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Rapid Repair of Severed Mammalian Axons *via* Polyethylene Glycol – Mediated Cell Fusion

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Rapid Repair of Severed Mammalian Axons *via* Polyethylene Glycol – Mediated Cell Fusion

by

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Dedication

This dissertation is dedicated to my parents for their limitless love, support, and sacrifice…all of which afforded me the opportunity to follow my dreams.

Epigraph

“Those who are free of resentful thoughts surely find peace.” - Gautama Buddha

Acknowledgements

I would like to thank my friends and family for their support during the good times and more importantly, the bad times. I would not have had the fortitude to withstand the difficulties I have experienced in my life without their love and encouragement.

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Lastly, I have a great deal of gratitude for the faculty I was fortunate enough to work with over the years. The skillset I have acquired working on so many different projects is something I value and would not have been possible had they not taken the time to teach me.

Rapid Repair of Severed Mammalian Axons *via* Polyethylene Glycol – Mediated Cell Fusion

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The University of Texas at Austin, 2014

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The ability to repair damaged mammalian axons to re-establish functional connections continues to be a goal for neuroscientists. Following axonal severance, proximal segments of mammalian axons seal themselves rapidly at the lesion site. Distal segments of severed mammalian axons undergo Wallerian degeneration within 24-72 hours. Prior to the onset of degeneration, distal axonal segments remain electrically excitable.

The work described in this dissertation demonstrates that polyethylene glycol (PEG), a hydrophilic polymer, can rapidly repair severed axons by fusing the plasmalemmas of two closely apposed distal and proximal axonal segments.

This plasmalemmal fusion restores morphological integrity of severed axons and their ability to conduct action potentials across the injury site. The ability to fuse proximal and distal severed axonal segments using PEG is improved when the axonal segments are exposed to antioxidants, such as melatonin and methylene blue, and also when microsutures provide additional support in transected sciatic nerves. The restoration of axonal continuity by PEG-fusion restores function, improving behavioral recovery in rats with crush-injured sciatic nerves, as well as those in which the sciatic is complete transected.

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GENERAL INTRODUCTION

For over a century, biologists have known that distal segments of severed mammalian PNS axons undergo Wallerian degeneration within 12-48 hours (Waller, 1850). If proximal axonal segments survive axotomy, regeneration by outgrowth can occur a rate of 1-2 mm/day. Such PNS axonal regeneration may take months to years to reach denervated target tissues in larger mammals such as humans (Ingoglia and Murray 2001). Furthermore, if target tissues are not reached, behavioral recovery is typically poor.

In the last several decades, various procedures have been used to increase the number of PNS axons that re-establish connections following severance, but not the rate at which PNS axons re-establish those connections. For example, nerve grafts (Lago et al. 2007), connective tissue matrices (Bozkurt et al. 2007), and nerve growth guides (Kalbermatten et al. 2009) have all been reported to improve the extent of regeneration by severed PNS axons.

Our lab has developed a technique to improve the speed of PNS repair using solutions of polyethylene glycol (PEG) to rapidly (within minutes) and directly re-establish physiological and morphological continuity. Initially, it was demonstrated that PEG solutions could induce fusion *in vitro* between the severed ends of a myelinated medial giant axon in the CNS of earthworms or unmyelinated medial giant axons in the CNS of crayfish (Krause and Bittner, 1990; Krause et al., 1991). Because such large-diameter invertebrate axons are

uniquely identifiable and have well-characterized behaviors associated with their function, we have been able to demonstrate that PEG-fusion can produce long-lasting *in vivo* behavioral recovery (in addition to morphological and functional continuity) (Lore et al., 1999).

In contrast to these large-diameter invertebrate axons, mammalian nerves have large numbers of small diameter axons that run in parallel and it has not been definitively shown whether direct application of PEG to severed mammalian PNS nerves *in vivo* will result in rapid and long-lasting repair. Recently we have shown that PEG fusion re-establishes the continuity of crush-severed rat sciatic axons *in vitro* as measured by the restored conduction of compound action potentials (CAPs) and the intra-axonal diffusion of fluorescent dye across the lesion site. These assessments demonstrate axonal continuity of repaired mammalian axons.

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- or cut-severance 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 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 axons in Ca^{2+}-free hypotonic saline opens severed axonal ends, flushes out most previously formed vesicles, and prevents new vesicle formation. PEG applied in pure H_2O 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 (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. The subsequent application of Ca^{2+}-containing isotonic saline to the lesion site induces vesicles to seal any remaining plasmalemmal holes. Crush-severed nerves that are PEG-fused may be mechanically weak at the lesion site because severance disrupts the extracellular matrix (ECM) that normally prevents intact axons from tearing when stretched or stressed by joint or muscle movements (schematic of PEG-fusion rationale shown in Fig. A1.1).

Chapter one presents evidence that local application of the antioxidant melatonin to reduce oxidative stress following axonal injury increases the

success of PEG-fusion *in vitro* and *in vivo*. As oxidative stress following injury can lead to Ca^{2+} - induced apoptosis, we chose to apply melationin *in vitro* and *in vivo* to severed mammalian PNS axons because melatonin acts as an antioxidant (Liu et al., 2004) and combats oxidative stress brought about by free radicals produced following injury (Fujimoto et al., 2000). Furthermore, melatonin decreases lipid peroxidation following injury to nervous tissue – thus providing protection from secondary injury (Kaptanoglu et al., 2000). Melatonin also inhibits nitric oxide production and may have neuroprotective effects (Rogerio et al., 2002). Results from the experiments outlined in this section suggest that the application of melatonin increases the survival of severed distal axonal segments, enhancing the success of PEG-fusion in severed mammalian PNS axons.

In chapter 2 we investigate whether PEG-fusion of crush-injured mammalian PNS axons *in vivo* restores not only axonal continuity, but appropriate behavioral function. In the human PNS, crush injuries are the most common type of traumatic axonal injury. Following crush injury of peripheral nerves, the ECM is not completely disrupted and the severed ends of axons are closely apposed within their endo-, peri-, and epineurial sheaths (Bozkurt et al., 2007, 2008). Through a series of experiments we attempt to show that PEG-fusion is a useful strategy for rapidly repairing crush-severed axons, providing lasting behavioral recovery following traumatic nerve injury.

Finally, in chapter three we outline a detailed approach to treat nerve severance injuries, combining microsutures, application of antioxidants, and PEG-fusion. Cut injuries completely disrupt the ECM, severing endo-, peri-, and epineurial sheaths. This may account for observed differences in recovery of function between crush and cut injuries (de Medinaceli, 1982; Carlton and Goldberg, 1986). Cut-severed axons PEG-fused in mammals *in vivo* have poor mechanical strength at the lesion site and may immediately pull apart after animals recover from anesthesia, thereby preventing chronic recovery (Lore et al. 1999; Bittner and Fishman, 2000). Using microsutures to bring proximal and distal severed axonal segments in close apposition, we attempt to show that local application of the antioxidant methylene blue as well as PEG can fuse adjacent axonal ends and rapidly restore behavioral function *in vivo.*

In summary, prognosis for those recovering from traumatic nerve injury has traditionally been poor. Frequently, what minimal amount of function does return happens very slowly over time, limited by a slow rate of axonal outgrowth from the site of injury. It is hoped that this work will introduce new and novel approaches in the treatment of traumatic nerve injury; treatments that encourage a focus on rapid repair in addition to target-oriented regeneration strategies.

Chapter One: Melatonin enhances PEG-fusion repair of severed rat sciatic axons

INTRODUCTION

For almost a century, injury to peripheral nerve axons in mammals and other vertebrates has been known to result in Wallerian degeneration of the severed distal stumps and slow (1 mm/day) outgrowths from the severed proximal stumps that can produce functional recovery (Ingoglia & Murray, 2001). It is less widely known that the hydrophilic polymer polyethylene glycol (PEG) can repair severed axons by fusing the plasmalemmas of the two closely apposed axonal ends. Such plasmalemmal fusion (PEG-fusion) rapidly (within seconds to minutes) restores axonal morphological integrity, axonal conduction of action potentials across the lesion site and therefore also rapidly restores behavioral functions mediated by severed CNS and PNS axons in invertebrates and mammals *in vitro* and *in vivo* (Bittner et al., 1986; Borgens & Shi, 2000).

We now report the ability of four experimental compounds [melatonin (MEL), cyclosporin A, (CsA), glial-derived neurotrophic factor, (GDNF), and methylprednisolone, (MP)] to alter the ability to PEG-fuse severed sciatic axons in rats compared to PEG-fusion of severed sciatic axons in control Krebs saline (CTL) that contains calcium. These experimental compounds were chosen because all have been reported to have antioxidant effects and/or to enhance axonal regeneration in the doses utilized (MEL = 100 _M, CsA = 4.2_M,

GDNF= 3.4×10−5 _M, MP= 10_M) (Dumont et al., 2001; Fine et al., 2002). We also tested PEG-fusion success when extracellular calcium was removed from the CTL (CTL-Ca) *in vivo* because PEG fusion success *in vitro* is increased by reducing extracellular calcium in similar physiological saline (Lore et al., 1999).

