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Rapid, Effective, and Long-Lasting Behavioral Recovery Produced by Microsutures, Methylene Blue, and Polyethylene Glycol After Completely Cutting Rat Sciatic Nerves

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Behavioral function lost in mammals (including humans) after peripheral nerve severance is slowly (weeks to years) and often poorly restored by 1–2-mm/day, nonspecifically directed outgrowths from proximal axonal stumps. To survive, proximal stumps must quickly repair (seal) plasmalemmal damage. We report that, after complete cut- or crush-severance of rat sciatic nerves, morphological continuity, action potential conduction, and behavioral functions can be consistently (>98% of trials), rapidly (minutes to days), dramatically (70–85% recovery), and chronically restored and some Wallerian degeneration prevented. We assess axoplasmic and axolemmal continuity by intra-axonal dye diffusion and action potential conduction across the lesion site and amount of behavioral recovery by Sciatic Functional Index and Foot Fault tests. We apply well-specified sequences of solutions containing FDA-approved chemicals. First, severed axonal ends are opened and resealing is prevented by hypotonic Ca^{2+}-free saline containing antioxidants (especially methylene blue) that inhibit plasmalemmal sealing in sciatic nerves in vivo, ex vivo, and in rat B104 hippocampal cells in vitro. Second, a hypotonic solution of polyethylene glycol (PEG) is applied to open closely apposed (by microsutures, if cut) axonal ends to induce their membranes to flow rapidly into each other (PEG-fusion), consistent with data showing that PEG rapidly seals (PEG-seals) transected neurites of B104 cells, independently of any known endogenous sealing mechanism. Third, Ca^{2+}-containing isotonic saline is applied to induce sealing of any remaining plasmalemmal holes by Ca^{2+}-induced accumulation and fusion of vesicles. These and other data suggest that PEG-sealing is neuroprotective, and our PEG-fusion protocols that repair cut- and crush-severed rat nerves might rapidly translate to clinical procedures.

Key words: axotomy; nerve regeneration; nerve repair

Cut- or crush-severed peripheral nerves are common injuries in humans that often produce significant behavioral deficits (Bozkurt et al., 2008; Campbell, 2008; Wolfe et al., 2010). Severed distal axonal segments in mammals degenerate within 1–3 days (Ramón y Cajal, 1928; Tsao et al., 1999; Bittner and Fishman, 2000; Bittner et al., 2000). Functional recovery involves 1–2-mm/day axonal outgrowths from surviving proximal stumps that often only partially and nonspecifically

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reinnervate distal denervated target tissues, producing minimal restoration of lost behaviors, especially after complete cut-severance (Bozkurt et al., 2008; Campbell, 2008). For over a century, improving the rate and extent of mammalian axonal regeneration has been a research goal for many neuroscientists, with all repair strategies to date, including nerve grafts, nerve growth guides, and microsutures, relying on axonal outgrowths to reinnervate target tissues. Such techniques have slightly improved the number and specificity of regenerating axons but have little effect on the time course of behavioral recovery or prevention of Wallerian degeneration of severed distal axons (Burdick et al., 2006; Lago et al., 2007; Campbell, 2008; De Ruiter et al., 2008; Kalbermatten et al., 2009; Welin et al., 2009).

After injury, mammalian axons that do not rapidly (within minutes) normally repair plasmalemmal damage do not survive (Schlaepfer and Bunge, 1973; Bittner and Fishman, 2000; Detrait et al., 2000a; Yoo et al., 2004; Nguyen et al., 2005), much less regenerate; i.e., rapid repair of plasmalemmal damage can be neuroprotective (Lago et al., 2007; Radogna et al., 2009; Spaeth et al., 2010). Rapid plasmalemmal sealing of small holes or complete axonal transections in mammalian neurons and other eukaryotic cells is normally produced by a Ca^{2+}-induced accumulation of membrane-bound structures (mostly vesicles) mediated by various protein isomers, many of which are Ca^{2+}-dependent and involved in membrane fusion at synapses or the Golgi apparatus (Spaeth et al., 2010). Antioxidants such as melatonin (MEL) or methylene blue (MB) and various toxins such as botulinum toxins, befeldin A, or N-ethylmaleimide that interfere with vesicle fusion or trafficking have been reported to decrease endogenous plasmalemmal sealing (Spaeth et al., 2010, 2011a,b). Oxidizing agents (H_2O_2, thimerosal [TH]) can increase plasmalemmal sealing in neurons and muscles (Cai et al., 2009; Spaeth et al., 2011a). MB also slightly improves behavioral recovery after damage to nerve or other tissues (Zhang et al., 2006; Radogna et al., 2009; Rojas et al., 2009; Britt et al., 2010).

In contrast to the slow and incomplete repair of mammalian axons, axonal fusion of severed axonal ends is an endogenous mechanism in many invertebrates that rapidly (within days) and completely restores behavior, because regenerating proximal outgrowths need grow only a few millimeters to fuse with (or otherwise activate) their surviving distal axons that do not degenerate for weeks to years (Hoy et al., 1967; Deriemer et al., 1983; Bittner and Fishman, 2000; Bittner et al., 2000; Neumann et al., 2011). Intrigued by these phenomena (Hoy et al., 1967; Bittner, 1973; Bittner and Brown, 1981), we used the fusogen polyethylene glycol (PEG) to induce exogenous (artificial) reconnection of cut-severed proximal and distal halves of unmyelinated crayfish medial giant axons (MGAs) by PEG-fusion in vitro, succeeding in about 3% of all trials (Bittner et al., 1986). Modified protocols produced PEG-fusion of myelinated earthworm MGAs (Krause and Bittner, 1990) in 80% or more of all trials in vitro. Long-lasting restoration of specific behavioral functions of earthworm MGAs in vivo in almost all trials was subsequently induced by PEG-fusion and PEG hydrogels to provide mechanical strength (Lore et al., 1999), although the hydrogels proved toxic to mammalian axons.

Most recently, we reported (Britt et al., 2010) that PEG solutions in almost 100% of all trials could significantly (but modestly, 25–30%) improve behavioral deficits resulting from crush (but not cut)-severance injuries of rat sciatic nerves at 2–3 weeks. Significant recovery at earlier time points was not observed.

Using concepts and results for natural endogenous sealing (especially for MB) and for artificially induced PEG-sealing described in the companion paper (Spaeth et al., 2011b), we report PEG-fusion protocols using MB and microsutures that greatly enhance our ability to repair cut- or crush-severed axons in vivo (Fig. 1). PEG-fused axons exhibit morphological continuity as assessed by intra-axonal dye diffusion and electrophysiological continuity as assessed by conduction of extracellularly recorded compound action potentials (CAPs) across the lesion site. These PEG-fusion protocols for cut- or crush-severed sciatic nerves consistently (over 98% of trials), rapidly, and more completely (7–44% at 24–72 hr after surgery, 66–85% at 12 postoperative weeks), and chronically (at least 12 weeks postoperatively) restore sciatic-mediated behaviors up to 13-fold better, compared with nontreated or conventionally treated animals as assessed by two standard behavioral tests, the Sciatic Functional Index (SFI) and foot fault (FF).

MATERIALS AND METHODS

In Vivo or Ex Vivo Preparations of Rat Sciatic Nerves

All procedures were approved by the University of Texas at Austin's Institutional Animal Care and Use Committee. All animals were housed in groups of three in polycarbonate cages with sawdust bedding, maintained on a 12:12-hr dark/light cycle, and given food and water ad libitum.

Surgical Procedures

Sprague Dawley rats were anesthetized with intraperitoneal injections of ketamine (90 mg/kg) and xylazine (10 mg/kg). The sciatic nerve was exposed by an incision about 1.5 cm long in posterior thigh muscles of the hind limb. All exposed sciatic nerves were cleaned of connective tissue and bathed with hypotonic Ca^{2+}-free Krebs physiological saline perfused from a Pasteur pipette. This Ca^{2+}-free saline contained in mM: 0.5 EGTA, 99 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 26 $NaHCO_3$, 10 Na ascorbate, 10 dextrose, pH 7.35, 319 mOsm double distilled H_2O (ddH_2O). We confirmed in anesthetized animals that exposed, intact sciatic nerves in this hypotonic Ca^{2+}-free saline conducted action potentials across the site of any intended lesion (see CAPs below). Animals in the sham group received no nerve injury following exposure of the sciatic nerve via a skin incision.

