciatic nerves recorded preoperatively (white and black bars)
and postoperatively (blue and red bars) for each group (cut,
crush, distilled water-crush, PEG-crush, and sham) used in dye
diffusion (black and red bars) or behavioral (white and blue
bars) assays. Colored bars on the x-axis indicate a CAP ampli-
tude of 0 mV for all animals. All CAP and other data in this and
other figures (except Fig. 3) are plotted as means ± SE.

1 The online version of this article contains Supplemental data.
scientific nerves and immediately assessed these nerves for morphological continuity by observing intraaxonal dye diffusion in vitro (Fig. 2). As previously described, sham-operated animals received no neural injury and only one CAP measurement was taken during each sham operation. Sham-operated CAP data are shown as identical pre- and postoperative means. Prior to any cut or crush injury, in vivo CAP amplitudes of these scientific nerves ranged from 1 to 5 mV and the mean preoperative CAP across all treatment groups was 2.4 ± 0.09 mV. Preoperative CAP amplitudes were not significantly different between treatment groups. No postoperative CAP was detectable immediately following crush injury, cut injury, or treatment with distilled water following crush injury (Fig. 2). The mean postoperative CAP following PEG-fusion of crush-severed axons was 2.0 ± 0.22 mV. This average postoperative CAP was significantly (P < 0.005) reduced compared with the preoperative CAP amplitude for this PEG-fused group or the average preoperative CAP amplitude pooled for all experimental control CAPs, respectively, again indicates that the two halves of many crush-severed scientific nerves are joined by PEG application.

We also observed preoperative CAP amplitudes ranging from 1 to 6 mV whose average values were not significantly different between any two groups (Fig. 2) in recordings from animals (n = 40) that were subsequently tested for behavioral recovery at 24 h to 8 wk postoperatively. The mean preoperative CAP across all treatment groups was 3.2 ± 0.22 mV (cut, n = 4; crush, n = 9; distilled water crush, n = 9; PEG-crush, n = 13; sham, n = 4). CAPs from sham-operated animals were treated as described earlier. CAPs conducted across the lesion site were not detected immediately following cut or crush injuries in the absence of PEG application. Crush-severed nerves did not have a detectable CAP with or without subsequent treatment with distilled water. After a crush injury, all 32 PEG-treated nerves, except one, had successful PEG-fusion as measured by conduction of CAPs of ≥0.5 mV through the lesion site (Fig. 2).

The mean postoperative CAP after successful PEG-fusion of crush-severed scientific nerves was 1.9 ± 0.23 mV (Fig. 2) and was significantly (P < 0.001) reduced compared with the preoperative CAP for this PEG-fused group (2.87 ± 0.29 mV) or the average preoperative CAP pooled for all experimental groups (3.27 ± 0.36 mV). The significant reduction in postoperative mean CAP amplitude for PEG-treated nerves indicates that not all crush-severed scientific axons are immediately repaired by PEG application. The observation that the postoperative CAP amplitude is 57 or 50% of preoperative or control CAPs, respectively, again indicates that the two halves of many crush-severed scientific axons are joined by our PEG-fusion technique, although the specificity of those connections is not tested by this measure.

Intraaxonal dye diffusion assessments of axonal continuity

For one set of experimental and control groups for which in vivo CAPs were measured (Fig. 2, black and red bars), we evaluated the morphological continuity of axons in the scientific nerve in vitro by observing the intraaxial diffusion of Texas Red, a hydrophilic fluorescent dye (Fig. 3). The lesion site was readily visible as a distinct gap in low-power fluorescence images. Small amounts of dye-labeled connective tissue elements at the cut edges and some extraneous autofluorescence were sometimes visible in distal nerve segments. In all sham (uninjured) nerve segments (n = 31, Fig. 3A), the dye was visible intraxonally throughout the entire segment (Fig. 3B). Dye did not diffuse intraaxonally across the lesion site in any nerves following crush injury (n = 21, Fig. 3, A and C), cut injury (n = 6, Fig. 3, A and E), or crush injury with distilled water treatment (n = 4, Fig. 3A; image not shown). In contrast, for 17 of 18 total nerves (94%) that were crushed and subsequently treated with PEG, dye diffused across the lesion site (Fig. 3, A and D). All these CAP and intraaxonal dye data are consistent with the interpretation that crush- or cut-severance completely disrupts physiological and morphological continuity between axonal segments proximal and distal to the lesion site and that PEG application usually rapidly restores physiological and morphological continuity to many crush-severed axons.