METHODS

In vitro preparations

For *in vitro* preparations, segments of sciatic nerves 3.5 cm in length were dissected from the hindlimbs of adult Sprague–Dawley rats (~200 g) immediately after sacrifice by an i.p. injection of sodium pentobarbital (60–80 mg/kg) and an injection of saturated KCl into the heart. Dissected sciatic nerve segments were incubated for 18 h at 37–38 ◦C under oxygen (95% O2, 5%CO2) in CTL (Krebs physiological saline in mM; 124 NaCl, 5 KCl, 1.2KH2PO4, 1.3MgSO4, 26 NaHCO3, 10Na ascorbate, 10 dextrose, 2 CaCl2, pH 7.35) or CTL containing an experimental compound (either CsA, MEL, GDNF, or MP). When testing each experimental compound, segments bathed in CTL were always run simultaneously. All experimental compounds were obtained from Sigma (St. Louis, MO).

Electrophysiology

Axonal viability and continuity was determined electrophysiologically by removing the nerve segments from the oxygenated, temperature-controlled vials and placing them in a 5-chamber sucrose gap recording device as previously described (Lore et al., 1999). The central chamber was filled with CTL, the end chambers with 0.12M KCl, and the two middle chambers with 0.23M sucrose. Compound action potentials (CAPs) were produced by supramaximal stimuli (1/s 0.4 ms duration, 4.5V pulses) to one end chamber. CAPs were recorded from the other end chamber, conventionally displayed on an oscilloscope, and peak CAP amplitudes were measured.

PEG-fusion

The procedure for PEG-fusion was as follows. CTL was removed from the central chamber following the recording of the CAP and replaced with CTL-Ca. PEG-fusion was attempted on nerve segments that exhibited a CAP of 1.5mV or greater (about four times greater than the smallest CAP that was easily detectable) by crushing a 1–2mm portion of the nerve segment in the middle chamber with fine forceps until a CAP was no longer detected (Marzullo et al., 2002). This procedure insured that the crush injury completely severed the sciatic axons (Lore et al., 1999). PEG (2000 Da) obtained from Aldrich (Milwaukee, WI) and dissolved in distilled water (50%, w/w) was applied for 2 min to the crush-injury site in the central chamber, which remained bathed in the applied PEG for an additional minute (3 min total exposure to PEG). The CTL-Ca containing PEG

that bathed the injury site in the central chamber was then replaced by washing several times with oxygenated CTL. After bathing for 10 min in CTL, the sciatic nerve was again stimulated in the end chambers as described above to record CAPs conducted across the crush-lesion site following PEG-induced fusion of the severed axon ends in the central chamber (Marzullo et al., 2002). Axons severed by crush or cut lesions do not conduct CAPS in the absence of PEG application (Shi & Borgens, 1999).

In vivo preparations

In *in vivo* preparations, sodium pentobarbital (40 mg/kg) i.p., was used as anesthesia, preceded by an injection of atropine (0.5 ml/kg) s.c. When supplemental anesthesia was required, halothane was administered by inhalation. An incision ~2.5 cm long was made in the mid-thigh and surgical spreaders were used to expose the sciatic nerve, which was cleaned of connective tissue, and the sheath nicked. Two pairs of hook electrodes were placed under the sciatic nerve, one pair to stimulate and the second to record the CAP, which was conventionally displayed on an oscilloscope. Using Dumont forceps (#5), the sciatic nerve was severed (pinched three times) and again stimulated to confirm that CAPs were no longer conducted across the lesion site A narrow stream of 100_M MEL, 100_M MEL in CTL-Ca (MEL-Ca), 10 _M MP, CTL, or CTL-Ca was applied to the lesion site. Next, PEG dissolved in distilled water as described above was applied to the lesion site for 1 min and allowed to

remain for an additional 2 min. The PEG was then pipetted off the injury site, which was washed with CTL. The nerve was again stimulated and recorded using hook electrodes to determine if the severed ends had been PEG-fused as evidenced by their ability to conduct CAPs >0.4mV across the lesion site. After attempting to record CAPs for approximately 5 min (repositioning the hook electrodes if no signal was initially recorded), the rat was euthanized by an injection of saturated KCl into the heart.

All experimental procedures were conducted in accordance with state and federal guidelines and with the approval of our institution's animal care and use committee (IACUC).

RESULTS

In-vitro Experiments

As previously reported peak CAP amplitudes of ≥0.4mV were easily detectable in the sucrose gap chamber (Marzullo et al., 2002). Peak CAP amplitudes were typically 2–11mV prior to PEG-fusion and 0.5–2.0mV after successful PEG-fusion. No CAP was detectable in cases of unsuccessful PEG-fusion. Differences in CAP amplitudes were assessed by one-way ANOVA, with significance set at $p < 0.05$. Differences in PEG-fusion percentages were assessed by $\chi 2$ tests, with significance set at $p < 0.05$.

Electrophysiological viability of sciatic nerve segments maintained at 37–38 ◦C in MEL, CsA, GDNF, MP or CTL *in vitro* was assessed at 18 h post-

severance by measuring peak CAP amplitudes in the sucrose gap chamber. A significantly ($\chi 2 = 11.62$, $p < 0.025$) higher percentage of severed nerve segments could be PEG-fused following 18 h exposure to MEL compared to CTL (Fig. 1A), as assessed by their ability to conduct CAPs >0.4mV through the lesion site after PEG application. Exposure to the other compounds did not result in significantly greater percentages of PEG-fusion compared to CTL. Furthermore, ANOVA showed significant differences in the fusion CAP amplitude across all groups ($F[4,73] = 4.95$, $p < 0.001$) with post-hoc assessments demonstrating that only MEL produced significantly ($p < 0.001$) higher CAP amplitudes conducted through the lesion site after PEG-fusion compared to CTL or any other experimental compound (Fig. 1B). To insure that these CAPs were due to repaired axons conducting through the lesion site, PEG-fused sciatic segments that displayed peak CAP amplitudes >0.4mV were again crushed halfway between the stimulating and recording electrodes by pinching three times with #5 Dumont forceps. This re-crushing always eliminated our ability to record a CAP conducted across the lesion site.

In-vivo Experiments

Given these *in vitro* data, we examined whether exposure to MEL *in vivo* also significantly increased the ability to fuse sciatic axons following severance, compared to CTL or MP (one of the compounds that had no acute effect to enhance PEG-fusion *in vitro*). Analyses comparing the results across the three

experimental groups indicated that the application of MEL *in vivo* (Fig. 2) produced a significantly higher percentage of PEG-fusions ($\chi 2 = 7.65$. $p < 0.025$) than CTL. All these *in vitro* and *in vivo* data suggest that MEL has beneficial effects to PEG-fuse severed axons.

Previous reports have shown that the application of PEG in calcium-free salines significantly increases the success of PEG-fusion *in vitro* (Lore et al., 1999). The current study shows that CTL-Ca also significantly increases the success of PEG-fusion *in vivo* (compare CTL with CTL-Ca in Fig. 2). When nerves were bathed in CTL prior to PEG-fusion, the percentage of fusion success was significantly ($\chi 2 = 7.93$, $p < 0.01$) lower than when axons were exposed to CTL-Ca prior to PEG-fusion. However, the ability of MEL to increase PEG-fusion success (Fig. 2) is so effective that it appears to overwhelm the effect of the absence of calcium in MEL-Ca to enhance PEG fusion. Specifically, there is no significantly greater effect of MEl-Ca to enhance PEG-fusion success compared to MEL (compare MEL with MEL-Ca in Fig. 2).

DISCUSSION

Our data strongly suggest that the effects of MEL to enhance PEG-fusion are relevant to both *in vitro* and *in vivo* preparations. In contrast, one other compound (CsA) that we have found to be efficacious *in vivo* is not effective in vitro, and may only be beneficial *in vivo* following a 5-day pre-treatment (Sunio & Bittner, 1997). Our data potentially may have substantial clinical ramifications.

That is, we have demonstrated that MP, currently the “gold–standard” of treatment for injuries to the CNS, has no beneficial effects on axon survival or PEG-fusion success either *in vitro* or *in vivo*. Further, MP can have substantial negative side effects (Dumont et al., 2001). In contrast, MEL appears to have relatively few, if any, deleterious side effects at the dosage (100 _M) we have used to enhance PEG-fusion (Karasek et al., 2002). PEG-fusion in turn, has been shown to rapidly repair severed axons *in vitro* and *in vivo* in a variety of invertebrate and mammalian species: neurites of mammalian cell lines in culture, earthworms, crayfish, rats, and guinea pigs (Bittner et al., 1986; Lore et al., 1999; Borgens & Shi, 2000). Hence, PEG combined with MEL might well be effective to rapidly repair severed axons following crush-type injuries to sciatic nerves and spinal cords in humans.