Sciatic cut-severance injuries (Fig. 1) were made with microdissection scissors. The cut ends were separated by 1–3 mm, completely severing all axons and their endo-, peri-, and epineural sheaths, as viewed through a ×10–50 dissecting microscope. The proximal and distal nerve segments were

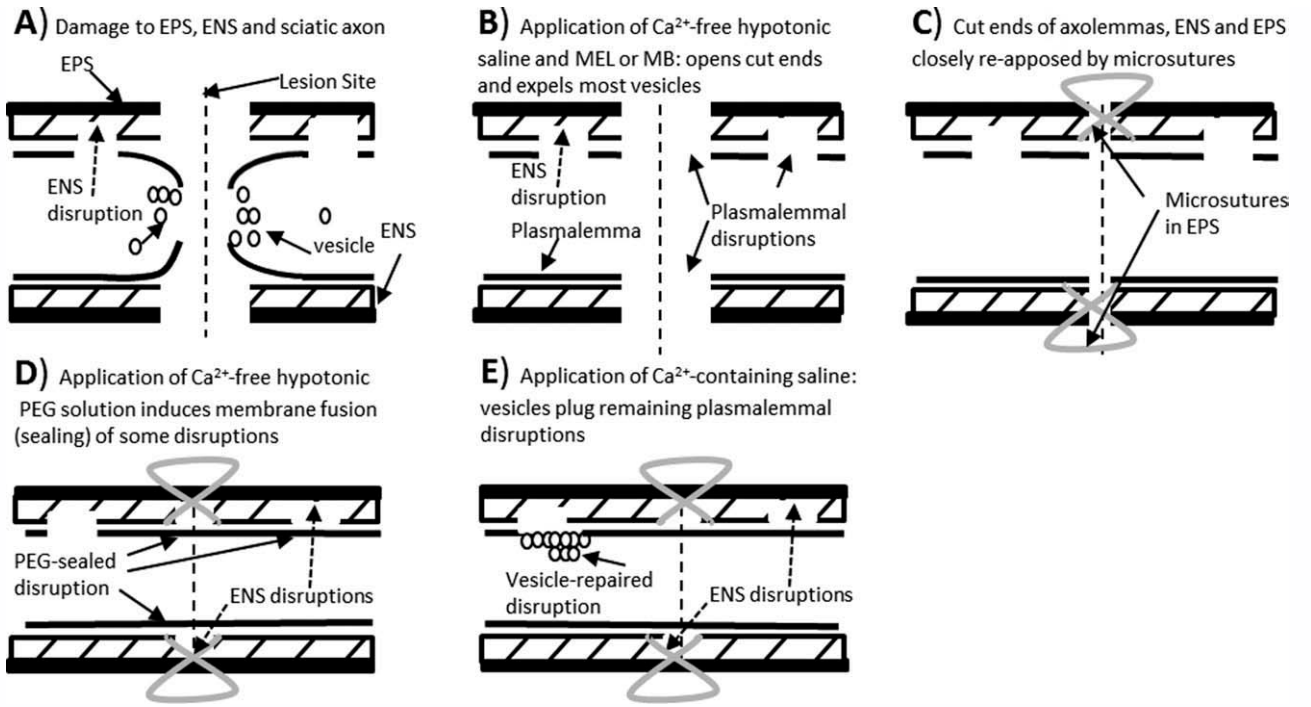

Fig. 1. PEG-fusion protocol for cut-severance repair. Hypothesized effects of each solution used in sequence to produce PEG-fusion of cut-severed axons. Cut-severance completely disrupts the axolemma as well as the endoneural (ENS), perineural and epineural (EPS) sheaths at the lesion site so that the cut ends typically separate by >1 mm. Microsutures placed in the EPS bring the proximal and distal cut axonal ends of sciatic nerves in close apposition. In rat sciatic and other mammalian nerve bundles, the PEG-fused cut ends are probably not the two halves of the same axon in most cases. Crush-severance PEG-fusion protocols use the same sequence of solutions, but microsutures are not needed or used because endo-, peri-, and/or epineural sheths remain sufficiently intact so that cut ends are opened and closely approximated and vesicles eliminated or reduced by Ca^{2+}-free hypotonic saline containing MB. Concentrations were 2 mM MEL, 100 μM MB, 500 mM 2 kDa PEG.

realigned so that slight abnormalities in shape of the two cut ends matched as closely as possible and were surgically reapposed with 10-0 vinyl microsutures (S&T). The microsuture needle was carefully inserted through the epineural sheath using a microneedle holder to avoid further damage to the axons. Surgeons tied six to eight slightly loose, microsuture knots, so that solutions containing MB or PEG in hypotonic Ca^{2+}-free saline or isotonic Ca^{2+}-containing saline could be delivered more easily to the axons during PEG-fusion repair. Isotonic Ca^{2+}-containing (Krebs) saline contains in mM; 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 26 $NaHCO_3$, 10 Na ascorbate, 10 dextrose, 2 $CaCl_2$, pH 7.35, 351 mOsm in ddH_2O. Throughout all procedures, the nerve was moistened with the appropriate saline.

For animals treated with 100 μM MB (Faulding, Aguadilla, Puerto Rico), or 2 mM MEL (Biomol, Plymouth Meeting, PA) the antioxidant was dissolved in hypotonic Ca^{2+}-free saline and applied from a micropipette positioned several millimeters above the nerve so that the solution flowed in a narrow stream (about 1 mm wide) for 1–3 min over the cut or crushed axons at the lesion site. Treatments of 500 mM PEG (Sigma Aldrich, St. Louis, MO) dissolved in ddH_2O were then applied to the lesion site via a similar micropipette so that the denser PEG-containing solution flowed for 1.5–2 min over the lesion site. After such treatments, a second pipette was used to perfuse the sciatic nerve with an appropriate saline.

Sciatic crush-severance injuries were made with Dumont No. 5 forceps, applying enough force to sever every axon. The crush-severance site about 1 mm long was much less opaque than adjacent uninjured sites viewed at ×20–50 through a dissecting microscope. After crush injury, the sciatic nerve was again assessed for CAP conduction across the lesion site. If any CAP was detected, the nerve was crushed again. This condition occurred in only two of over 185 crush-severed nerves. To apply treatment solutions in the sequence described above for cut-severed sciatic nerves, the epineural sheaths of crush-severed sciatic nerves were nicked at the lesion site with microdissection scissors to allow better access of solutions to the crush-severed axons.

For animals (n = 300) to be examined behaviorally for 8–12 weeks postoperatively, the skin over the lesion site was closed with sutures and surgical staples. The rats received a 5 mg/kg subcutaneous injection of ketoprofen after surgery. The effects of the anesthesia lasted for about 24 hr. Alternatively (n = 120 animals), a 2–4-cm length of sciatic nerve was removed for ex vivo assays of morphological integrity by intra-axonal dye diffusion.

Treatment Groups and Their Rationale

Cut-severed rat sciatic nerves were assayed in vivo or ex vivo. Some were left untreated (cut), some were treated

with microsutures only (cut + suture) to appose proximal and distal ends closely, and some were treated with MB only (cut + MB) or PEG only (cut + PEG). Some cut-severed sciatic nerves were treated either with microsutures and PEG (cut + suture + PEG) or microsutures and MB (cut + suture + MB), and others were treated with microsutures, PEG, and MB (cut + suture + MB + PEG). Sham-treated animals received an incision to expose the sciatic nerve whose epineurium was nicked but received no crush-severance injury or drug treatment. The sham-injury group was used as the intact nerve group to which all other treatments were compared for complete recovery. Sciatic nerves with cut-severed axons that did not receive any treatment (cut group) were used to assess both the extent of the injury and any spontaneous recovery that might occur following injury. All treated groups were compared with the cut-only group to assess possible behavioral recovery. Sciatic nerves with cut-severed axons whose cut ends were closely apposed with microsutures (cut + sutures) were used to compare the effect of a similar standard clinical treatment to untreated cuts (cut group) and to the effects of several PEG protocols (cut + suture + PEG and cut + suture + MB + PEG).

Crush-severed rat sciatic nerves were assayed in vivo or ex vivo. Some were left untreated, some were treated with either MEL (crush + MEL) or MB (crush + MB), some were treated with PEG (crush + PEG), and some were treated with PEG together with MEL (crush + MEL + PEG) or MB (crush + MB + PEG). Sciatic nerves with crush-severed axons that did not receive any treatment (crush) were used to assess both the extent of the sciatic nerve injury and any spontaneous recovery that might occur following that injury. All treated groups were compared with the crush treatment group to assess possible behavioral recovery. Sciatic nerves with crush-severed axons treated only with PEG (crush + PEG) were used to compare the effects of those treated with PEG plus either MEL (crush + MEL + PEG) or MB (crush + MB + PEG).

Electrophysiological Recording of CAPs

Electrophysiological recordings of CAPs conducting across a lesion site in vivo were made by two pairs of nickel-tipped hook electrodes placed on or beneath the sciatic nerve to stimulate and record CAPs, which were displayed conventionally on an oscilloscope (Lore et al., 1999; Marzullo et al., 2002; Britt et al., 2010). All lesions were made between the stimulating and recording pairs of hook electrodes. Complete crush or cut-severance of all axons was confirmed by an inability to record a detectable CAP conducted through the lesion site. The sciatic nerve was always stimulated after any treatment to determine whether CAPs conducted through the lesion site. During preoperative and postoperative CAP recordings in vivo, the nerve was continuously moistened with Ca^{2+}-free saline.

For some animals ($n > 100$), to correlate a measure of electrophysiological continuity with a measure of morphological continuity, we recorded CAPs from both left and right sciatic nerves in adult male Sprague Dawley rats (250–350 g) assigned to each of the crush and cut treatment groups described above. Following all such treatments, we assessed morphological continuity with an intra-axonal dye diffusion assay (see description in the following section).

In another set of rats ($n > 200$), to correlate our measure of electrophysiological continuity with our measures of behavioral recovery for each rat, we recorded CAPs from the left sciatic nerves before in vivo and after any injury and/or subsequent treatment. We then postoperatively tested the rats behaviorally for 6 (FF) or 12 (SFI) weeks. To reduce use of animals for the three crush treatment groups (crush, crush + MEL, crush + MB), data were examined for animals previously reported (Britt et al., 2010) and additional animals sampled thereafter. Because the previously reported vs. more recently collected CAP, SFI, and FF means or curves did not differ significantly ($P > 0.05$) for any of the three treatment groups, we combined the data for these groups to generate Figure 3B,D and Tables II and V. Cut treatment groups did not contain any previously reported data.