FIG. 3. Graph showing percentage of scientific nerve preparations exhibiting intraaxonal dye diffusion across a lesion site following cut, crush, distilled water crush, PEG-crush, or sham operations (A). Fluorescence images showing intraaxonal dye diffusion across a lesion site following (B) sham, (C) crush, (D) PEG-crush, and (E) cut operations in scientific nerves. In C and D, dotted lines are drawn to show the location and extent of the lesion. In E, the dotted line indicates the edge of the distal segment (not visible because it contained no dye). In each image the proximal portion of scientific nerve is on the left and the distal portion of scientific nerve is on the right. Scale bar: 200 μm for B–E.
from 72 h through 8 wk postoperation (continued to significantly improve relative to cut group animals similarly to crush group animals (Fig. 4). Crush group animals at 48 and 72 h postoperation, cut group animals performed

ior relative to cut animals at 24 h following surgery (PEG-crush animals showed improved hindlimb motor behav-

n

n

significantly (P < 0.05) for animals with crush-severed sciatic nerves that were not PEG-treated (video SV2). In these videos, cut group animals occasionally refrained from using the injured limb for portions of the trial (video SV3) and missteps of the cut-injured limb were almost all exclusively full faults. Sham-operated animals did not show any deficit throughout the FF test in video SV4 and other video recordings. In videos of open field trials, behavioral recovery of animals with PEG-treated crush-severed sciatic nerves (video SV6) was noticeably better than that of animals with crush-

nerve behaviors (Fig. 4). Animals with cut sciatic nerves exhibited a mean FF asymmetry score of −72 ± 6.5 at 24 h following surgery and did not improve over the course of the study. The FF asymmetry scores did not differ significantly (P > 0.05) for animals with crush-severed sciatic nerves that received no further treatment (n = 9) compared with that of animals that subsequently received distilled water as a vehicle control (n = 9; data not shown). Thus FF asymmetry scores were pooled from crush and crush nerves treated with distilled water (n = 18).

PEG-crush animals performed better than crush animals when measured at 24 h after surgery and this difference persisted until postoperative week 4. One-way ANOVA showed significant differences in behavior function (as measured by FF) across all groups [F(3,24) = 29.4, P < 0.001], with post hoc assessments demonstrating that PEG-crush animals performed significantly (P < 0.05) better between 24 h and 3 wk postoperation compared with crush group animals. At 4–8 wk after surgery, PEG-crush and crush group animals did not differ significantly in their behavior; i.e., recovery was so complete that the behaviors of intact PEG-fused and sham-operated animals were indistinguishable (P > 0.05). Crush and PEG-crush animals showed improved hindlimb motor behav-

ior relative to cut animals at 24 h following surgery (P < 0.05). At 48 and 72 h postoperation, cut group animals performed similarly to crush group animals (Fig. 4). Crush group animals continued to significantly improve relative to cut group animals from 72 h through 8 wk postoperation (P < 0.01). Sham-

operated animals performed significantly better from 24 h through 3 wk postoperation compared with cut, crush, and PEG-crush group animals (P < 0.01).

We also compared the time course of recovery of FF asymmetry scores for different groups (lines connecting data points in Fig. 4) by regression analysis (see METHODS). The rate of recovery of crush and PEG-crush animals was significantly [t(18) = 3.64, P < 0.001; F(13) = 3.69, P < 0.01] faster compared with cut group animals, which did not show any improvement for 8 wk postoperation. The time course of recovery for PEG-crush animals was also significantly improved compared with untreated crush animals [t(27) = 2.13, P < 0.05; Fig. 4].