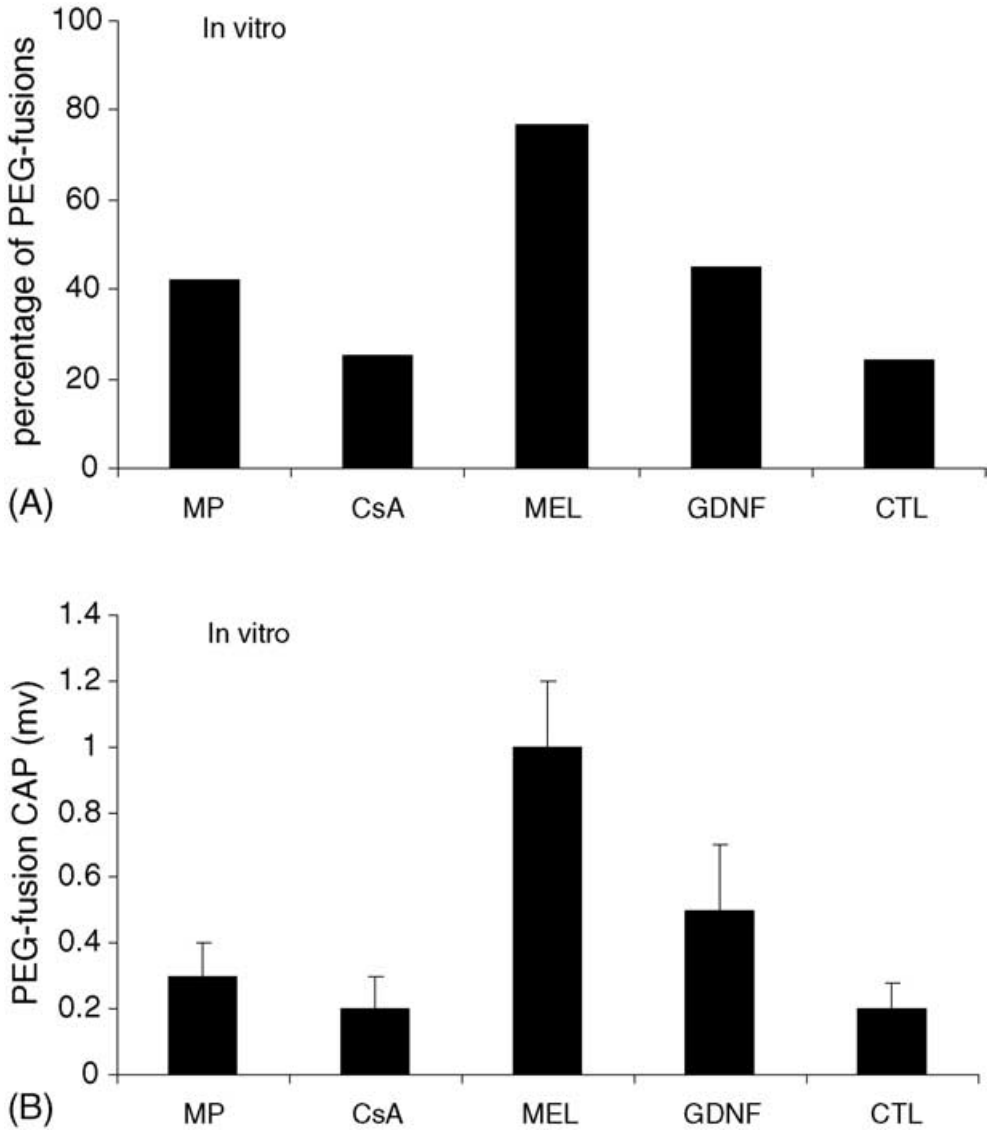

Fig. 1.1: MEL enhances PEG-fusion (A) and peak CAP amplitude (B) *in vitro*. (A) The percentage of PEG-fusion success of severed sciatic nerve segments maintained at 37–38 ◦C *in vitro* for 18 h post-severance in MEL, CsA, GDNF, MP, or CTL. $p < 0.025$ for MEL vs. CTL and $p > 0.05$ for all other experimental compounds vs. CTL by $\chi 2$ analyses and (B) the peak CAP amplitude of severed sciatic nerve segments maintained at 37–38 ◦C *in vitro* are significantly higher in those segments exposed to MEL compared to the other compounds, $F[4,73] = 4.95$, $p < 0.001$. n = total number of animals sampled in each treatment group for Figs. 1 and 2. For A and B MEL: $n = 12$, CsA: $n = 11$, GDNF: $n = 19$, MP: $n = 11$, CTL: $n = 25$.

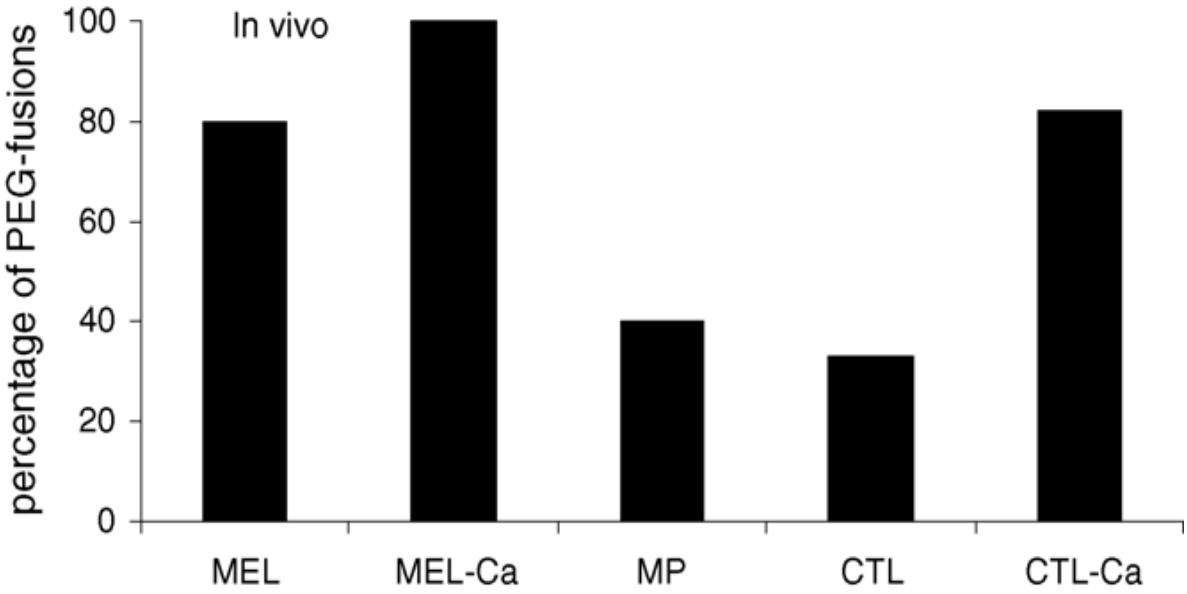

Fig. 1.2: MEL or reduced extracellular calcium enhances PEG-fusion *in vivo*. MEL *in vivo* produces a significantly higher percentage of PEG-fusions compared to CTL ($\chi 2 = 7.65$. $p < 0.025$). CTL-Ca enhances PEG-fusion success compared to CTL. PEG-fusion success in CTL-Ca was 80% compared to 33% in CTL ($\chi 2 = 7.93$, $p < 0.01$). There was no significant difference between the two MEL groups (MEL, MEL-Ca), probably because of the strong effect of MEL to enhance PEG-fusion. MEL: $n = 15$, MEL–Ca: $n = 15$, MP: $n = 15$ CTL: $n = 15$, CTL–Ca: $n = 17$.

Chapter Two: PEG rapidly restores axonal integrity and improves the recovery rate after sciatic nerve crush injury

INTRODUCTION

Crush-severance is the most common form of traumatic injury to PNS axons in humans (Bozkurt et al, 2007, 2008). Crush-severance injuries completely disrupt the axolemma at the lesion site and also produce smaller plasmalemmal holes proximal and distal to the severance site (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 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 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. A1.1).

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 with sufficient specificity to enable the restoration of some or all of their original functions.

METHODS

Animals

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

Our experimental group consisted of animals with crush-severed axons that receive a PEG treatment. Control groups and their rationales were as follows: 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 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_2PO_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_2PO_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

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.

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.

Electrophysiological recording of CAPs across a lesion site

As one measure of axonal continuity through a lesion site, conventional *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 *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 *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).

Intra-axonal dye diffusion across a lesion site

As described above and in Table1, 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 epineural 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 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 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 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):

$$SFI = \left(\frac{NPL - EPL}{EPL} + \frac{ETS - NTS}{NTS} + \frac{EIT - NIT}{NIT} \right) x73$$

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.

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 ± 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 ± 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 ± 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 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 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 ($p<0.01$).

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).

Sciatic Functional Index

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 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 asymmetry scores is consistent with our measures of physiological and morphological continuity.

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.

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 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). The 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 a basic problem before now considering the clinical use of PEG to repair bundles of crush-severed PNS axons (e.g., sciatic or other peripheral nerves). 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.

Dye diffusion groups

Treatment group	*Number of rats, CAP confirmation assays*
PEG-crush	9, 18
Crush	11, 21
Distilled water crush	2, 4
Cut	3, 6
Sham-operated	15, 31
	Total = 40 rats, 80 CAP confirmation assays

Behavioral groups

Treatment group	*Number of rats, CAP confirmation assays*
PEG-crush	14, 13
Crush	9, 9
Distilled water crush	9, 9
Cut	4, 4
Sham-operated	4, 4
	Total = 40 rats, 39 CAP confirmation assays

Table 2.1.

Table 2.1: 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.