Intra-Axonal Dye Diffusion Across a Lesion Site

To correlate CAP conduction in vivo with intra-axonal diffusion of dye through the lesion site ex vivo, after recording CAPs, we excised a 2–4-cm length of the sciatic nerve (including any lesion site) from animals not used for behavioral studies (Lore et al., 1999; Britt et al., 2010). The proximal end of the sciatic nerve segment was placed within a petroleum jelly (Vaseline) ring containing Ca^{2+}-free saline and 20 μl hydrophilic dye (2 kDa Texas red dextran; Molecular Probes, Eugene, OR). The remainder of the nerve, including any cut- or crush-severance site, was bathed in Ca^{2+}-containing saline. The Petri dish containing the nerve in the Vaseline well was refrigerated for 14 hr at 4°C. Nerves were examined for intra-axonal diffusion of fluorescent dye beyond the cut or 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 treatment conditions, during the dark portion of each animal's daily light cycle, during which rats are more active. Rats were handled daily for 7 days prior to the start of behavioral testing. Baseline behavior scores for SFI and FF were obtained 1–2 days prior to surgery. After surgery, all rats were evaluated for behavioral recovery at 24, 48, and 72 hr postoperatively and then at weekly intervals for 12 weeks (SFI) or 6 weeks (FF). Animals were first tested at 24 hr postoperatively to allow them to recover from the effects of surgery and anesthesia that would most affect behavioral measures.

SFI. The SFI is probably the most commonly used test for evaluating recovery of behaviors mediated by the sciatic nerve in rats, especially for rat models of Parkinson's disease (Schallert et al., 1978) and sciatic nerve severance (de Medinaceli et al., 1982; Britt et al., 2010). The SFI uses footprints to measure gait quality to indicate how well sciatic axons innervate more distal muscle groups (de Medinaceli et al., 1982; Britt et al., 2010). Rats were trained to traverse a wooden beam positioned to lead up to their home cage, as previously reported (Britt et al., 2010). Three consecutive footprints from each limb (for a total of six consecutive prints)

were used to measure (in millimeters) the following: normal footprint length (NPL), experimental footprint length (EPL), normal toe spread between toes one and five (NTS), experimental toe spread (ETS), normal intermediary toe spread between toes two and four (NIT), and experimental intermediary toe spread (EIT). Baseline and postoperative SFI scores were then computed for each animal as previously described (de Medinaceli et al., 1982; Britt et al., 2010).

Foot fault (FF) test. Compared with SFI scores, FF scores indicate how well sciatic axons innervate more proximal muscle groups (Britt et al., 2010). After sciatic nerve damage, more proximal muscle groups are likely innervated before more distal muscle groups (Wolfe et al., 2010). Animals were allowed to roam freely on a wire mesh grid (45×30 cm^2, with 2.5×2.5 cm^2 openings) elevated 1.5 cm above a solid base floor. Baseline and postoperative trials for each animal were recorded for 50 total steps per hind limb. A FF was scored when a misstep resulted in the hind limb falling through an opening in the grid. If the hind limb 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 1. A full fault occurred when the animal's hind limb touched the floor beneath the grid for support and was given a fault score of 2. Baseline and postoperative FF asymmetry scores were then calculated, as previously reported (Britt et al., 2010).

Statistical Analyses

Student's *t*-test was used to assess differences ($P < 0.05$) in preoperative CAP amplitudes vs. postoperative CAP amplitudes. Mixed-subject 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 (Agresti, 1996; Detrait et al., 2000a,b; Britt et al., 2010). Statistically significant differences ($P < 0.05$) between mean values of curves fitted (GraphPad Prism 5.0) to SFI and FF data for cut treatment groups were determined by using analysis of covariance (ANCOVA). GraphPad Prism and curvefit.com were used to compare overall curves of data points from separate treatment groups. Mixed-subject ANCOVA was then used to compare curve parameters between treatment groups of interest.

RESULTS

Intra-Axonal Diffusion of Fluorescent Dye in Intact and PEG-Fused Nerves

Because PEG induces sealing (PEG-sealing) of transected neurites of B104 cells (Spaeth et al., 2011a), we investigated whether PEG could seal cut ends of sciatic axons. Excised (cut) sciatic nerves were placed in a Petri dish containing 10 mM PEG dissolved in Ca^{2+}-free hypotonic saline for 1 min. The nerves were placed in another Petri dish containing Texas red dextran dissolved in Ca^{2+}-free hypotonic saline and assayed for dye uptake or exclusion. Dye was always excluded at severed axonal ends treated with 10 mM PEG (Fig. 2A). These data are consistent with previous data (Spaeth et al., 2011b) showing that PEG rapidly seals cut sciatic axons or B104 neurites.

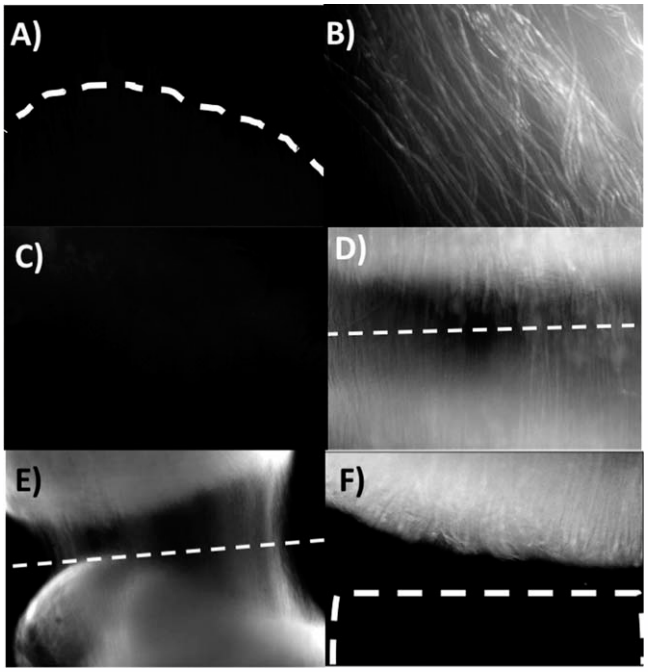

Fig. 2. Dye diffusion through intact or injured sciatic nerves. **A:** Fluorescent image showing dye exclusion by the cut end of a sciatic nerve (indicated by thick dashed lines). This nerve was placed in a Petri dish containing 10 mM PEG dissolved in Ca^{2+}-free hypotonic saline for 1 min. The nerve was then placed in another Petri dish containing Texas red dextran and Ca^{2+}-free isotonic saline. **B:** Fluorescent image showing intra-axonal dye diffusion through an intact (sham operated) sciatic nerve. The proximal end of the nerve was placed in a water-tight Vaseline well filled with Texas red dextan dissolved in Ca^{2+}-free hypotonic saline. The distal end of the nerve extended outside the well and was bathed in Ca^{2+}-containing isotonic saline. **C:** Fluorescent image showing no dye uptake by an intact (sham operated) sciatic nerve. The proximal end of the nerve was placed in a well filled with Texas red dextran and Ca^{2+}-containing isotonic saline. The distal end of the nerve extended out of the well and was bathed in Ca^{2+}-free hypotonic saline. **D,E:** Fluorescent images showing intra-axonal dye diffusion through the lesion site of crush-severed nerves treated with 2 mM MEL and then PEG-fused (D) or 100 μM MB and then PEG-fused (E). The lesion site is indicated by thin dashed lines. The proximal end of these nerves was placed in a well filled with Texas red dextran and Ca^{2+}-free hypotonic saline. The distal end of these nerves, including the lesion site, extended outside the well and was bathed in Ca^{2+}-containing isotonic saline. **F:** Fluorescent image of a sciatic nerve segment cut-severed at its midpoint showing that dye does not cross this lesion site that is not PEG-fused. The very proximal (cut) end was placed in a well filled with Texas red dextran and Ca^{2+}-free hypotonic saline. The cut-severance lesion site was outside the well and was bathed in Ca^{2+}-containing isotonic saline solution. The distal cut end (indicated by thick dashed lines) of this lesion site was in close apposition to the proximal end but was not PEG-fused. Proximal portion of the sciatic nerve is always located at the top of the image.

We next investigated whether PEG and/or antioxidants could restore morphological continuity to crush- or cut-severed axons. After recording CAPs before injury, after injury, and after any experimental treatment

TABLE I. Dye Diffusion Data for Crush- and Cut-Severed Sciatic Axons*

	Cut-severed	
Cut-severed treatment group	Cut end in Ca^{2+}-free; cut lesion site in Ca^{2+} (n)	Cut end in Ca^{2+}; cut lesion site in Ca^{2+} (n)
Cut	No (10)	No (5)
Cut + PEG	No (10)	No (5)
Cut + suture	No (10)	Not tested
Cut + suture + MB	No (4)	Not tested
Cut + suture + PEG	Yes (8)	Not tested
Cut + suture + MB + PEG	Yes (10)	Not tested
Sham	Yes (10)	No (3)
Crush-severed treatment group	Cut end in Ca^{2+}-free; crush lesion site in Ca^{2+} (n)	Cut end in Ca^{2+}; crush lesion site in Ca^{2+} (n)
Crush	No (20)	No (5)
Crush + MEL	No (10)	Not tested
Crush + MB	No (10)	Not tested
Crush + PEG	Yes (20)	No (5)
Crush + MEL + PEG	Yes (10)	Not tested
Crush + MB + PEG	Yes (10)	Not tested
Sham	Yes (10)	No (5)
Crush + PEG-sealed end	No (5)	Not tested

*Injury and treatment performed on sciatic nerve. n, Number of sciatic nerves tested for each cut- or crush-severed treatment group. For all dye diffusion assays, lesion sites were always maintained in Ca^{2+}-containing saline outside the well containing dye. Proximal ends were bathed in Ca^{2+}-free (column 1) or Ca^{2+}-containing saline (column 2). Yes: Dye diffused past the lesion site intra-axonally for each sample (100% of the time) for that treatment group. No: Dye did not diffuse past the lesion site for any sample of that treatment group (0% of nerves with dye past the lesion site). Not tested: Dye diffusion assay not performed for that treatment group and injury type.

if necessary, as described in the next section detailing CAP protocols, we excised 2–4-cm lengths of sciatic nerves on either side of the lesion site from rats in each treatment group (Table I; n = 4–20 for each group). As previously reported (Lore et al., 1999; Britt et al, 2010) for intra-axonal dye diffusion assays, the most proximal excised end was placed in a watertight Vaseline well containing dye (Texas red dextran) dissolved in Ca^{2+}-free hypotonic saline or Ca^{2+}-containing isotonic saline; the lesion site was outside the well and was maintained in Ca^{2+}-containing isotonic saline or Ca^{2+}-free hypotonic saline, as specified below.

