Polyethylene glycol rapidly restores axonal integrity and improves the rate of motor behavior recovery after sciatic nerve crush injury

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Abstract

The inability to rapidly (within minutes to hours) improve behavioral function after severance of PNS 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 \textit{in vitro} and \textit{in vivo}. We now report that PEG fusion not only re-establishes the integrity of crush-severed rat sciatic axons as measured by the restored conduction of compound action potentials (CAPs) and the intra-axonal 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 post-operative weeks for the foot fault (FF) asymmetry test and between weeks 2-3 for Sciatic Functional Index (SFI) based on analysis of footprints. That is, the foot fault test was the more sensitive indicator of early behavioral recovery, showing significant post-operative improvement of motor behavior in PEG-treated animals at 24-48 hours. In contrast, the SFI more sensitively measured longer-term post-operative behavioral recovery and deficits at 4-8 weeks, 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.

\textit{Key words: axotomy; axonal regeneration; membrane fusion; neurotrauma; nerve repair; polyethylene glycol; sciatic functional index; foot fault; rat}
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 in 12-72 hours (Waller 1850; Ramon y Cajal 1928). If proximal PNS axonal segments survive axotomy, regeneration by outgrowth can occur at rates of 1-2 mm/day (Ramon y Cajal 1928; Hadlock et al. 2005). Hence, acute (within days) recovery in mammals is non-existent. Chronic behavioral recovery is delayed and often very inadequate or non-existent because PNS outgrowths take months-to-years to reach denervated target tissues in larger mammals such as humans and those target tissues are often non-specifically re-innervated (Das and Wallace 1986; Ingoglia and Murray 2001; Bozkurt et al. 2007, 2008).

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

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

This PEG-fusion technique and its rationales are as follows: Ca\(^{2+}\) 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 minutes (Bittner and Fishman 2000). Eventually, the plasmalemma is completely repaired (Fig. 1A) and vesicles are no longer observed 24 hours 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 similar time courses as in invertebrates (Bittner and Fishman 2000; Detrait et al. 2000a,b; Yoo et al. 2003, 2004; Nguyen et al. 2005).

Bathing recently-severed invertebrate or mammalian axons in Ca\(^{2+}\)-free hypotonic salines, containing EGTA opens severed axonal ends, flushes out
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most previously formed vesicles, and prevents new vesicle formation (Fig. 1B-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 Ca²⁺-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 hours) 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
re-connected proximal and distal axonal ends of individual mammalian axons
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with sufficient specificity to enable the restoration of some or all of their original
functions.

Materials and Methods

Subjects

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

Our experimental group is: Crush-severed axons that receive a PEG
treatment. Our control groups and their rationales are: Crush [-severed]
controls treated with distilled water examine whether our vehicle affects any of
our three measures of nerve repair. Sham controls examine whether the injuries
to skin and adjacent muscles impair any measure of nerve repair. Cut [-severed]
controls examine 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 ~ 2mm and are not
surgically re-apposed.

One set of experimental and control groups totaling 40 adult male
Sprague-Dawley rats (250-350 g) received in vivo 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 intra-axonal dye diffusion assessments of axonal continuity (Table 1).

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
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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 eight weeks 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 dark:light cycle and given food and water ad libitum.

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 milli-osmolar), 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\(_3\), 110Na ascorbate, 10 dextrose, 2 CaCl\(_2\), pH 7.35, 345 milli-osmolar) 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 post-crush treatment to eliminate possibility of bias in the force used to make the crush in the PEG vs. non-PEG treatments. Following crush injury, the severed ends of the crushed sciatic axons remained closely apposed within their endo-, peri- and
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epineural sheaths. The epineural 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 kD 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 micro-dissection scissors so that the proximal and distal ends retracted for 1-2 mm and were not re-apposed or further treated, which produced a complete severance of all axons and their epineural sheaths. After assessing CAP conduction (see below), 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 hours (Kantor 1986). Our first behavioral analysis was conducted at 24 hours post-operation and therefore ketoprofen should have very little, if any, residual effect on behavioral performance.
As one measure of axonal continuity through a lesion site, conventional \textit{in vivo} extracellular stimulation and recording of CAPs (extracellular recordings of action potentials generated by sciatic nerve axons) was performed for animals (n=40) immediately used to examine morphological continuity by \textit{in vitro} intra-axonal dye diffusion (Lore et al. 1999) as well as for animals (n=39) used to examine behavioral measures of re-innervation specificity for 8 post-operative weeks. Nickel-tipped hook electrodes were placed beneath the sciatic nerve to stimulate and record CAPs, which were visualized on an oscilloscope display. Pre-operative electrophysiological assessments of axonal continuity were made by recording CAPs of at least 0.5 mV conducted through the site of the proposed cut or crush injury.