Video recordings SV1–SV4 of representative animals from each of the experimental groups and three control groups at 3 postoperative weeks showed that experimental animals with PEG-treated crush-severed sciatic nerves navigated an FF grid noticeably better (video SV1) than control animals with crush-

severed sciatic nerves that were not PEG-treated (video SV2). In these videos, cut group animals occasionally refrained from using the injured limb for portions of the trial (video SV3) and missteps of the cut-injured limb were almost all exclusively full faults. Sham-operated animals did not show any deficit throughout the FF test in video SV4 and other video recordings. In videos of open field trials, behavioral recovery of animals with PEG-treated crush-severed sciatic nerves (video SV6) was noticeably better than that of animals with crush-

severed sciatic nerves that were not PEG-treated (video SV5).

Sciatic Functional Index

Mean SFI scores ± SE (see METHODS) were obtained three times for each animal prior to any operative procedures and at postoperative times of 24 h, 48 h, 72 h, 1 wk, and weekly thereafter for 8 wk (Fig. 5). Baseline preoperative scores did not differ significantly between any experimental or control groups. Sham-operated animals did not show any behavioral deficit at any postoperative time as measured by the SFI, indicating that muscle injury during surgery did not result in behavioral impairment (Fig. 5). Cut group animals exhibited a

FIG. 4. Mean foot fault (FF) asymmetry scores from 0 to 8 postoperative weeks for cut (solid line, diamonds), crush (dashed line, circles), PEG-crush (solid line, triangles), or sham-operated (dotted line, squares) groups. Baseline scores were obtained twice for each animal prior to surgery and are plotted at the 0 wk time point. SE values are so close to the plotted mean values at some time points that the error bars are within the space occupied by the symbol showing the mean value.

FIG. 5. Sciatic Functional Index (SFI) results from 0–8 postoperative wk for cut (solid line, diamonds), crush (dashed line, circles), PEG-crush (solid line, triangles), or sham-operated (dotted line, squares) groups. Baseline scores were obtained 3 times for each animal prior to surgery and plotted at the 0 wk time point.
mean SFI score of \(-92 \pm 0.95\) and did not show any improvement in behavior for the duration of the study, as previously reported (de Medinaeli et al. 1982; Hare et al. 1992).

We observed no significant difference at any postoperative time between animals with crush-severed sciatic nerves that received no treatment \((n = 9)\) compared with animals that received distilled water treatment \((n = 9;\) data not shown). These results were consistent with results from electrophysiological measures \((\text{CAPs}; \text{Fig. 2})\), morphological measures \((\text{intraaxonal dye diffusion}; \text{Fig. 3})\), and FF asymmetry measures \((\text{Fig. 4})\). Therefore data from crush and distilled water crush group animals were pooled.

One-way ANOVA of SFI results showed differences between groups were significant \([F(3,40)=18.78, P < 0.001]\), with post hoc assessments demonstrating no significant difference in the time course of recovery between PEG-crush and crush group animals, although further inspection did show that PEG-crush animals performed significantly \([t(27) = 2.12, P < 0.05]\) better 3 wk postoperation compared with crush group animals. Over the 8-wk postoperative testing period, crush group animals showed significant improvement in behavior as measured by the SFI compared with cut group animals \((P < 0.05)\). Additionally, sham-operated animals performed significantly better on the SFI test than did both crush group animals \((P < 0.01)\) and PEG-crush animals \((P < 0.01)\) throughout the study.

We compared the time course of recovery of SFI scores for the experimental and various control groups \((\text{lines connecting data points in \text{Fig. 5}})\) by regression analysis \((\text{see METHODS})\). No significant difference was found between the recovery of PEG-crush and crush group animals. Both crush and PEG-crush animals showed significant improvement \([t(18) = 3.35, P \leq 0.01; t(13) = 3.69, P < 0.01]\) in behavior relative to animals with sciatic nerve cuts.