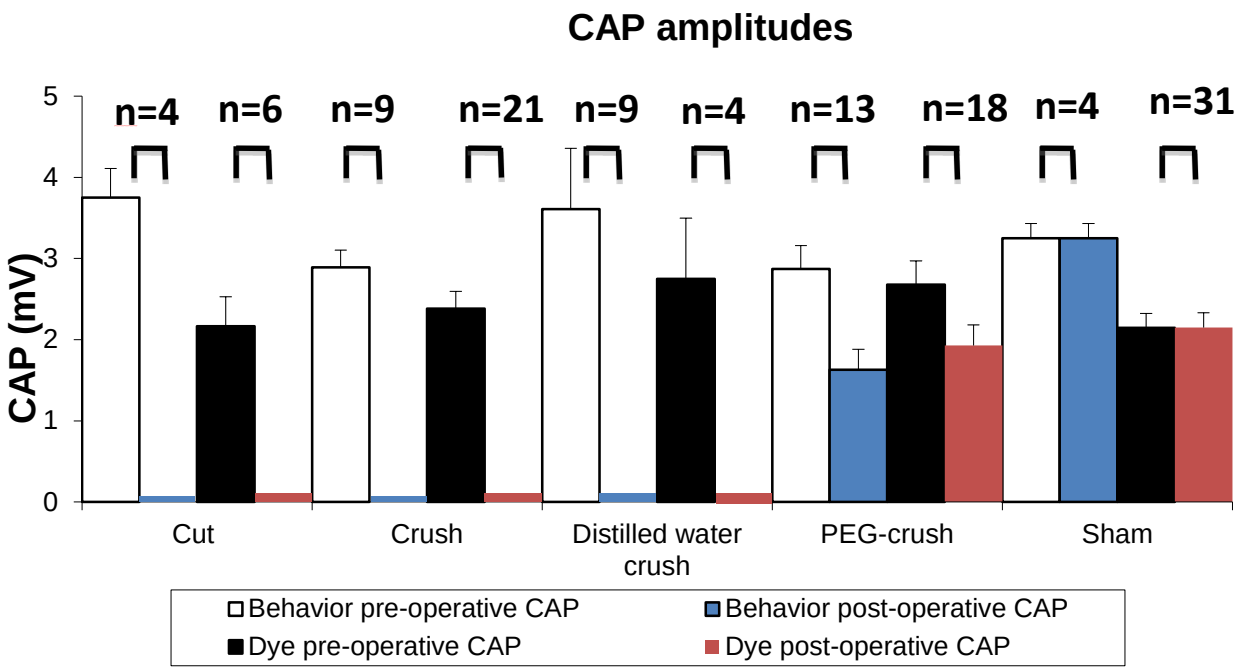

Fig. 2.2. CAP amplitudes of rat sciatic nerves recorded pre-operatively (white and black bars) and post-operatively (blue and red bars) for each group (cut, crush, distilled water-crush, PEG-crush and sham) used in dye diffusion (black and red bars) or behavioral (white and blue bars) assays. Colored 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.

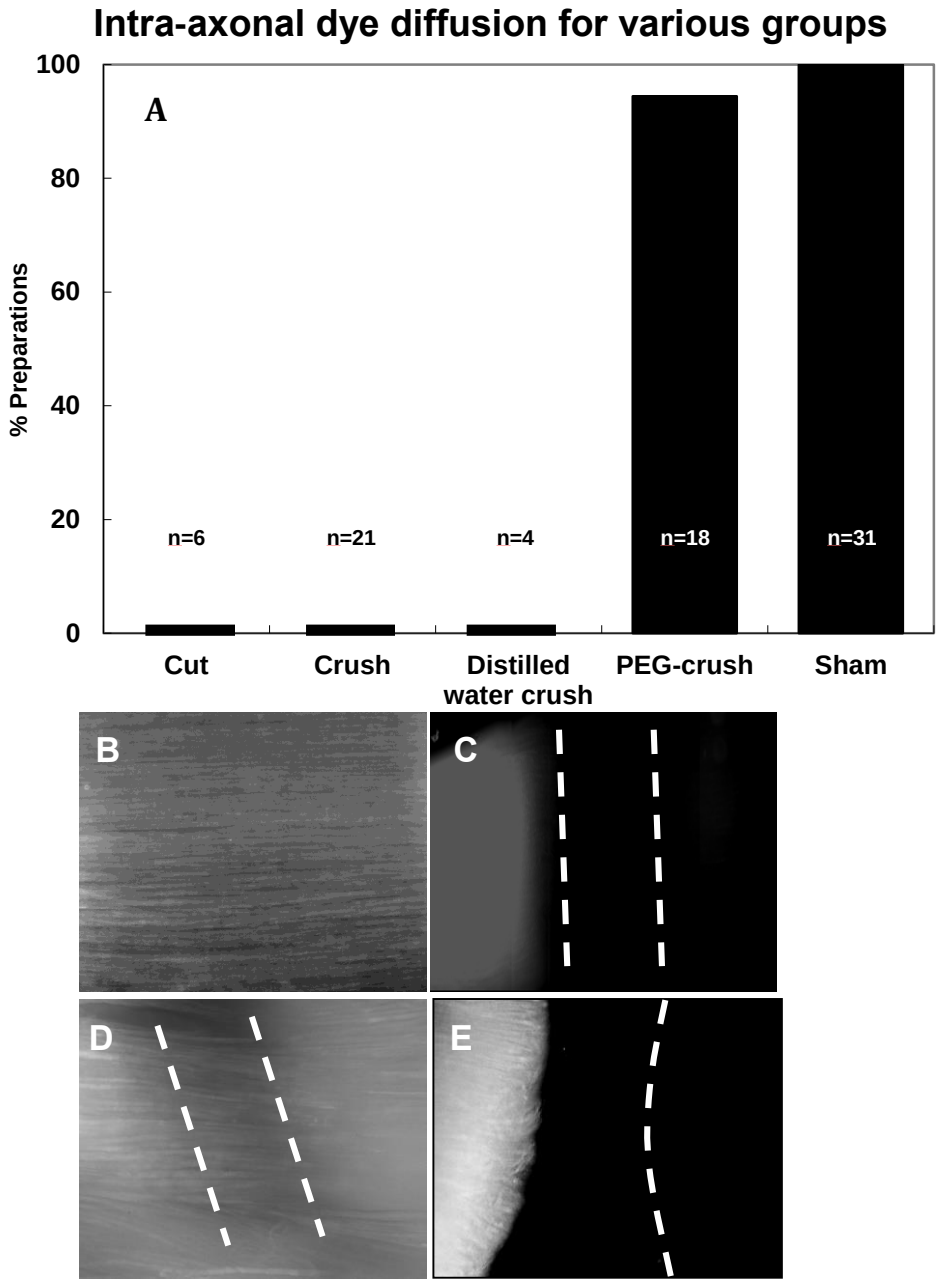

Fig. 2.3. Graph showing percent of sciatic nerve preparations exhibiting intra-axonal dye diffusion across a lesion site following cut, crush, distilled water crush, PEG-crush, or sham operations (Fig. 3A). Fluorescence images showing intra-axonal dye diffusion across a lesion site following B) sham, 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.

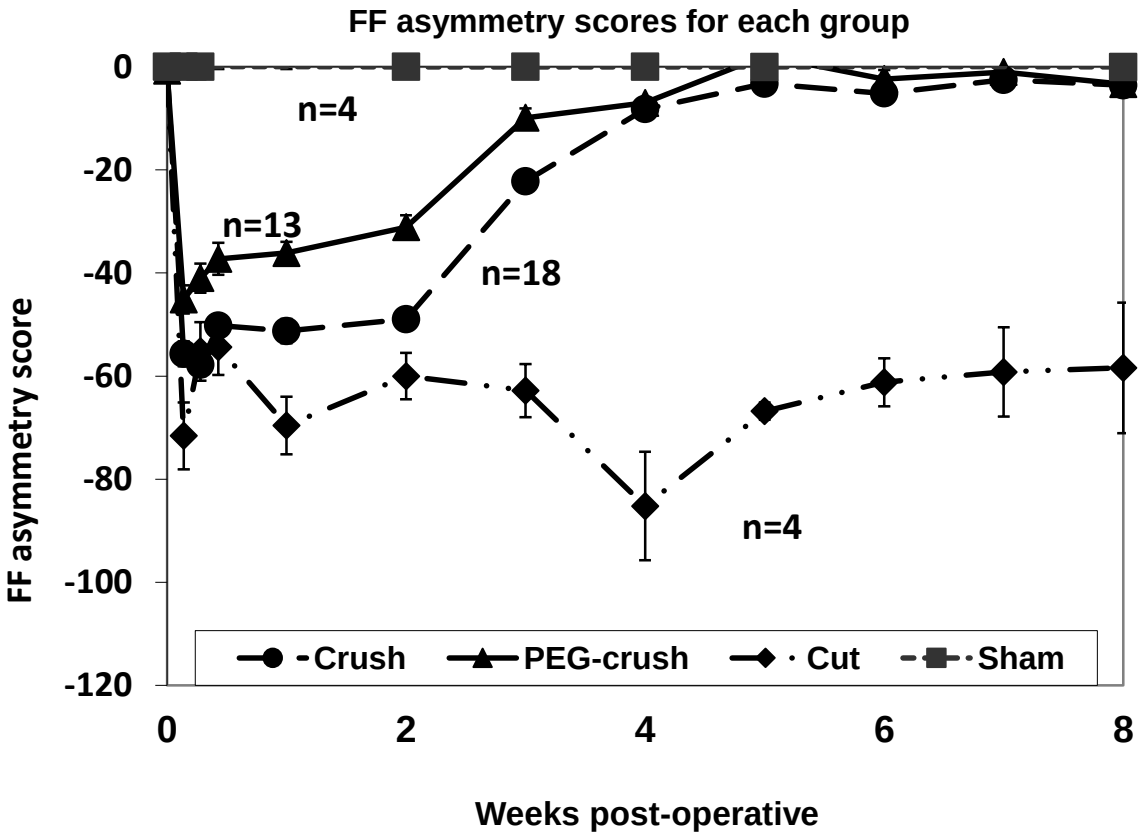

Fig. 2.4. Mean foot fault asymmetry scores from 0 – 8 post-operative weeks for cut (solid line, diamonds), crush (dashed line, circles), PEG-crush (solid line, triangles) or sham-operated (dotted line, squares) groups. Baseline scores were obtained twice for each animal prior to surgery and are plotted 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.