Dye always diffused intra-axonally throughout the entire length of uninjured (sham-operated) sciatic nerves if the proximal ends of sciatic axons in the well were bathed in Texas red dextran dissolved in Ca^{2+}-free hypotonic saline with the distal end of the nerve outside the well bathed in either Ca^{2+}-containing saline (Fig. 2B) or Ca^{2+}-free saline (not shown). Dye never diffused intra-axonally if the proximal cut axonal ends in the well were bathed in Texas red dextran in Ca^{2+}-containing isotonic saline and the cut distal ends outside the well were bathed in either Ca^{2+}-free hypotonic saline (Fig. 2C) or Ca^{2+}-containing isotonic saline (not shown). These data are consistent with data from B104 cells and other preparations (Xie and Barrett, 1991; Spaeth et al., 2010, 2011b) showing that severed neuritic or axonal ends do not seal in Ca^{2+}-free saline but do seal in Ca^{2+}-containing saline.

Dye always diffused intra-axonally across the lesion site for PEG-fused crush-severed nerves (MEL + PEG [Fig. 2D] or MB + PEG [Fig. 2E]) if the proximal end in the well was exposed to Texas red dye in Ca^{2+}-free hypotonic saline and the lesion site, which might also contain 2 mM MEL or 100 μM MB. The distal nerve segment outside of the well was exposed to Ca^{2+}-containing isotonic saline, and 500 mM 2 kDa PEG was applied to the lesion site by a micropipette to PEG-fuse axons at the lesion site. These data are consistent with our hypothesis that PEG-fusion of closely apposed severed axonal halves restores axonal continuity at the site of PEG-fusion (Britt et al., 2010) and that MEL or MB applied before PEG application does not impede (and may assist) PEG-fusion.

Dye never diffused past the lesion site in cut- (Fig. 2F)- or crush (not shown)-severed sciatic nerves if the proximal end was placed in a well filled with Texas red dextran in Ca^{2+}-free hypotonic saline, and the distal end, including the lesion site, was placed outside of the well in Ca^{2+}-containing isotonic saline. These data are consistent with the hypotheses that cut or crush injuries completely sever all axons within the lesion site (see CAP data), that (in the absence of PEG) Ca^{2+} is necessary for endogenous sealing of severed axons, and that severed axons do not exhibit morphological continuity in the absence of PEG-fusion.

Taken together, these intra-axonal dye diffusion data as a measure of morphological continuity of some PEG-fused axons at the lesion site are consistent with interpretations that: 1) Ca^{2+} prevents dye uptake ex vivo because it initiates sealing of severed axonal ends (see Spaeth et al., 2011b); 2) Ca^{2+}-free hypotonic saline opens axonal ends and allows uptake of dye that diffuses intra-axonally throughout axons having morphological continuity; 3) cut- or crush-severance completely disrupts axonal continuity between proximal and distal axonal segments; and 4) if cut- or crush-severed axonal ends are open and in close apposition, solutions containing PEG can establish morphological continuity to distal and proximal cut- or crush-severed ends (of unknown specificity) of many sciatic axons. These dye diffusion data are the first describing morphological repair after PEG fusion in vivo for any mammal.

CAP Conduction in Intact and PEG-Fused Nerves

We first confirmed in anesthetized rats that exposed, intact sciatic nerves conducted CAPS across the site of any intended lesion (see Materials and Methods). We then bathed the nerves in Ca^{2+}-free hypotonic saline and completely cut or crush-severed them. Complete cut- or crush-severance was demonstrated by confirming that

TABLE II. CAP Data for Cut- or Crush-Severed Groups★

Treatment group	Mean prelesion CAP (mV)	Mean postlesion CAP (mV)	Postlesion CAP/prelesion CAP (%)	Average preinjury CAP (mV)	Average postinjury CAP (mV)
Pretreatment cuts in vivo, n = 120 rats	4.1 ± 0.17				
Cut					
Dye n = 10	3.9 ± 0.24	0.0	0	4.0 ± 0.22	0.0
Beh n = 10	4.1 ± 0.20	0.0	0		
Cut + PEG					
Dye n = 10	3.5 ± 0.25	0.0	0	3.7 ± 0.22	0.0
Beh n = 10	3.9 ± 0.19	0.0	0		
Cut + suture					
Dye n = 10	3.6 ± 0.34	0.0	0	3.7 ± 0.31	0.0
Beh n = 10	3.7 ± 0.28	0.0	0		
Cut + suture + MB					
Dye n = 4	3.0 ± 0.21	0.0	0	3.1 ± 0.25	0.0
Beh n = 4	3.6 ± 0.29	0.0	0		
Cut + suture + PEG					
Dye n = 8	3.4 ± 0.22	1.8 ± 0.15	53	3.8 ± 0.20	2.1 ± 0.22
Beh n = 4	4.2 ± 0.17	2.4 ± 0.29	57		
Cut + suture + MB + PEG					
Dye n = 10	4.1 ± 0.20	3.1 ± 0.18	75†††	4.3 ± 0.23	3.3 ± 0.23
Beh n = 10	4.5 ± 0.26	3.5 ± 0.27	78†††		
Sham					
Dye n = 10	3.2 ± 0.13	3.2 ± 0.13	100	3.6 ± 0.16	3.6 ± 0.16
Beh n = 10	3.9 ± 0.19	3.9 ± 0.19	100		
Pretreatment crushes in vivo n = 90 rats	3.1 ± 0.29				
Ex vivo, n = 90 nerves; 45 rats	3.4 ± 0.21				
Crush					
Dye n = 20	2.9 ± 0.27	0.0	0	3.0 ± 0.25	0.0
Beh n = 20	3.1 ± 0.23	0.0	0		
Crush + MEL					
Dye n = 10	3.1 ± 0.33	0.0	0	3.2 ± 0.31	0.0
Beh n = 10	3.3 ± 0.28	0.0	0		
Crush + MB					
Dye n = 10	3.6 ± 0.46	0.0	0	3.5 ± 0.34	0.0
Beh n = 10	3.4 ± 0.23	0.0	0		
Crush + PEG					
Dye n = 20	3.0 ± 0.21	1.6 ± 0.21	53	3.1 ± 0.25	1.7 ± 0.23
Beh n = 20	3.2 ± 0.29	1.7 ± 0.25	52		
Crush + MEL + PEG					
Dye n = 10	3.9 ± 0.20	2.3 ± 0.18	56	3.7 ± 0.24	1.9 ± 0.20
Beh n = 10	3.5 ± 0.28	1.5 ± 0.22	43		
Crush + MB + PEG					
Dye n = 10	4.0 ± 0.33	3.1 ± 0.24	73†,##	4.1 ± 0.32	3.1 ± 0.29
Beh n = 10	4.1 ± 0.31	3.0 ± 0.34	78†††,###		
Sham					
Dye n = 10	3.2 ± 0.08	3.2 ± 0.08	100	2.9 ± 0.13	2.9 ± 0.13
Beh n = 10	2.5 ± 0.18	2.5 ± 0.18	100		

★CAP amplitudes ± SEMs for different cut and crush treatment groups. Preoperation: Prior to crush-severance and any subsequent treatment, prelesion CAP amplitudes were recorded in vivo using hook electrodes placed directly on the sciatic nerve. Student's *t*-test with Bonferroni's correction for multiple comparisons was used to compare CAP data. Cut + suture + MB + PEG had a significantly ($P < 0.001$) better % postlesion CAP compared with cut + suture + PEG, for both Dye and Beh treatment groups. Daggers indicate significant difference for crush + MB + PEG vs. crush + PEG treatment groups (†$P < 0.05$; †††$P < 0.001$).
Number signs indicate significant difference for crush + MB + PEG vs. crush + MEL + PEG treatment groups (##$P < 0.01$, ###$P < 0.001$).

sciatic axons did not conduct CAPs across the severance site and that cut-severed sciatic nerves had no physical connections between cut ends. Some cut- or crush-severed nerves were then PEG-fused at the lesion site with or without treatment with MB or MEL.

Table II gives CAP amplitude data recorded in vivo from cut-severed sciatic nerves used for ex vivo dye diffusion studies (Dye) and for in vivo behavioral studies (Beh). CAPs were always detected in intact sciatic nerves and sham-operated nerves and never detected across the lesion site immediately after cutting the sciatic nerve (for n values see Table I). CAPs were always subsequently detected postcut for cut + suture + PEG and cut + suture + MB + PEG-treated nerves, i.e., when

cut nerves were PEG-fused. Cut + suture + MB + PEG-treated nerves had the greatest recovery of postcut CAP amplitudes of any treatment group ($P < 0.001$). CAPs were never detected postcut across the lesion site for cut-, cut + PEG-, cut + suture-, or cut + suture + MB-treated sciatic nerves, i.e., in the absence of PEG-fusion. The presence or absence of CAP conduction across the lesion was also always associated with the presence or absence, respectively, of dye diffusion across the lesion site. These CAP data are the first describing through-conduction for successful PEG-fusion after cut-severance in vivo for any mammal.