**Discussion**

Assessments of CAP amplitude \((\text{Fig. 2})\) and intraaxonal dye diffusion \((\text{Fig. 3})\) show that direct application of PEG to crush-severed sciatic nerves usually \((97\% \text{ of all attempts})\) rapidly restores physiological and morphological continuity to at least some axons in the sciatic nerve. In fact, continuity may be restored to many proximal and distal axonal halves \((\text{with unknown specificity})\) since CAP amplitudes of PEG-fused nerves on average are 50 to 89\% of CAP amplitudes of intact-control or sham-operated nerves. FF asymmetry scores \((\text{Fig. 4})\) provide quantitative assessment of hindlimb motor behavior and show significantly faster recovery of functional behavior associated with direct application of PEG to crush-severed sciatic nerves. This finding for FF asymmetry scores is consistent with our measures of physiological and morphological continuity.

Video observations of FF trials and of open field trials also show rapid behavioral improvement associated with direct application of PEG to crush-severed sciatic nerves. That is, all data are consistent with the interpretation that PEG rejoins proximal and distal axonal halves \((\text{PEG-fusion})\) with sufficient specificity to allow significantly faster improvement in hindlimb motor behaviors mediated by the sciatic nerve.

Direct PEG application to crush-severed axons produces significant reversal of behavioral deficits within 24–48 h and does not prevent further improvement in behaviors at later \((3 \text{ to 8 weeks})\) postoperative times. The shorter-term behavioral recoveries at 24–48 h are likely produced by PEG-fused axons. Longer-term behavioral recoveries at 3 to 8 wk may well be produced by crush-severed sciatic axons that were not PEG-fused and then grew out at 1–2 mm/day to appropriately reinnervate denervated muscles.

One of the difficulties in assessing behavioral outcome and treatment success following neural injury is that animals readily adopt compensatory behavioral strategies that can mask their true deficits \((\text{Schallert et al. 2000, 2002, 2006})\). A modified FF test eliminates much of this problem \((\text{Hernandez and Schallert} 1988)\). When injured rats walk on a grid surface, the impaired hindlimb frequently slips through the openings. In the absence of a platform underlying the grid surface, rats typically learn motor strategies that reduce the number of slips, which obscure adequate evaluation of the degree of deficit. Previous reports have indicated such compensation occurs, often involving a shift in the burden of weight support and locomotion to the uninjured limbs \((\text{Dellon and Dellon} 1991)\). Placing a solid platform just beneath the grid surface provides a “crutch” floor that the rats can use for support when their impaired hindlimb slips through the grid openings, allowing for a more sensitive detection of deficits \((\text{Schallert et al. 2002; Yang et al. 2006})\). Unoperated or sham-operated rats rarely use the underlying solid floor surface during exploration of the grid. In contrast, rats with sciatic nerve injuries frequently use the floor for support.

The SFI is a computational assessment of three variables that measure print length, intermediary toe spread \((\text{digits 2–4})\), and total toe spread \((\text{digits 1–5})\). Previous studies have shown that the most useful parameter for overall evaluation of sciatic function is total toe spread \((\text{Bain et al. 1989; Bervar 2000})\). This factor deviates the most from control values, making it highly sensitive to long-term deficits in fine distal control. Proximal muscles are innervated more quickly than distal muscles and SFI scores may be more heavily influenced by toe use and toe spread controlled by distal muscle groups compared with FF asymmetry scores. Thus the SFI might be expected to show significant behavioral improvement later than FF asymmetry scores and detect more chronic deficits, as we have reported.