After cutting or crushing the sciatic nerve between the stimulating and recording electrodes, complete severance of all axons was confirmed by an inability to record any detectable CAPs conducted through the lesion site (Lore et al. 1999). After any treatment, the sciatic nerve was always stimulated to determine if any detectable CAPs conducted through the lesion site. During pre-operative and postoperative CAP recordings, the nerve was frequently moistened with Ca\(^{2+}\)-free saline. Some animals received a sham operation consisting of exposure of the sciatic nerve \textit{via} incision and application of Ca\(^{2+}\)-containing saline. Since these control animals in a sham-operated group received no neural injury, one CAP measurement was taken during each sham operation, and that CAP amplitude was plotted as both a pre- and post-operative CAP (see Fig. 2 in Results).
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Intra-axonal dye diffusion across a lesion site

As described above and in Table 1, one group of experimental animals received crush-severance injury followed by treatment with PEG and other groups of control animals received a sham, cut-severance, crush-severance or crush-severance injury plus distilled water. The effectiveness of all these procedures was assessed by a CAP confirmation assay.

To examine intra-axonal diffusion of dye through the lesion site after performing a CAP confirmation assay, we excised a 3-4 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 water-tight well made of Vaseline ejected from a 20 cc syringe on a 60x15 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), while 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 hours at 4°C, and usually examined for intra-axonal diffusion of fluorescent dye beyond the crush or
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transection site using a Zeiss ICM-405 inverted fluorescence microscope. Some
nerves were imaged using a Leica DM IRBE with a 20X 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 seven 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 24, 48, and 72 hours after surgery, and then at weekly post-
operative intervals for 8 weeks. Animals were first tested at 24 hours post-
operatively to allow animals to recover from anesthesia.

Foot-fault Test

Animals were allowed to roam freely on a wire mesh grid (45 cm x 30 cm,
with 2.5 cm x 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 equations below) for each of the
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injured and uninjured hindlimbs of every animal at each post-operative 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 uninjured hindlimb, yielding an asymmetry score for each animal at a given post-operative time according to the following three equations:

1. Composite FF Score = (# Partial Faults x 1) + (# Full Faults x 2)
2. %FF = Composite FF Score/50 (total number of steps) x 100%
3. FF Asymmetry Score = % FF (uninjured limb) – %FF (injured limb)

FF asymmetry tests were conducted two times at 1-5 days prior to surgery and their scores averaged to obtain pre-operative baseline values plotted at 0 post-operative 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 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 post-operative time). Animals had their injured and uninjured hind paws inked with black and red ink, respectively, and were placed near the end of the beam.
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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: experimental footprint length; NTS: normal toe spread between toes one and five; ETS: experimental toe spread; NIT: normal intermediary toe spread between toes two and four; EIT: experimental intermediary toe spread (Carlton and Goldberg 1986). SFI scores were then computed for each animal at a given post-operative time using the following formula (Carlton and Goldberg 1986):

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

SFI scores of about −100 indicate complete impairment of behaviors 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 pre-operative baseline values plotted at 0 post-operative days.

Video Recordings

We recorded foot fault tests using a Canon XL1 with a shutter speed of 1/420 s (all videos available in Supplemental Data online). 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 (SV1-4). Open field observations were recorded in the dark using the camera’s night vision setting,
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sacrificing some resolution in the videos for increased locomotor activity (SV5-6).

The video recordings show qualitative differences in behavior between experimental groups.

**Statistical Analyses**

Students’ t-test was used to assess differences (p<0.05) in pre-operative CAP amplitudes vs. post-operative 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 eight-week 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 post-injury 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 sciatic nerves, and immediately assessed these nerves for morphological continuity by observing intra-axonal 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 post-operative means. Prior to any cut or crush injury, in vivo CAP amplitudes of these sciatic nerves ranged from 1-5 mV, and the mean pre-operative CAP across all treatment groups was $2.4 \pm 0.09$ mV. Pre-operative CAP amplitudes were not significantly different between treatment groups. No post-operative CAP was detectable immediately following crush injury, cut injury or treatment with distilled water following crush injury (Fig. 2). The mean post-operative CAP following PEG-fusion of crush-severed axons was $2.0 \pm 0.22$ mV. This average post-operative CAP was significantly ($p<0.005$) reduced compared to the pre-operative CAP amplitude for this PEG-fused group or the average pre-operative CAP amplitude pooled for all experimental groups used to assess intra-axonal dye diffusion. The observation that the post-operative CAP is 72% or 89% of pre-operative or control CAPs, respectively, indicates that the two halves of many crush-severed sciatic axons are joined by PEG application.