Table II also gives CAP amplitude data recorded in vivo from crush-severed sciatic nerves subsequently used for ex vivo dye diffusion studies (Dye) or from nerves subsequently used for in vivo behavioral studies (Beh). CAPs were always detected in all intact axons prior to crushing the entire sciatic nerve. CAPs were rarely detected distal to the lesion after crushing the entire sciatic nerve. In those rare cases (two of 185) when a CAP was detected distally after a crush, the nerve was completely crushed again at the same site. CAPs were then never detected. CAPs were always detected posttreatment in the sham group and across the lesion site in crush + MEL + PEG-, crush + MB + PEG-, and crush + PEG-treated nerves, i.e., if crush-severed ends were PEG-fused. CAPs were never detected postoperatively across the lesion site in crush-, crush + MEL-, or crush + MB-treated sciatic nerves, i.e., if PEG was not applied. CAP data from crush-severed nerves again correlated perfectly with dye diffusion data; i.e., the presence or absence of CAP conduction across the lesion site was always associated with the presence or absence, respectively, of intra-axonal dye diffusion across the lesion site (compare Tables I and II).

For sciatic nerves used for ex vivo dye diffusion studies, treatment with crush + MB + PEG restored an average of 73% of their initial CAP amplitudes, which was significantly greater than crush + PEG (53%; $P < 0.05$) or crush + MEL + PEG (56%; $P < 0.01$) CAP amplitudes. For sciatic nerves used for in vivo behavioral studies, treatment with MB + PEG restored an average of 78% of the initial CAP amplitude, which was significantly greater compared with treatment with crush + PEG (52%; $P < 0.001$) or crush + MEL + PEG (43%; $P < 0.001$). That is, treatment of cut or crush lesions with PEG + MB produced the largest postoperative CAP amplitudes, consistent with the hypothesis that larger CAP amplitudes are due to a greater number of axons conducting APs across the lesion site.

Behavioral Recovery of Cut-Severed Nerves

To assess the restoration of sciatic-mediated behaviors after complete cut-severance, we used two conventional behavioral assays: the SFI and a modified FF asymmetry test. The SFI is more sensitive to more distal sensory-motor functions, and the FF is more sensitive to more proximal sensory-motor functions and earlier behavioral recovery following sciatic nerve cut- or crush-severance (Britt et al., 2010). We plot these behaviors as different panels without SEM bars in Figure 3A–D to allow easier and immediate comparison with each other and with the more detailed data analyses given in Tables III–V.

One-way mixed-subject ANOVA followed by post hoc analyses of SFI data (Fig. 3A, Table III) showed that each data point for the cut + suture + MB + PEG group was significantly ($P < 0.01$) greater compared with each data point at the same postoperative time for all other treatment groups (except for cut + suture + PEG) from 1 to 12 weeks postoperation. Although no pair of data points at a given postoperative time was significantly different, curve-fit analyses (see Materials and Methods) showed that SFI behavioral recovery for the cut + suture + MB + PEG group was significantly ($P < 0.01$) greater and faster than the 1 day to 12 weeks of recovery of the cut + suture + PEG group and $P < 0.001$ when compared with any other cut-severance group. The cut + suture + PEG curve was also significantly ($P < 0.01$) better than the cut + suture curve (Fig. 3A). After 2 or 12 weeks postcut severance, cut and cut + suture animals showed 0–4% and 0–21% recovery, respectively, compared with cut + suture + PEG and cut + suture + MB + PEG, which showed 22% and 41% recovery at 2 weeks or 60% and 70% at 12 weeks, respectively. Similar analyses of FF for cut- severance groups (Fig. 3C, Table IV) showed that each data point for the cut + suture + MB + PEG group was significantly ($P < 0.05$ or $P < 0.01$) greater compared with each data point at the same postoperative time for any other treatment group (except cut + suture + PEG) from 1 to 12 weeks postoperation. Although no pairs of data points at a given postoperative time were significantly different, comparison of curve-fit parameters revealed tha the FF behavioral recovery for the cut + suture + MB + PEG group was significantly ($P < 0.01$) greater and faster than the 1 day to 12 weeks of recovery of the cut + suture + PEG group and $P < 0.001$ compared with any other group. After 2 weeks vs. 12 postcut severance, cut or cut + suture animals showed 9% vs. 14% or 8% vs. 43% recovery, respectively, compared with cut + suture + PEG or cut + suture + MB + PEG, which showed 8% or 8% recovery at 2 weeks and 53% or 79% at 12 weeks, respectively.

There was no statistically significant ($P > 0.05$) difference between curves of data points for cut + suture + MB + PEG generated by C.P.K. (a neurosurgeon) and J.M.B. (a graduate student). Successful PEG-fusions have also been produced by undergraduates, postdoctoral fellows, and faculty. All these statistical analyses also strongly suggested that the rate of recovery of behavioral function for cut-severed nerves was fastest when cut nerves were treated with suture + MB + PEG, followed by suture + PEG and, finally, suture.

For either SFI or FF tests, data for cut, cut + MB, or cut + PEG groups did not differ significantly at any time point or for the curves generated by the entire set of data points for each group. That is, in the absence of close apposition of cut ends, neither MB nor PEG had a

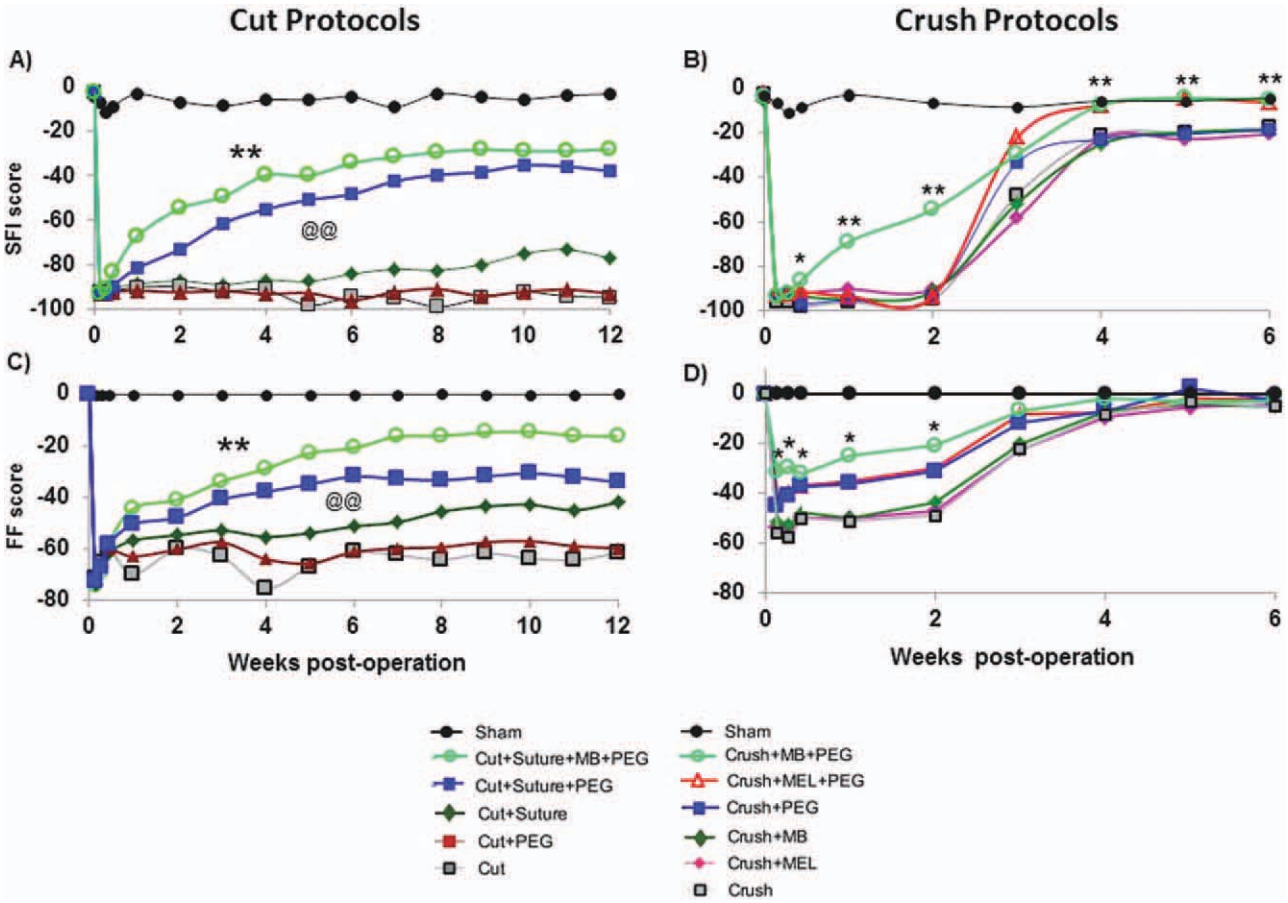

Fig. 3. Mean SFI scores (**A,B**) and mean FF asymmetry scores (**C,D**) for cut (**A,C**) or crush (**B,D**) treatment groups vs. postoperative week. Tabulated means ± SEM and detailed statistical comparisons see Table III for A, Table V for B, Table IV for C, and Table V for D. For cut protocols, ** label the cut + suture + MB + PEG curve and @@ labels the cut + suture + PEG curve that both significantly ($P < 0.01$) differ from the cut curve. For the crush protocol, asterisks label individual postoperative data points for crush + MB + PEG treatment group that differ significantly from data points for the crush group at the same postoperative time. *$P < 0.05$, **$P < 0.01$.

beneficial effect. Furthermore, we observed no significant difference between cut + suture and cut + suture + MB. That is, with close apposition of cut ends by microsutures, but not PEG-fusion, the antioxidant MB did not enhance behavioral recovery. Cut + suture treatment showed some limited and slow improvement compared with cut alone ($P < 0.05$), as previously reported. Unlike those for the comparable crush-severance groups (crush + PEG and crush + MB + PEG) described below, FF scores for cut + suture + MB + PEG or for cut + suture + PEG did not recover to levels recorded for sham-operated or intact sciatic nerves. However, our cut + suture + MB + PEG protocol for PEG-fusion of completely cut nerves did produce much better, long-lasting recovery of sciatic nerve function as measured by the SFI, which was up to 13-fold better, i.e., 63–70% within 6–12 weeks compared with 2–16% recovery for cut or cut + suture protocols (Fig. 3A,C).