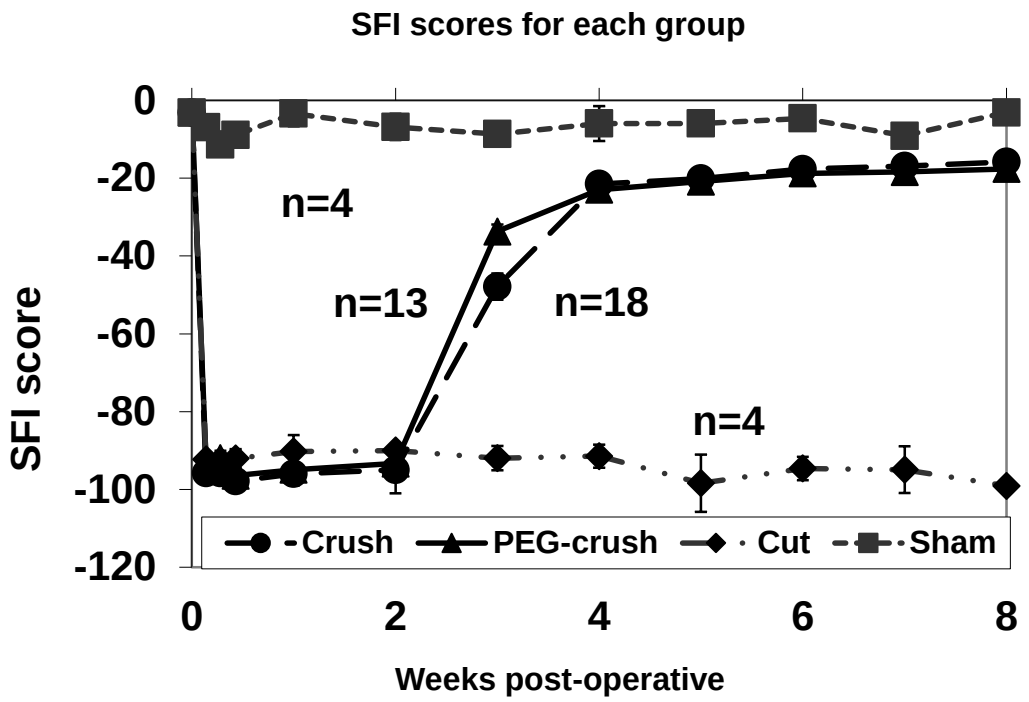

Fig. 2.5. SFI results from 0 – 8 post-operative weeks for cut (solid line, diamonds), crush (dashed line, circles), PEG-crush (solid line, triangles) or sham-operated (dotted line, squares) groups. Baseline scores were obtained three times for each animal prior to surgery and plotted at the 0 week time point.

Chapter 3: Rapid behavioral recovery after axotomy: mechanisms of axonal repair by PEG and methylene blue

INTRODUCTION

Cut- or crush-severed peripheral nerves are common injuries in humans that often produce significant behavioral deficits. Severed distal axonal segments in mammals degenerate within 1-3 days, and functional recovery involves 1-2 mm/day axonal outgrowths from surviving proximal stumps that often only partially and non-specifically re-innervate distal target tissues. Denervated target tissues may permanently atrophy before slowly regenerating motor axons reach them weeks-to-years post-operatively in large mammals (Ramon y Cajal, 1928; Lago et al., 2007; Bozkurt et al., 2008; Campbell, 2008; Kalbermatten et al., 2009). Consequently for over a century, improving the rate and extent of mammalian axonal regeneration has been a major research goal for many neuroscientists, with all repair strategies to date, including nerve grafts, nerve growth guides, and microsutures, relying on axonal outgrowths to re-innervate target tissues. Such techniques have slightly improved the number and specificity of regenerating axons, but never the time course of behavioral recovery (Lago et al., 2007; Kalbermatten et al., 2009) – or prevented Wallerian degeneration of severed distal axons.

After injury, mammalian axons that do not repair plasmalemmal damage do not survive (Sclaepfer & Bunge, 1973; Detrait et al., 2000; Yoo et al., 2003;

Nguyen et al., 2005), much less regenerate, i.e., repair of plasmalemmal damage can be neuroprotective. 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 (Appendix, Fig.A2). Antioxidants (melatonin: MEL) have been reported to decrease plasmalemmal sealing (Spaeth et al., 2010) and slightly improve behavioral recovery (methylene blue: MB) after damage to nerve or other tissues (Zhang et al., 2006; Rojas et al., 2009). Additionally, recent studies have shown that pretreating mice with MB significantly reduced cochlear damage from intense broad-band noise (Park et al., 2014).

In contrast to the slow and incomplete repair of severed mammalian axons, for many invertebrates complete behavioral function is often restored quickly (within days) after axonal severance because regenerating proximal outgrowths need grow only a few mm to specifically fuse with (or otherwise activate) their surviving distal axon that does not degenerate for weeks to years (Hoy et al., 1967). The membrane fusogen polyethylene glycol (PEG) has also been used to rapidly (1-2min) and specifically reconnect (PEG-fuse) the severed proximal and distal halves of individually-identified invertebrate giant axons *in vitro*, as measured by intra-axonal dye diffusion, restoration of action potential conduction across the lesion site *in vitro* or *in vivo* and permanent restoration of

their specific behavioral functions *in vivo* (Krause & Bittner, 1990; Lore et al., 1999). PEG has also been used to rapidly restore morphological and physiological continuity *in vitro*, *ex vivo* and *in vivo* to cut- or crush-severed mammalian sciatic and spinal axons (Lore et al., 1999; Marzullo et al., 2002; Stavisky et al., 2005; Britt et al., 2010). This PEG-fusion technique has recently been used to significantly (but modestly) improve behavioral deficits due to crush (but never cut) severance injuries of rat sciatic nerves at 2-3 weeks, but not earlier (Britt et al., 2010).

We now describe for the first time well-specified PEG-fusion procedures and their rationale that rapidly and dramatically repair both cut- severed (Fig. A3) or crush-severed rat sciatic nerves (Fig. A1) to permanently restore well-studied behavioral functions and prevent Wallerian degeneration of many axons. In brief, completely severed sciatic axons are first treated with Ca^{2+}-free hypotonic saline to increase axoplasmic volume, open any sealed axonal ends, expel vesicles- and closely appose crush-severed ends remaining within endo-, peri- or epineural sheaths. Microsutures through epineural sheaths closely appose cut-severed ends. MEL or MB is applied to inhibit vesicle formation and decrease sealing probability *before* applying PEG to artificially induce closely-apposed membranes of severed, open, vesicle-free axonal ends to flow into each other (Lentz, 2007), producing a partial rejoining of the plasmalemmal membranes of proximal and distal axonal segments of unknown specificity. PEG-fused axons are then exposed to Ca^{2+}-containing saline to induce vesicles to accumulate and

seal any remaining holes near the lesion site. Such PEG-fused axons exhibit morphological continuity (assessed by intra-axonal dye diffusion) and electrophysiological continuity (assessed by conduction of action potentials) across the lesion site. These PEG-fusion protocols for cut- or crush-severed sciatic nerves rapidly, more completely (40-60% at 24-72 hours after surgery), and permanently restore sciatic-mediated behaviors compared to non-treated or conventionally-treated animals, as assessed by two standard behavioral tests: Sciatic Functional Index (SFI) and Foot Fault (FF).

We have also examined possible cellular/molecular mechanisms by which MB and PEG produce behavioral recovery. Our *in vitro* and *ex vivo* data suggest that plasmalemmal leaflets at severed ends of rat hippocampal B104 cells or excised sciatic nerves collapse and fuse within seconds when treated with PEG (PEG-seal, Fig.A4). Such PEG-sealing is not affected by substances that affect previously-reported sealing pathways (Spaeth et al., 2010) and may have neuroprotective effects.

METHODS

All experimental 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 dark:light cycle and given food and water *ad libitum*.

Surgical procedures

Rats were anesthetized with intra-peritoneal 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 Kreb's physiological saline perfused from a Pasteur pipette. This hypotonic Krebs solution 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, 295 mOsm). 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 below).

Animals in the Sham group received no nerve injury following exposure of the sciatic nerve *via* a skin incision.

Animals in cut-severance treatment groups received a sciatic nerve transection with microdissection scissors so that the proximal and distal ends retracted for 1–2 mm, completely severing all axons and their endo-, peri- and epi-neural sheaths. The cut severed ends separated by 1-3mm as viewed through a 20-50x dissecting microscope. For groups treated with microsutures, proximal and distal nerve segments were surgically re-apposed 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. Approximately 6-8 microsuture knots were tied during any suture repair. The surgeons intentionally tied slightly loose microsuture knots, so that the MB

and/or PEG could be more easily delivered to the axons during the repair. Throughout this procedure, the nerve was moistened with hypotonic Ca^{2+}-free saline perfused from a Pasteur pipette as described below.