We also observed pre-operative CAP amplitudes ranging from 1-6 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 hours to 8 weeks postoperatively. The mean pre-operative CAP across all treatment groups was $3.2 \pm 0.22$ 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 above. CAPs conducted across the lesion site were not detected immediately following cut or crush injuries in the
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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 at least 0.5 mV through the lesion site (Fig.
2).

The mean post-operative CAP after successful PEG-fusion of crush-
severed sciatic nerves was 1.9 ± 0.23 mV (Fig. 2) and was significantly (p<0.001)
reduced compared to the pre-operative CAP for this PEG-fused group (2.87 ±
0.29 mV) or the average pre-operative 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 sciatic
axons are immediately repaired by PEG application. The observation that the
post-operative CAP amplitude is 57% or 50% of pre-operative or control CAPs,
respectively, again indicates that the two halves of many crush-severed sciatic
axons are joined by our PEG-fusion technique, although the specificity of those
connections are not tested by this measure.

Intra-axonal 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 sciatic nerve in vitro by observing the intra-axonal
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 extra-axonal autofluorescence was sometimes visible in distal nerve segments. In all sham (uninjured) nerve segments (n=31, Fig. 3A), the dye was visible intra-axonally throughout the entire segment (Fig. 3B). Dye did not diffuse intra-axonally across the lesion site in any nerves following crush injury (n=21, Fig. 3A, C), cut injury (n=6, Fig. 3A, 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. 3A, D). All these CAP and intra-axonal 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.

Foot Fault Asymmetry Scores

FF asymmetry scores (averages +/- SEM, see Methods) were obtained twice for each animal prior to any operative procedures and at post-operative times of 24, 48, and 72 hours, and weekly for eight weeks. We observed no significant differences in baseline scores between any experimental groups. Sham-operated animals did not show any obvious behavioral deficit as measured by FF asymmetry score at any post-operative time, indicating that muscle injury during surgery did not produce detectable impairment in hindlimb motor behaviors (Fig. 4). Animals with cut sciatic nerves exhibited a mean FF asymmetry score of -72 +/- 6.5 at 24 hours following surgery, and did not improve
PEG improves rate of recovery following sciatic injury 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 to 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 hours after surgery and this difference persisted until post-operative week four. 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 hours to 3 weeks post-operation compared to crush group animals. At 4-8 weeks 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 behavior relative to cut animals at 24 hours following surgery (p<0.05). At 48 and 72 hours post-operation, 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 hours through eight weeks post-operation (p<0.01). Sham-operated animals performed significantly better from 24 hours through three weeks post-operation compared to cut, crush, and PEG-crush group animals (p<0.01).
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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 to cut group animals, which did not show any improvement for eight weeks post-operation. The time course of recovery for PEG-crush animals was also significantly improved compared to untreated crush animals ($t\ [27] = 2.13, p < 0.05$; Fig. 4).

Video recordings (SV1-4) of representative animals from each of the experimental group and three control groups at three post-operative weeks showed that experimental animals with PEG-treated crush-severed sciatic nerves navigated a FF grid noticeably better (SV1) than control animals with crush-severed sciatic nerves that were not PEG-treated (SV2). In these videos, cut group animals occasionally refrained from using the injured limb for portions of the trial (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 this (SV4) and other video recordings. In videos of open field trials, behavioral recovery of animals with PEG-treated crush-severed sciatic nerves (SV6) was noticeably better than animals with crush-severed sciatic nerves that were not PEG-treated (SV5).

Sciatic Functional Index
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Mean SFI scores +/- SEM (see Methods) were obtained three times for each animal prior to any operative procedures and at post-operative times of 24 hours, 48 hours, 72 hours, one week and weekly thereafter for eight weeks (Fig. 5). Baseline pre-operative scores did not differ significantly between any experimental or control groups. Sham-operated animals did not show any behavioral deficit at any post-operative 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 mean SFI score of -92 +/- 0.95, and did not show any improvement in behavior for the duration of the study, as previously reported (de Medinaceli et al. 1982; Hare et al. 1992).