The sciatic nerves of a few (n = 5) animals were also cut-severed in vivo, and the wound site was closed and/or moistened with Ca^{2+}-containing isotonic Krebs for at least 30 min. [Sealing of mammalian neurites and nerves is typically complete in 10–20 min in Ca^{2+}-containing isotonic saline (Xie and Barrett, 1991; Lore et al., 1999; Spaeth et al., 2010).] The sciatic nerve was then bathed in Ca^{2+}-free hypotonic saline, and our cut + suture + MB + PEG treatment was applied. All five animals had an easily detectable CAP before cutting and no detectable (0 mV) CAPs after cutting, and all exhibited a readily detectable CAP after this PEG-fusion protocol. The mean CAP was 2.55 ± 0.37 mV prior to cutting and 2.45 ± 0.44 mV after PEG-fusion, i.e., a greater than 90% recovery.

Behavioral Recovery of Crush-Severed Nerves

One-way mixed-subject ANOVA followed by post hoc analyses of SFI data (Fig. 3B, Table V) showed that crush + MB + PEG groups had significantly ($P < 0.05$ or $P < 0.01$) greater and more rapid recovery at each postoperative time point compared with all other groups (except sham) from 3 days to 2 weeks postoperation.

TABLE III. SFI Data for Cut-Severed Groups

Treatment group	Cut	Cut + PEG	Cut + suture	Cut + suture + PEG	Cut + suture + MB + PEG	Sham
Baseline	−3.1 ± 0.5	−2.5 ± 0.8	−3.3 ± 0.7	−2.4 ± 1.0	−2.63 ± 1.8	−4.3 ± 1.8
24 Hours	−95.8 ± 1.9	−93.3 ± 2.9	−92.5 ± 3.2	−93.3 ± 1.3	−93.3 ± 1.1	−6.9 ± 2.5
48 Hours	−93.8 ± 3.8	−92.7 ± 1.4	−91.7 ± 3.1	−93.1 ± 1.9	−91.2 ± 2.0	−11.5 ± 1.4
72 Hours	−94.3 ± 3.2	−94.5 ± 2.8	−90.5 ± 2.7	−89.6 ± 2.2	−83.8 ± 1.8	−8.8 ± 2.7
1 Week	−92.8 ± 3.1	−92.2 ± 1.4	−88.7 ± 2.2	−81.7 ± 2.8	−67.2 ± 3.0[cc,ee,ff]	−3.5 ± 1.2
2 Weeks	−90.3 ± 3.1	−91.7 ± 2.2	−87.4 ± 3.1	−73.1 ± 3.2[a,bb,dd]	−54.8 ± 2.9[cc,ee,ff]	−6.1 ± 1.6
3 Weeks	−91.8 ± 1.8	−88.4 ± 4.1	−89.1 ± 1.9	−61.5 ± 1.6[a,bb,dd]	−49.5 ± 1.7[cc,ee,ff]	−8.3 ± 2.4
4 Weeks	−91.4 ± 2.5	−89.9 ± 3.2	−87.8 ± 2.5	−55.8 ± 2.9[a,bb,dd]	−39.8 ± 2.1[cc,ee,ff]	−5.2 ± 3.1
5 Weeks	−98.0 ± 1.2	−91.2 ± 2.1	−87.4 ± 2.9	−50.9 ± 1.5[a,bb,dd]	−40.0 ± 1.9[cc,ee,ff]	−5.2 ± 2.9
6 Weeks	−94.2 ± 3.3	−89.1 ± 3.4	−84.2 ± 3.4	−48.7 ± 1.2[a,bb,dd]	−34.4 ± 1.9[cc,ee,ff]	−4.9 ± 1.1
7 Weeks	−94.3 ± 2.2	−92.8 ± 2.9	−82.1 ± 1.6	−42.4 ± 2.6[a,bb,dd]	−31.5 ± 3.2[cc,ee,ff]	−9.0 ± 2.5
8 Weeks	−99.9 ± 2.6	−91.0 ± 1.6	−82.5 ± 2.8	−39.9 ± 1.9[a,bb,dd]	−29.5 ± 1.9[cc,ee,ff]	−3.8 ± 1.4
9 Weeks	−95.2 ± 1.9	−94.1 ± 1.2	−80.2 ± 2.2	−38.1 ± 2.3[a,bb,dd]	−28.3 ± 2.0[cc,ee,ff]	−4.7 ± 3.2
10 Weeks	−92.7 ± 2.0	−92.2 ± 2.6	−75.5 ± 2.7	−35.6 ± 1.8[a,bb,dd]	−29.0 ± 2.1[cc,ee,ff]	−5.6 ± 1.7
11 Weeks	−94.8 ± 3.0	−91.3 ± 2.1	−73.3 ± 1.8	−35.8 ± 3.3[a,bb,dd]	−29.0 ± 3.1[cc,ee,ff]	−3.9 ± 2.7
12 Weeks	−94.7 ± 3.1	−93.5 ± 3.3	−77.4 ± 3.0	−37.9 ± 2.6[a,bb,dd]	−28.1 ± 2.8[cc,ee,ff]	−3.0 ± 2.2

*SFI scores for all cut treatment groups reported as mean SFI score ± SEMs for data shown in Figure 3A. Single points at the same postoperative time for cut treatment SFI data were compared using mixed-subject ANOVA. A single lower case letter indicates $P < 0.05$ and two letters $P < 0.01$, for the following comparisons: a, cut + suture + PEG vs. cut; b, cut + suture + PEG vs. cut + suture; c, cut + suture + MB + PEG vs. cut + PEG; d, cut + suture + PEG vs. cut + PEG; e, = cut + suture + MB + PEG vs. cut; f, cut + suture + MB + PEG vs. cut + suture.

Crush + MB + PEG and crush + MEL + PEG groups had significantly ($P < 0.01$) improved SFI performance during postoperative weeks 4–6 compared with those groups treated only with PEG.

Similar analyses of crush-severance FF data (Fig. 3D, Table V) showed that crush + MB + PEG groups had significantly greater and more rapid recovery at 1 day to 3 weeks postoperation compared with crush + PEG and crush + MEL + PEG ($P < 0.05$) and crush, crush + MEL, and crush + MB ($P < 0.01$) groups. At 4–6 weeks postoperation, crush + MEL + PEG and crush + MB + PEG groups did not differ significantly ($P > 0.05$) in their FF score compared with the sham group (Fig. 3B,D, Table V). For either SFI or FF tests, data for crush, crush + MB, and crush + MEL groups did not differ significantly at any time point or for the curves generated by the entire set of data points for each group; i.e., in the absence of PEG-fusion, these antioxidants did not enhance behavioral recovery. Recovery after crush injury was much more rapid and complete than after cut or cut + suture. Finally, the SFI, FF, and CAP scores for cut- or crush-severance groups having successful PEG-fusion were always in the order from highest to lowest: MB (or MEL) + PEG treatment then treatment by PEG alone which, in turn, was much better than any treatment lacking PEG-fusion.

Video Recordings of Animals With Cut- or Crush-Severed Nerves

We recorded SFI and FF trials during the dark phase of the animals' reverse dark/light cycle using a Canon XL1 with a shutter speed of 1/420 sec. Both behavioral tests were scored by experimenters who were blind to the treatment conditions. FF tests assessed the animals' behavior in real time, whereas SFI footprints were analyzed at a later time. The video recordings showed marked differences in behavior between treatment groups (see Supporting Information video online).

Supporting Information Video S1A–E shows a representative video-recorded SFI trial at 1 week postoperation for an animal from each of the following treatment groups: 1) sham-operated, 2) crush, 3) crush + PEG, 4) crush + MB + PEG, and 5) cut + suture + MB + PEG. The sham-operated animal (Supp. Info. Video S1A) keeps its hind limbs under its body during locomotion, fully supporting its body weight while plantar stepping, easily maintaining its balance while traversing a beam. This gait with alternating use of the hind limbs and other hind limb behavior is essentially the same as that seen for an unoperated animal. The crush animal (Supp. Info. Video S1B) swings its injured hind limb out from beneath its body and is unable to use this limb to support its body weight. This animal does not exhibit plantar stepping and is unable to maintain its balance while traversing the beam. Cut or cut + suture animals showed similar gaits (video not shown). The crush + PEG animal (Supp. Info. Video S1C) exhibits balanced weight-bearing locomotion and plantar stepping, although its gait is a "gallop" in which both hind limbs move at the same time and bear weight together, rather than on alternate steps. Cut + suture + PEG animals showed similar gaits (video not shown). The crush + MB + PEG animal (Supp. Info. Video S1D) exhibits behavior very similar to the sham-operated animal and unoperated animals, quickly traversing the beam using a normal gait with no loss of balance. Cut + suture + MB + PEG animals showed similar behaviors (Supp. Info. Video S1E).