Sciatic crush-severance injuries were made with Dumont #5 forceps, applying enough force to sever every axon. The crush-severance site of the sciatic nerve was much less opaque than adjacent uninjured sites viewed at 20-50x through a dissecting microscope. Following crush injury, a gap of about 1mm was visible at the crush site. After crushing, the sciatic nerves were again assessed for CAP conduction across the lesion site. If any CAP was detected, the nerve was crushed again. This condition occurred only 2 times out of over 185 crush-severed nerves.

To apply treatment solutions, the epineurial sheath of the sciatic nerve was nicked at the lesion site with microdissection scissors to allow better access of solutions containing PEG (Sigma-Aldrich), MB (Faulding, Aguadilla, PR), and/or MEL (Sigma-Aldrich) to cut- or crush-severed axons. For animals in groups treated with MB, MEL and/or PEG, 100µM MB or 2mM MEL dissolved in ddH_2O was applied from a micropipette positioned several mm above the lesion site in the sciatic nerve so that a solution flowed in a narrow stream (about 1 mm wide) over the cut or crushed axons at the lesion site. The constantly flowing solution was applied for 1-2min. Treatments of 500mM PEG dissolved in ddH_2O were applied to the lesion site *via* a similar micropipette so that the denser PEG-containing solution flowed for 1.5-2min over the lesion site. MEL or MB solutions

(if any) were applied before PEG solutions. After any such treatments, a second Pasteur pipette was used to bathe the sciatic nerve with isotonic Ca^{2+}-containing Krebs saline (in mM; 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 26 $NaHCO_3$, 110Na ascorbate, 10 dextrose, 2 $CaCl_2$, pH 7.35, 345 mOsm). The skin over the lesion site was closed with surgical staples in all rats that later received behavioral tests. All animals used for behavioral testing received a 5mg/kg subcutaneous injection of ketoprofen after surgery. The effects of the anesthesia lasted about 24 hours.

A 2-3cm length of sciatic nerve was removed for *ex vivo* assays of morphological integrity by intra-axonal dye diffusion.

Treatment groups and their rationale

Rat sciatic nerves (n=120 total; see Table S1 for detailed listing of n values for each treatment group) assayed *in vivo* or *ex vivo* were cut-severed (Cut group). Some were left untreated (Cut), some were treated with microsutures only (Cut+Suture) to closely appose proximal and distal ends, 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). The sham group received an incision to expose the sciatic nerve whose epineurium was nicked, but received no crush-severance injury or drug treatment. The sham

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 to the cut 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 standard clinical treatment to untreated cuts (Cut group) and to the effects of several PEG protocols (Cut+Suture+PEG and Cut+Suture+MB+PEG).

Rat sciatic nerves (n= 180 total; see Table S1 for detailed listing of n values for each treatment group) assayed *in vivo* or *ex vivo* were crush-severed (Crush group). 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). Other rats (sham-operated) received an incision to expose the sciatic nerve whose epineurium was nicked, but received no crush-severance injury or drug treatment. The intact nerves of this sham group were used as the control group to which all other treatments were compared for complete recovery. Sciatic nerves with crush-severed axons that did not receive any treatment (Crush) were used to assess both the extent of our sciatic nerve injury and any spontaneous recovery that might occur following that injury. All treated groups were compared to the crush treatment group to assess possible behavioral

recovery. Sciatic nerves with crush-severed axons treated with PEG only (Crush+PEG) were used to compare the effects of those treated with PEG plus either MEL (Crush+MEL+PEG) or MB (Crush+MB+PEG).

Note in all cases the order of CAP or behavioral recovery is almost always that given in the key to symbol usage in Figure 1. The order of decreasing recovery for cut treatments is: (1) Sham, (2) Cut+Suture+MB+PEG, (3) Cut+Suture+PEG, (4) Cut+Suture. Furthermore, Cut+PEG and Cut+MB show no improvement compared to cut alone. That is, 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: (1) Sham, (2) Crush+MB+PEG, (3) Crush+MEL+PEG, (4) Crush+PEG. Furthermore, Crush+MEL and Crush+PEG show no improvement compared to crush alone.

Electrophysiological recording of CAPs

Electrophysiological recording of CAPs from each rat sciatic nerve across a lesion site *in vivo* was followed by *ex vivo* intra-axonal dye diffusion assessments of morphological integrity. Two pairs of nickel-tipped hook electrodes were placed on or beneath the sciatic nerve to stimulate and record CAPs, which were displayed conventionally on an oscilloscope. Any 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 if CAPs conducted through the lesion site. During pre-operative and post-operative CAP recordings *in vivo*, the nerve was often moistened with Ca^{2+}-free saline.

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 (250-350g) 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 following section).

In another set of rats, 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 and after any injury and/or subsequent treatment. We then post-operatively tested the rats behaviorally for six (FF) or twelve weeks (SFI). To reduce use of animals for 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. Since the previously reported versus more recently collected CAP, SFI and FF means did not differ significantly ($p < 0.05$) for any of these three treatment groups, we combined the respective data for these groups to generate Figure 3.1. No animals in cut treatment groups used any previously reported data. Our CAP data are first describing through-conduction for successful PEG-fusion after cut-severance *in vivo* for any mammal.

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 seven 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 post-operative hours and then at weekly intervals for 12 weeks (SFI) or 6 weeks (FF). Animals were first tested at 24 post-operative hours to allow them to recover from most effects of surgery and anesthesia that would affect behavioral measures.

Sciatic Functional Index (SFI)

The SFI uses footprints to measure gait quality, adapted from rat models of Parkinson's disease (Schallert et al., 1978), and sciatic nerve severance (de Medinaceli, 1982; Britt et al., 2010). Such measurements indicate how well sciatic axons innervate more distal muscle groups (Britt et al., 2010). Rats were trained to traverse a wooden beam positioned to lead up to 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 a given post-operative time). For all

baseline and post-operative trials, rats always had their left (surgically operated) and right (uninjured) hind paws inked with black and red ink, respectively, and were placed near the end of the wooden beam farthest from the home cage. Three consecutive footprints from each limb (for a total of six consecutive prints) were used to measure (in millimeters) the following: 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 post-operative SFI scores were then computed for each animal as previously described (Schallert et al., 1978; de Medinaceli, 1982).

Foot Fault (FF) Test

Compared to SFI scores, foot fault scores indicate how well sciatic axons innervate more proximal muscle groups (Britt et al., 2010). Following sciatic nerve damage, more proximal muscle groups are expected to be innervated before more distal muscle groups (Bittner et al., 2000). Animals were allowed to roam freely on a wire mesh grid (45x30cm^2, with 2.5x2.5cm^2 openings) elevated 1.5cm above a solid base floor. Baseline and post-operative 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 and was given a fault score of two. Baseline and post-operative foot fault asymmetry scores were then calculated as previously reported (Britt et al., 2010).

RESULTS

First, we confirm in anesthetized rats that exposed, intact sciatic nerves conduct action potentials across the site of any intended lesion. We then bathe the nerves in Ca^{2+}-free hypotonic saline and completely cut-sever them using microdissection scissors or completely crush-sever them using fine-forceps. Complete cut- or crush- severance is demonstrated by confirming that sciatic axons do not conduct action potentials across the severance site and that cut-severed sciatic nerves have no physical connections between cut ends. Cut-severed nerves are re-apposed by microsutures. Then, 2mM MEL or 100µM MB dissolved in Ca^{2+}-free hypotonic saline is applied to the injury site for 1-2 min. Next, 500mM 3kDa PEG in Ca^{2+}-free double distilled water (ddH_20) is applied for 1-2min. Finally, PEG-fused axons are bathed with isotonic Ca^{2+}-containing physiological saline to initiate sealing of any remaining axolemmal disruptions.

Intra-axonal diffusion of fluorescent dye in intact and PEG-fused nerves

To assess morphological continuity *ex vivo*, we used conventional (Lore et al., 1999; Britt et al., 2010) confocal or fluorescence photomicroscopic

assessments of intra-axonal diffusion of Texas Red dye. We excised 1-2 cm lengths of sciatic nerves on either side of the lesion site from rats in each sham-operated, cut- or crush-severed treatment group. 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 maintained in Ca^{2+}-containing isotonic saline.

Dye always diffused intra-axonally throughout the entire length of axons inside and outside the well when: (1) proximal cut ends were maintained in the well in Ca^{2+}-free hypotonic saline in sham-operated animals, (2) cut-severed axonal ends were first closely apposed with microsutures and then treated with PEG (3) crush-severed axons were treated with PEG. Dye never diffused intra-axonally throughout the entire length of axons inside and outside the well when: (1) proximal cut ends of sham or any treatment group were maintained in Ca^{2+}-containing isotonic saline or first PEG-sealed, (2) proximal cut ends were maintained in Ca^{2+}-free hypotonic saline, but PEG was not applied to crush-severed nerves or to cut-severed nerves whose cut ends were not closely apposed by microsutures.

These intra-axonal dye-diffusion data as a measure of morphological continuity of some PEG-fused axons at the lesion site are consistent with conclusions that: (1) Ca^{2+} prevents dye uptake *ex vivo* because it initiates sealing of severed axonal ends; (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; (4) If severed axonal ends of unknown specificty are open and in close apposition, solutions containing PEG can restore morphological continuity to many severed sciatic axons.