Our data suggesting that PEG-fusion can more rapidly improve behavioral recovery following a crush injury to the sciatic nerve in the rat extend previous studies indicating that PEG induces both physiological \((\text{measured by the restored conduction of action potentials through the lesion site})\) and morphological continuity \((\text{intraaxonal diffusion of fluorescent dyes across the lesion site})\) between the cut or crushed ends of mammalian myelinated axons \((\text{Lore et al. 1999})\). Other recent studies assessing behavioral recovery following PEG treatment of cut- or crush-severed axons have focused on injury models of CNS axons. Subcutaneous injections of PEG have been reported to partially restore the cutaneous trunci muscle reflex in guinea pigs following a crush injury to the midthoracic spinal cord \((\text{Borgens and Bohnert 2001})\). Rats receiving an intravenous injection of PEG following a spinal cord compression injury at T4 showed improved locomotor performance relative to saline-treated control groups as measured by the Basso–Beattie–Bresnahan open-field hindlimb motor scale \((\text{Ditor et al. 2007})\). A computer-managed open-field behavioral test has
also been used in a recent study reporting improved exploratory behavior in animals receiving a subcutaneous injection of PEG following traumatic brain injury compared with untreated animals (Koob et al. 2008).

The PEG-fusion technique modified for in vivo use may have immediate clinical implications to improve the acute and chronic repair of PNS crush-severance injuries as measured by physiological, morphological, and behavioral assays described herein. For example, after nerve crush functional recovery is often poor if regenerating motor axons are too far from the denervated target tissue. Prolonged denervation of muscle leads to a deterioration of the intramuscular nerve sheaths, which are the normal target pathways for regenerating motor axons (Fu and Gordon 1995; Mackinnon et al. 1991). The loss of intramuscular nerve sheaths greatly reduces the number of motor axons that are able to innervate muscle fibers. In addition, muscle fibers do not fully recover from atrophy due to prolonged denervation (Fu and Gordon 1995). It is possible that the repair of at least some axons by PEG-fusion can have a trophic integrity-maintenance effect on target muscles that might keep them more receptive to reinnervation for a longer time, which could promote a more optimal chronic outcome. That is, initial functional recovery mediated by PEG-fusion of some axons might later be increased by reinnervation of denervated muscle fibers by newly formed synapses made by other, non-PEG-fused regenerated axons that reach the muscle at 4–8 wk.

Finally, we have worked on solving two basic problems before now considering the clinical use of PEG to repair bundles of crush-severed PNS axons (e.g., sciatic or other peripheral nerves). First, severed distal stumps need be induced to survive until they can be PEG-fused. We can now consistently (80–100% success rate) induce severed mammalian CNS or PNS axons to survive for 3–10 days by cooling (Marzullo et al. 2001; Sea et al. 1995) or injections of cyclosporin A (Sunio and Bittner 1997) and such surviving axons can be PEG-fused (Lore et al. 1999; Marzullo et al. 2001; Stavisky et al. 2003, 2005). The ability to extend the time needed to use PEG-fusion techniques on crush-severed PNS axons is important because such injuries usually occur in a nonclinical setting and medical treatment may be delayed for hours to days.

Second, crush-severed axons that are PEG-fused in mammals in vivo have poor mechanical strength at the lesion site (Fig. 1E) and may pull apart after animals recover from anesthesia, thereby preventing chronic recovery (Bittner and Fishman 2000; Lore et al. 1999). Crush-severed axons PEG-fused in invertebrates in vivo show both acute and chronic repair of severed axons if the animal is immobilized by cooling for 24–48 h or if a PEG hydrogel is applied to add mechanical strength to the ECM at the lesion site (Lore et al. 1999). Unfortunately, this hydrogel by itself has acute cytotoxic effects in mammalian neurons and behavior-impairing effects (unpublished observations). Other tissue adherents (e.g., fibrin glues or biogels) applied to the ECM at the site of PEG-fusion might provide additional mechanical strength in vivo (Fig. 1F). Alternatively, techniques to immobilize the joints crossed by the affected axons might enhance the PEG-fusion technique. If successful, such techniques would also be immediately applicable for clinical use, although a concern might be that this procedure could be detrimental because optimal behavioral outcome may require adequate motor experience (Bittner et al. 2000; Kleim et al. 2003). However, intense motor rehabilitation targeting movements impaired by the injury could be combined with PEG and other axon-mending or degeneration-preventing interventions to improve restoration of function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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