We observed no significant difference at any post-operative time between animals with crush-severed sciatic nerves that received no treatment (n=9) compared to animals that received distilled water treatment (n=9; data not shown). These results were consistent with results from electrophysiological measures (CAPs: Fig. 2), morphological measures (intra-axonal dye diffusion: Fig.3), and FF asymmetry measures (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 three weeks post-operation compared to crush group animals. Over the eight week
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post-operative testing period, crush group animals showed significant improvement in behavior as measured by the SFI compared to cut group animals (p<0.05). Additionally, sham-operated animals performed significantly better on the SFI test than 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 (lines connecting data points in Fig. 5) by regression analysis (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 (t [18] = 3.35, p < 0.01; t [13] = 3.69, p < 0.01) improvement in behavior relative to animals with sciatic nerve cuts.

Discussion

Assessments of CAP amplitude (Fig. 2) and intra-axonal dye diffusion (Fig. 3) show that direct application of PEG to crush-severed sciatic nerves usually (97% 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 (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 (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
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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 (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 hours, and does not prevent further
improvement in behaviors at later (three to eight weeks) post-operative times.
The shorter-term behavioral recoveries at 24 - 48 hours are likely produced by
PEG-fused axons. Longer-term behavioral recoveries at three to eight weeks
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 re-innervate 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 (Schallert et al. 2000, 2002,
2006). A modified FF test eliminates much of this problem (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
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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 (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 (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 (digits 2-4) and total toe spread (digits 1-5). Previous studies have shown that the most useful parameter for overall evaluation of sciatic function is total toe spread (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 to FF asymmetry scores. Hence 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 extends previous studies indicating that PEG induces both physiological (measured by the restored
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Conduction of action potentials through the lesion site) and morphological continuity (intra-axonal diffusion of fluorescent dyes across the lesion site) between the cut or crushed ends of mammalian myelinated axons (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 cutaneus trunchi muscle reflex in guinea pigs following a crush injury to the midthoracic spinal cord (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, and Bresnahan open-field hindlimb motor scale (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 to 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 (Mackinnon et al. 1991; Fu and Gordon 1995).
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loss of intramuscular nerve sheaths greatly reduces the number of motor axons that are able to reinnervate 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 weeks.

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 (Sea et al. 1995; Marzullo et al. 2001) 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 non-clinical 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 (Lore et al. 1999; Bittner and Fishman 2000). 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 hours 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.

**Acknowledgements**
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**Figure Legends**

**Fig. 2.** CAP amplitudes of rat sciatic nerves recorded pre-operatively (white and black bars) and post-operatively (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 amplitude of 0mV for all animals. All CAP and other data in this and other figures (except figure 3) are plotted as means +/- SEM.

**Fig. 3.** Graph showing percent of sciatic nerve preparations exhibiting intra-axonal dye diffusion across a lesion site following cut, crush, distilled water crush, PEG-crush, or sham operations (Fig. 3A). Fluorescence images showing intra-axonal dye diffusion across a lesion site following B) sham, C) crush, D) PEG-crush and E) cut operations in sciatic 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 sciatic nerve is on the left and the distal portion of sciatic nerve is on the right. Scale bar: 200 μm for B-E.

**Fig. 4.** Mean foot fault asymmetry scores from 0 – 8 post-operative 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 week time point. SEM 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.
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Fig. 5. SFI results from 0 – 8 post-operative 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 three times for each animal prior to surgery and plotted at the 0 week time point.

Tables

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<thead>
<tr>
<th>Treatment group</th>
<th>Number of rats, CAP confirmation assays</th>
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<tr>
<td>PEG-crush</td>
<td>9, 18</td>
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<tr>
<td>Crush</td>
<td>11, 21</td>
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<tr>
<td>Distilled water crush</td>
<td>2, 4</td>
</tr>
<tr>
<td>Cut</td>
<td>3, 6</td>
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<tr>
<td>Sham-operated</td>
<td>15, 31</td>
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<td><strong>Total</strong></td>
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<th>Treatment group</th>
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<td>Sham-operated</td>
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<td><strong>Total</strong></td>
<td><strong>40 rats, 39 CAP confirmation assays</strong></td>
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</table>

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. 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. Hence more rats were assayed for CAPs and dye diffusion than 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 these 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.
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Fig. 1: Mechanisms of axonal repair by PEG and increasing strength of ECM by fibrin glue
Intra-axonal dye diffusion for various groups

A 100

% Preparations

0 20 40 60 80

n=6  n=21  n=4  n=18  n=31

Cut  Crush  Distilled water crush  PEG-crush  Sham

B

C

200 um

D

E