Supporting Information Video S1F–H shows a representative video-recorded FF trial at 1 day postoperation for an animal from each of the following treatment groups: 6) sham-operated, 7) crush, and 8) crush + MB

TABLE IV. FF Data for Cut-Severed Groups*

Treatment Group	Cut	Cut + PEG	Cut + suture	Cut + suture + PEG	Cut + suture + MB + PEG[cc,ee,ff]	Sham
Baseline	−0.2 ± 0.04	−0.2 ± 0.01	−0.20 ± 0.03	−0.25 ± 0.07	−0.37 ± 1.8	−4.3 ± 1.8
24 Hours	−71.6 ± 3.1	−71.3 ± 2.4	−73.4 ± 3.5	−72.8 ± 2.9	−73.0 ± 1.1	−6.9 ± 2.5
48 Hours	−65.2 ± 1.7	−64.8 ± 3.1	−67.2 ± 1.9	−66.9 ± 2.2	−67.5 ± 2.0	−11.5 ± 1.4
72 Hours	−59.4 ± 2.4	−59.9 ± 3.7	−61.4 ± 2.6	−58.4 ± 3.3	−56.0 ± 1.8	−8.8 ± 2.7
1 Week	−69.6 ± 3.7	−62.8 ± 2.5	−56.6 ± 2.8	−50.3 ± 3.2[bb,d]	−45.5 ± 3.0[cc,ee,ff]	−3.5 ± 1.2
2 Weeks	−60.0 ± 1.9	−60.7 ± 2.9	−54.6 ± 3.1	−47.8 ± 1.6[bb,d]	−40.5 ± 2.9[cc,ee,ff]	−6.1 ± 1.6
3 Weeks	−62.8 ± 2.5	−57.4 ± 2.3	−52.8 ± 4.2	−40.8 ± 1.9[bb,d]	−33.3 ± 1.7[cc,ee,ff]	−8.3 ± 2.4
4 Weeks	−75.2 ± 2.3	−63.8 ± 1.9	−55.2 ± 1.8	−37.8 ± 2.3[bb,d]	−27.8 ± 2.1[cc,ee,ff]	−5.2 ± 3.1
5 Weeks	−66.8 ± 1.2	−65.5 ± 3.3	−53.8 ± 1.3	−34.9 ± 2.8[bb,d]	−23.6 ± 2.4[cc,ee,ff]	−5.2 ± 2.9
6 Weeks	−61.2 ± 2.8	−61.8 ± 2.4	−51.2 ± 1.9	−31.9 ± 3.4[bb,d]	−21.1 ± 1.9[cc,ee,ff]	−4.9 ± 1.1
7 Weeks	−62.3 ± 3.4	−59.7 ± 3.6	−49.6 ± 3.6	−32.8 ± 3.1[bb,d]	−17.4 ± 3.2[cc,ee,ff]	−9.0 ± 2.5
8 Weeks	−64.1 ± 2.3	−59.2 ± 2.9	−45.4 ± 2.5	−33.4 ± 3.7[bb,d]	−15.5 ± 1.3[cc,ee,ff]	−3.8 ± 1.4
9 Weeks	−61.7 ± 2.7	−57.3 ± 1.6	−43.5 ± 3.0	−31.9 ± 2.7[bb,d]	−15.2 ± 2.0[cc,ee,ff]	−4.7 ± 3.2
10 Weeks	−63.6 ± 3.8	−56.9 ± 3.4	−42.8 ± 3.7	−30.7 ± 2.1[bb,d]	−15.5 ± 2.2[cc,ee,ff]	−5.6 ± 1.7
11 Weeks	−64.2 ± 3.2	−58.8 ± 1.6	−44.9 ± 2.6	−32.1 ± 3.8[bb,d]	−16.7 ± 3.1[cc,ee,ff]	−3.9 ± 2.7
12 Weeks	−61.5 ± 1.3	−59.7 ± 2.8	−41.9 ± 2.2	−34.1 ± 3.5[bb,d]	−15.5 ± 2.8[cc,ee,ff]	−3.0 ± 2.2

*FF asymmetry scores for all cut treatment groups reported as mean FF score ± SEMs shown in Figure 3C. Data at the same postoperative time for cut treatment FF data were compared using mixed-subject ANOVA. A single lower case letter indicates $P < 0.05$ and two letters $P < 0.01$, for the following comparisons: a, cut + suture + PEG vs. cut; b, cut + suture + PEG vs. cut + suture; c, cut + suture + MB + PEG vs. cut + PEG; d, cut + suture + PEG vs. cut + PEG; e, cut + suture + MB + PEG vs. cut; f, cut + suture + MB + PEG vs. cut + suture.

TABLE V. SFI and FF Data for Crush-Severed Groups*

	Crush	Crush + MEL	Crush + MB	Crush + PEG	Crush + MEL + PEG	Crush + MB + PEG	Sham
SFI Group							
Baseline	−3.1 ± 0.5	−2.5 ± 0.8	−5.9 ± 0.7	−4.4 ± 0.8	−2.8 ± 0.5	−4.4 ± 0.5	−3.2 ± 0.7
24 h	−95.8 ± 2.1	−92.8 ± 2.3	−93.3 ± 1.8	−92.5 ± 1.0	-93.3 ± 2.4	−93.3 ± 1.6	−6.9 ± 2.6
48 h	−95.8 ± 1.8	−93.1 ± 1.1	−92.3 ± 2.0	−92.0 ± 1.3	−92.1 ± 1.9	−90.2 ± 1.6	−11.4 ± 1.3
72 h	−97.9 ± 2.6	−92.2 ± 3.2	−93.3 ± 2.8	−96.5 ± 1.8	−91.2 ± 1.9	−86.3 ± 2.3[a]	−8.9 ± 0.6
1 wk	−96.0 ± 2.3	−90.3 ± 2.2	−94.5 ± 2.4	−95.0 ± 1.8	−93.3 ± 2.0	69.0 ± 3.0[a,b,c,d,e]	−3.4 ± 1.8
2 wk	−95.0 ± 3.0	−90.0 ± 3.2	−91.1 ± 3.1	−93.3 ± 2.8	−93.3 ± 3.2	−54.5 ± 4.4[aa,bb,cc,dd,ee]	−6.8 ± 3.3
3 wk	−47.9 ± 6.0	−58.4 ± 4.3	−51.7 ± 5.2	−33.7 ± 1.8	−22.3 ± 3.8[gg]	−29.8 ± 4.3[aa,bb,cc]	−8.6 ± 3.4
4 wk	−21.5 ± 3.4	−22.7 ± 4.2	−25.2 ± 3.9	−23.0 ± 1.8	−7.6 ± 3.6[ff]	−7.1 ± 2.8[aa,bb,cc,dd]	−5.8 ± 1.9
5 wk	−20.1 ± 1.9	−23.2 ± 2.6	−20.0 ± 3.1	−20.9 ± 2.3	−4.7 ± 3.4[ff]	−4.3 ± 3.2[aa,bb,cc,dd]	−5.7 ± 4.5
6 wk	−17.6 ± 2.1	−20.7 ± 1.8	−19.1 ± 2.0	−18.8 ± 2.3	−6.8 ± 1.9[ff]	−5.2 ± 2.2[aa,bb,cc,dd]	−4.6 ± 1.1
FF Group							
Baseline	0.4 ± 0.01	−0.2 ± 0.02	−0.3 ± 0.01	−0.7 ± 0.01	−0.1 ± 0.01	−0.4 ± 0.02	0.1 ± 0.01
24 h	−55.7 ± 0.5	−53.4 ± 0.4	−51.6 ± 0.4	−45.1 ± 0.6	−42.3 ± 0.5	−31.0 ± 0.4[aa,bb,cc,d,e]	−0.01 ± 0.01
48 h	−57.8 ± 0.4	−54.9 ± 0.5	−52.5 ± 0.4	−41 ± 0.6	−39.1 ± 0.6[f]	−29.6 ± 0.5[aa,bb,cc,d,e]	−0.03 ± 0.05
72 h	−50.2 ± 0.4	−49.9 ± 0.4	−47.9 ± 0.6	−37.3 ± 0.7	−36.9 ± 0.5[f]	−32.0 ± 0.5[aa,bb,cc,d,e]	0.04 ± 0.01
1 wk	−51.3 ± 0.5	−50.0 ± 0.5	−49.9 ± 0.5	−36.1 ± 0.5	−35.0 ± 0.4[f]	−25.0 ± 0.3[aa,bb,cc,d,e]	0.03 ± 0.01
2 wk	−49.0 ± 0.3	−47.2 ± 0.3	−43.4 ± 0.4	−31.1 ± 0.5	−30.0 ± 0.3[f]	−21.0 ± 0.3[aa,bb,cc,d,e]	−0.02 ± 0.02
3 wk	−22.2 ± 0.4	−22.5 ± 0.5	−20.0 ± 0.4	−11.9 ± 0.4	−8.0 ± 0.3[f]	−7.0 ± 0.3	−0.03 ± 0.02
4 wk	−8.2 ± 0.2	−9.6 ± 0.3	−7.1 ± 0.3	−7.0 ± 0.2	−7.3 ± 0.2	−2.0 ± 0.2	−0.03 ± 0.01
5 wk	−3.3 ± 0.2	−5.4 ± 0.2	−4.1 ± 0.1	2.1 ± 0.1	−1.7 ± 0.1	−3.0 ± 0.1	−0.04 ± 0.02
6 wk	−5.1 ± 0.1	-4.0 ± 0.2	−5.2 ± 0.2	−2.4 ± 0.1	−2.4 ± 0.1	−3.0 ± 0.1	0.01 ± 0.01

*Scores for all Crush-treatment groups reported as mean SFI or FF scores ± SEMs shown in Figure 3B and D, respectively. Crush-treatment SFI and FF data were compared using mixed ANOVA. For statistical significance, a single lower case letter indicates $P < 0.05$, two letters $P < 0.01$, and three letters $P < 0.001$, for the following comparisons between treatment groups at any given sampling time: a, crush + MB + PEG vs. crush; b, crush + MB + PEG vs. crush + MB; c, crush + MB + PEG vs. crush + MEL; d, crush + MB + PEG vs. crush + PEG; e, crush + MB + PEG vs. crush + PEG + MEL; f, crush + MEL + PEG vs. crush.