Conduction of action potentials in intact and PEG-fused nerves

To assess physiological continuity of sham-operated or cut- or crush-severed axons (n>300), we extracellularly stimulated and recorded the ability of sciatic axons to conduct compound action potentials (CAPs). We used two pairs of hook electrodes, one pair to stimulate and the other to record CAPs on an oscilloscope. All cut- or crush-severance lesions were made between the two electrodes. Figure 1A and *SI Appendix,* Table S2 give 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 all intact sciatic nerves and *never* detected across the lesion site immediately after cutting the sciatic nerve. CAPs were subsequently detected post-cut only for Sham, Cut+Suture+PEG and Cut+Suture+MB+PEG-treated nerves, i.e., only when cut nerves were PEG-fused. Cut+Suture+MB+PEG-treated nerves had the greatest recovery of post-cut CAP amplitudes of any treatment group ($p<0.001$). CAPs were *never* detected post-cut across the lesion site for Cut-, Cut+PEG-, Cut+Suture, 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.

Figure 3.1B shows 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 (2/185) 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 post-treatment 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 post-operatively across the lesion site in Crush-, Crush+MEL-, or Crush+MB-treated sciatic nerves, i.e., if PEG was not applied. Treatment with MB+PEG again produced by far the greatest (73%-78%, $p<0.05$-0.001) post-lesion recovery of CAP amplitude for any crush-severed treatment group. 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.

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 Sciatic Functional Index (SFI) and a modified Foot-Fault (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).

One-way ANOVA and post-hoc analyses of SFI data (Fig. 3.1C) showed that each data point for the Cut+Suture+MB+PEG group was significantly ($p<0.01$) greater compared to each data point at the same post-operative time for all other treatment groups (except Cut+Suture+PEG) from 1-12 weeks post-operation. Although no pair of data points at a given post-operative time were significantly different, curve-fit analyses showed that SFI behavioral recovery for the Cut+Suture+MB+PEG group was significantly ($p<0.01$) greater and faster than the 1 day-12 weeks recovery of the Cut+Suture+PEG group, and $p<0.001$ when compared to any other cut-severance group. In contrast, curve-fit analyses showed no significant difference between Cut+Suture+MB+PEG animals operated upon by CPK or JMB (Fig. 3.1C), i.e., successful PEG-fusion is possible for researchers with very different training. The Cut+Suture+PEG curve was also significantly ($p<0.01$) better than the Cut+Suture curve (Fig. 3.1C).

Similar analyses of FF cut-severance groups (Fig. 3.1E) showed that each data point for the Cut+Suture+MB+PEG group was significantly ($p<0.05$ or

$p<0.01$) greater compared to each data point at the same post-operative time for any other treatment group (except Cut+Suture+PEG) from 1-12 weeks post-operation. Although no pair of data points at a given post-operative time were significantly different, comparison of curve-fit parameters revealed the FF behavioral recovery for the Cut+Suture+MB+PEG group was significantly ($p<0.01$) greater and faster than the 1day-12 week recovery of the Cut+Suture+PEG group, and $p<0.001$ compared to any other group.

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 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 no PEG-fusion, the anti-oxidant MB did not enhance behavioral recovery. Cut+Suture treatment showed some limited and slow improvement compared to Cut alone ($p<0.05$), as previously reported (Bozkurt et al., 2008; Campbell, 2008). Unlike 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 permanent recovery of sciatic nerve function as measured

by the SFI, i.e., 60%-80% within 6-12 weeks compared to 0-25% recovery for Cut or Cut+Suture protocols (Fig. 3.1C).

Behavioral recovery of crush-severed nerves

One-way ANOVA and post-hoc analyses of SFI data (Fig. 3.1D) showed that Crush+MB+PEG groups had significantly ($p<0.05$ or $p<0.01$) greater and more rapid recovery at each post-operative time point compared to all other groups (except Sham) from 3 days-2 weeks post-operation. Crush+MB+PEG and Crush+MEL+PEG groups had significantly ($p<0.01$) improved SFI performance during post-operative weeks 4-6 compared to those groups treated only with PEG.

Similar analyses of crush-severance FF data (Fig. 3.1F) showed that Crush+MB+PEG groups had significantly greater and more rapid recovery at 1day-3weeks post-operation compared to Crush+PEG and Crush+MEL+PEG ($p<0.05$) and Crush, Crush+MEL, and Crush+MB ($p<0.01$) groups. At 4-6 weeks post-operation, Crush+MEL+PEG and Crush+MB+PEG groups did not differ significantly ($p>0.05$) in their FF score compared to the Sham group. 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 anti-oxidants 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.

We have also examined over 15 cut- or crush-severed nerves that were PEG-fused at 1-12 weeks post-operatively in sacrificed animals and noted that CAPs always transmitted, and dye always diffused, across the lesion site. Viewed with a dissecting microscope, the distal portion of the sciatic nerve was not obviously different from sham-operated or un-operated nerves, i.e., PEG-fusion prevented much Wallerian degeneration.

Cellular mechanisms of PEG-sealing and PEG-fusion

To begin to examine cellular mechanisms by which PEG, MB, MEL or other substances might influence membrane dynamics that affect PEG-fusion *in vivo*, we examined their effect on PEG-sealing *in vitro* of individually identified B104 cells transected >50 µM or <50µM from their soma in Petri dishes (Spaeth et al., 2010). Sealing probability was defined as the percent of B104 cells that exclude 3kDaTexas Red dextran. Sealing probabilities at each sampling time were compared for different treatments using a Cochran-Mantel-Haenszel χ^2 test (CMH) (Agresti, 1996); sealing rates for different treatments were compared using Fisher's Z test (FZT).

Since MB and MEL increase PEG-induced behavioral recovery *in vivo*, we first investigated the *in vitro* effects of 2mM MEL or 100µM MB on sealing of B104 cells with transected neurites. After 10 min transection in Ca^{2+}-free saline containing MEL or MB, the bathing solution was replaced with Ca^{2+}-containing saline (without MEL or MB). At various times after Ca^{2+} addition (post-Ca^{2+} addition time: PC time), Texas Red dextran dye was added and sealing assessed. Sealing probabilities and rates were significantly ($p<0.01$, CMH; $p<0.05$, FZT) decreased when B104 cells were transected in 2mM MEL or 100µM MB compared to cells transected in only Ca^{2+}-free saline (Fig. 3.2A,B). These effects of MEL and MB on plasmalemmal sealing were similar to the effects produced by other antioxidants (Spaeth et al., 2010)

To examine the effect of PEG on plasmalemmal sealing *in vitro*, we transected B104 neurites >50µm or <50µm from the cell body in Ca^{2+}-free saline and added various PEG concentrations for 1min to mimic our PEG application *in vivo* or *ex vivo* . Texas Red dextran (3kDa) was then added and dye uptake immediately (0min post-PEG) assessed. Sealing probability at 0min post-PEG increased sigmoidally from 0% to 100% sealing with PEG concentration (R^2 goodness of fit values > 0.97) when B104 neurites were exposed to 0.2-50mM PEG for 1min, and then maintained in Ca^{2+}-containing or Ca^{2+}-free saline (Fig. 3.2C). At lower concentrations (0-0.8mM), PEG did not induce sealing of cut ends. This PEG-sealing probability declined following 1min exposure to 100-

500mM PEG and uninjured cells also filled with dye (Fig. 3.2C). Similar data were obtained for B104 cells transected <50µm from the cell body.

Our *ex vivo* data also suggest that PEG can seal or disrupt the plasmalemma depending on concentration and duration of PEG application. For example, 1-2min applications of lower PEG concentrations *in vitro* produced PEG-sealing (Figs. 3.2C; 3.3A) and shorter application times *in vivo* or *ex vivo* produced PEG-fusion (Figs. 3.1, 3.3D). In contrast, higher concentrations on B104 cells *in vitro* (Fig. 3.2C) or longer application times on rat sciatic nerves *ex vivo* had deleterious effects (Fig. 3.3D).

Taken together, these data suggest that (1) PEG rapidly seals (PEG-seals) plasmalemmal damage in the absence of Ca^{2+}; (2) Sealing probability increases as PEG concentration increases up to 50mM PEG; and, (3) Higher PEG concentrations rapidly produce plasmalemmal damage.

Our *in vitro* PEG-sealing data described above also suggest that PEG concentrations of about 50mM most effectively and rapidly reconnect (PEG-fuse) crush-severed axonal halves *in vivo*. In our *in vivo* protocol, we use a microelectrode to apply 500mM PEG that is then diluted, perhaps to about 50mM, by extracellular and intracellular fluids between the microelectrode and the severed axons several millimeters away.

To examine the effects of Ca^{2+} on PEG-sealing, B104 neurites were transected in Ca^{2+}-free saline and then treated with 10mM PEG (Fig.3A). These transected neurites PEG-sealed with a significantly ($P<0.001$) greater probability

at 0-5min post-Ca^{2+} addition (PC) time compared to cells not treated with PEG and maintained in Ca^{2+}-containing saline. B104 cells do not usually seal in Ca^{2+}-free saline (Spaeth et al., 2010), but PEG-sealing of B104 cells occurred as rapidly as sealing measurements could be made in Ca^{2+}-free or Ca^{2+}-containing saline (Figs. 3.2A-B, 3.3A). However, PEG cannot substitute for all effects of Ca^{2+}, since sealing probability declined in transected (or intact) cells never exposed to Ca^{2+}, starting 20min after 10mM PEG exposure in Ca^{2+}-free saline (Fig. 3.3A).