+ PEG. The sham-operated animal (Supp. Info. Video S1F) passes from one side of the grid to the other, making few faults. The crush animal (Supp. Info. Video S1G) makes a full fault with nearly every step, hesitates before passing from one side of the grid to the other, and shows an adjusted gait with a high leg swing of the injured hind limb. The crush + MB + PEG animal (Supp. Info. Video S1H) walks freely across the grid without hesita-

tion, maintaining balanced weight-bearing locomotion while keeping its injured hind limb under its body.

Our SFI, FF, and video recording data are the first showing behavioral recovery after cut-severance followed by PEG-fusion for any mammal. Furthermore, our PEG-fusion protocol modified to use MB before applying PEG produces dramatically better behavioral recovery as assessed by SFI or FF scores after crush-severance compared with our previous PEG-fusion protocol (Britt et al., 2010).

DISCUSSION

As described in the accompanying paper (Spaeth et al., 2011b), our in vitro, ex vivo, and in vivo data are consistent with rationales and interrelated effects of our cut-severance or crush-severance PEG-fusion protocols as illustrated in Figure 1 and as follows.

Ca^{2+}-Free Hypotonic Saline and Sutures

Our current data (Spaeth et al., 2011b) and previously published data (Lore et al., 1999; Britt et al., 2010) are consistent with our hypothesis that Ca^{2+}-free hypotonic salines open cut axonal ends, extrude vesicles, and allow dye uptake (Figs. 1A, 2A,B). Cut-severed ends of sciatic nerves separate by 1–3 mm, whereas crush-severed ends remain within epi- and perineural sheaths (Lore et al., 1999). Viewed with dissecting microscopes, cut-severed ends can be brought into close apposition by sutures, and we hypothesize that close apposition of cut, open, vesicle-free ends is necessary for successful PEG-fusion (see below). In our PEG-fusion protocol, microsutures almost certainly never align cut nerve ends so perfectly that the same proximal and distal axonal ends are specifically reapposed and PEG-fused. Nevertheless, our PEG-fusion results for cut-severance repair are very comparable to our PEG-fusion results for crush-severance results in which crush-severed ends remain aligned within damaged, but not completely disrupted, sheaths (Lore et al., 1999).

MB Before Applying PEG

Application of an antioxidant (MB) before applying 500 mM PEG in Ca^{2+}-free ddH_2O decreases vesicle formation at cut or crushed ends and reduces the probability that such ends partially collapse (Spaeth et al., 2011b), which is consistent with our hypothesis that endogenous sealing mechanisms are similar in all eukaryotic cells (Spaeth et al., 2010). Antioxidants such as MB applied before PEG should decrease sealing and help keep axonal ends open and free of vesicles and possibly enhance PEG-fusion. In this study, our dye diffusion data (Fig. 2, Table I), CAP conduction (Table II), and behavioral data (Fig. 3, Tables III–V) are all consistent with these hypotheses.

PEG

Our current data (Fig. 3) are consistent with the hypothesis that PEG rapidly and directly induces membrane fusion by removing waters of hydration at closely apposed membranes at severed axonal ends and small holes, thereby allowing membrane lipids in plasmalemmal leaflets to collapse, fuse, and seal cut ends or to spread and seal smaller plasmalemmal holes (Lore et al., 1999; Bittner et al., 2000; Fishman and Bittner, 2003; Lentz, 2007; Spaeth et al., 2011a).

Ca^{2+}-Containing Isotonic Saline After Applying PEG

Our current data (Figs. 2–4) and previously published data (Bittner and Fishman, 2000; Spaeth et al., 2010, 2011a,b) are consistent with our hypothesis that applying Ca^{2+}-containing isotonic saline after applying PEG seals any remaining holes in PEG-fused sciatic axons. As described above, Ca^{2+} initiates the formation, accumulation, and fusion of vesicles to seal plasmalemmal damage in all eukaryotic cells, almost certainly including axons in rats or any other mammal (Spaeth et al., 2010, 2011b).

Results of Dye Diffusion, CAP Conduction, and Behavioral Analyses Are in Agreement

Our CAP, dye diffusion, and behavioral data (Figs. 2, 3, Tables I–V) are in agreement with the hypotheses given above and are also internally consistent. That is, in almost 200 trials, the presence or absence of dye diffusion across a lesion site is always associated with the presence or absence of CAP conduction across the same lesion site following cut- or crush-severance. Mean CAP amplitudes correlate well with amounts of behavioral recovery for each control or experimental treatment group.

Furthermore, the order of CAP or behavioral recovery is that given in the symbol key in Figure 3. For example, the order of decreasing recovery for cut treatments is sham (1), cut + suture + MB + PEG (2), cut + suture + PEG (3), and cut + suture (4); cut + PEG and cut + MB treatments show no improvement compared with cut alone; i.e., PEG, MB, and MEL have no significant effect unless applied under a well-specified PEG-fusion protocol. The order of decreasing recovery for crush treatments is: sham (1), crush + MB + PEG (2), crush + MEL + PEG (3), crush + PEG (4); crush + MEL and crush + PEG also show no improvement compared with crush alone.

Significance of Rapid, Effective, Consistent, and Long-Lasting Repair by PEG-Fusion

Cut- or crush-severance lesions of mammalian peripheral nerves occur frequently and often produce significant behavioral deficits (Bozkurt et al., 2008; Campbell, 2008; Wolfe et al., 2010) even months to years postinjury, including treatment with the most beneficial conventional techniques available today (microsurgery, nerve growth guides). In contrast, we report here that completely cut- or crush-severed rat sciatic nerves treated with our modified PEG-fusion protocols show

morphological and electrophysiological repair within minutes and dramatic behavioral recovery within 1–7 days that is maintained for at least 12 weeks, i.e., long-lasting repair. After several weeks of training, PEG-fusion is consistently obtained in over 98% of all trials by neurosurgeons, faculty, postdoctoral fellows, and graduate or undergraduate students.

We do not know how many proximal and distal, cut or crushed axonal segments are PEG-fused, the specificity of the motor or sensory proximal-distal axonal fusions, or the degree of rapidly or slowly occurring cortical or spinal plasticities that might compensate for any initial deficits. [Similar questions remain unanswered for regeneration by axonal outgrowths after cut or crush-severance of mammalian peripheral nerves (Nguyen et al., 2002; Bozkurt et al., 2008; Campbell, 2008).] However, the numbers and connection specificities of completely cut or crushed sciatic axons that are PEG-fused are sufficient to produce, within 1–7 days, dramatic recovery of behaviors mediated by this nerve, and behavioral tests are the best measures of functional recovery, as opposed to electromyograms, counts of axonal numbers, extent of myelin sheathing, etc. For example, high-magnification EM sections are required to identify the plasmalemma, and serial sections without loss are in practice impossible to obtain to follow single, small-diameter mammalian axons completely through a lesion site to determine definitively the existence or absence of plasmalemmal continuity (Xie and Barrett, 1991; Bittner et al., 1986; Ballinger et al., 1997). We also note that 10–15% of the originally innervating axonal number can produce very significant amounts of behavioral recovery (Eidelberg et al., 1977; Kakulis, 1999).

Adding a mild oxidizing agent to the Ca^{2+}-containing isotonic saline after applying PEG might further increase PEG-fusion success by enhancing the sealing of any remaining axolemmal holes in PEG-fused axons (Spaeth et al., 2011b). Longer term behavioral recovery might also be improved by applying trophic agents, nerve growth guides, or other techniques (Bozkurt et al., 2007; Campbell, 2008; De Ruiter et al., 2008; Kalbermatten et al., 2009; Kwon et al., 2009; Wolfe et al., 2010) that moderately enhance the extent or specificity of regeneration at 1–2 mm/day by axons that are not PEG-fused. Various exercise or training paradigms might also improve longer term behavioral recovery (Bittner at al, 2000).

Neuroprotective effects of MB, MEL, or PEG might enhance survival of severed axons (Bozkurt et al., 2007; Campbell, 2008; Cao et al., 2010), whether or not they have been PEG-fused, by increasing the number of PEG-fused axons and/or by improving survival of severed distal stumps or proximal axons not PEG-fused. In the latter case, rescued axons might subsequently regenerate by conventional outgrowths at 1–2 mm/day. These slowly regenerating outgrowths combined with relearning to use PEG-fused axons might account for the slow increase in SFI and/or FF behavioral recovery after the second postoperative week (Fig. 3).

The applicability of our current PEG-fusion protocols beyond treating very acute traumatic nerve injuries is further indicated by our current and previously published data showing that 1) cut-severed stumps of distal axons in PNS nerves in vivo (Sea et al., 1995) or spinal nerves ex vivo (Marzullo et al., 2002) can be maintained morphologically and functionally intact for 5–10 days by cooling to 10–23°C or by cyclosporin A injections (Sunio and Bittner, 1997); 2) distal axonal stumps of cut-severed rat spinal nerves maintained intact for days by cooling in vitro can be PEG-fused (Marzulo et al., 2002; Stavisky et al., 2005); and 3) previously cut- or crush-severed axons that have sealed in Ca^{2+}-containing saline can be apposed and successfully sealed (see Results; see also Lore et al., 1999). If need be, such previously cut nerves can also be recut and successfully PEG-fused (Lore et al., 1999). Finally, all these solutions required for successful PEG-fusion use FDA-approved chemicals (Ca^{2+}-free saline, MB, PEG), and all surgical techniques and microapplication of solutions are in standard clinical use.

Given these facts, our PEG-fusion protocols may indeed be quickly translatable to important clinical procedures that dramatically and chronically restore within minutes to days much behavior lost by cut or crush axonal severance, a result not obtained with any other chemical or surgical treatment described to date.

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