To examine whether PEG-sealing utilizes any previously reported pathways for membrane fusion in eukaryotic cells, we transected B104 cells in various substances at concentrations shown to significantly affect plasmalemmal sealing (Fig. 3.3C): N-ethylmaleimide (NEM) to inhibit the N-ethylmalemide sensitive factor (NSF), botulinum neurotoxin type A (BoNT A) to inhibit soluble N-ethylmaleimide sensitive factor attachment protein of 25kDa (SNAP-25), tetanus toxin (TeNT) to inhibit synaptobrevin, brefeldin A (BrefA) to inhibit Golgi-vesicular traffic, the small peptide protein kinase inhibitor (PKI) to inhibit the protein kinase activated by cAMP (PKA), di-thiothreotol (DTT), MB and MEL to decrease cytosolic oxidation, and di-butyryl cyclic adenosine monophosphate (db-cAMP) to increase activity of PKA and the exchange protein activated by cAMP (Epac) (Togo, 2004). PEG was added immediately after transection for 1min and dye was added immediately after PEG was washed out. Compared to controls, the sealing probability of B104 cells transected >50µm (Fig.3B) or <50µm from the

soma were significantly ($p<0.001$, CMH) and maximally enhanced by 10mM PEG. Such PEG-sealing was at or near 100% and was not significantly ($p>0.05$) affected by Ca^{2+} (Fig. 3.3B) or any other substance tested that enhanced or decreased sealing probability (Fig. 3.3C). All these data suggest that PEG directly produces membrane sealing (and thus PEG-sealing and PEG-fusion) independent of any known sealing pathway (Fig. A2).

DISCUSSION

Cut- or crush-severance lesions of mammalian peripheral nerves occur frequently and often produce significant behavioral deficits (Bozkurt et al., 2008; Campbell, 2008) even months-to-years post-injury even after treatment with the most-beneficial conventional techniques available today (microsurgery, nerve growth guides). In contrast, we now report for the first time that completely cut- or crush-severed rat sciatic nerves treated with our modified PEG-fusion protocols (Figs. A1, A3) show morphological and electrophysiological repair within minutes and dramatic behavioral recovery within 1-3 days that is maintained for at least 12 weeks, i.e., permanent repair. Our *in vitro, ex vivo*, and *in vivo* data are all consistent with rationales and effects of our cut or crush PEG-fusion protocols as follows: (1) Ca^{2+}-free hypotonic saline opens cut- or crush-severed axonal ends and brings them into close apposition in crush-severed nerves; open ends of cut-severed nerves are closely apposed by microsutures through the epineurium; (2) Application of an anti-oxidant (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 and seal; (3) Application of Ca^{2+}-containing isotonic saline seals any remaining plasmalemmal holes by enhancing vesicle formation, accumulation, interaction and fusion (Figs. A1, A3); (4) PEG-fusion prevents much Wallerian degeneration. (5) PEG 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 disruptions (Bittner & Fishman, 2000; Lentz, 2007; Fig. A4). That is, PEG-sealing and/or PEG-fusion protocols are not affected by Ca^{2+}, proteins or other substances in any other known membrane fusion or vesicle trafficking pathways (Figs.3.2-3). Rather, PEG-sealing (and also almost-certainly PEG-fusion) depends upon PEG concentration with a parabolic- shaped dose-response curve (Fig. 3.2C).

It is relevant for possible translation into clinical procedures that PEG-induced fusion is almost certainly possible for other mammalian axons (including humans), as it is for many giant axons from several invertebrate phyla (Krause & Bittner, 1990; Lore et al., 1999), since cut- or crush-severed PNS or CNS axons in other mammals including rat, rabbit, and guinea pig sciatic or spinal tract axons can also be PEG-fused (Lore et al., 1999; Marzullo et al., 2002; Stavisky et al., 2005; Britt et al., 2010). Furthermore, all drugs in our PEG-fusion protocols are benign, non-toxic in the concentrations used, readily available, and

are already FDA-approved for human use. The applicability of these protocols beyond treating very acute traumatic nerve injuries is further indicated by our previously published data showing that (1) Cut-severed stumps of distal axons in rat tail nerves can be maintained morphologically and functionally intact for 5-10 days by cooling this body part *in vivo* (Sea et al., 1995) or by cyclosporin A injections (Sunio & Bittner, 1997). (2) Distal axonal stumps of cut-severed rat spinal nerves maintained intact for days by cooling *in vitro* can be PEG-fused (Marzullo et al., 2002). (3) Previously cut- or crush-severed axons that have sealed in Ca^{2+}-containing saline can be recut, apposed and successfully sealed (Lore et al., 1999). Consequently, our PEG-fusion protocols may indeed be quickly translatable to important clinical procedures that dramatically permanently restore within minutes-to-days much behavior lost by cut- or crush-axonal severance, a result not obtained by any other chemical or surgical treatment published to date.

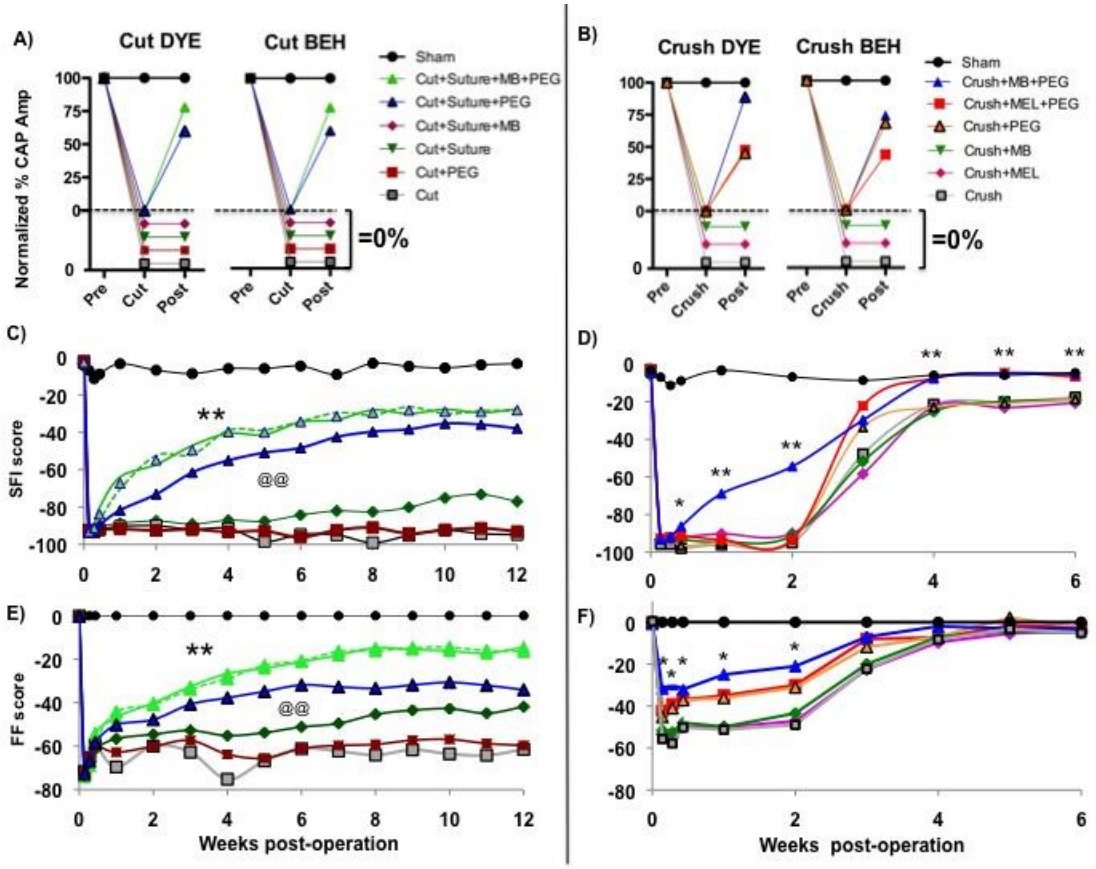

Fig. 3.1: (**A,B**), Mean CAP amplitudes normalized with respect to pre-operative amplitude recorded post-operatively from rat sciatic nerves *ex vivo* (Dye) or *in vivo* (Beh) for Cut (**A**) or Crush groups (**B**), as given by key in **A** or **B**. (**C,D)** Mean SFI scores and (**E,F)** mean FF asymmetry scores for Cut (**C,E**) or Crush **(D,F**) treatment groups vs. post-operative week. Statistical comparisons: Cut SFI and FF graphs, * = Cut+Suture+MB+PEG vs. Cut+ Suture+PEG,curves, @ = Cut+Suture+PEG vs. Cut+Suture curves; SFI and FF crush graphs, * = Crush+MB+PEG vs. Crush+PEG at the same post-operative time. Single symbol = $p<0.05$, double symbol = $p<0.01$. Green triangle symbols = combined data for Cut+Suture+MB+PEG treatment group; dotted green line= CPK data, solid green line = JMB data. Concentrations: 2mM MEL, 100µM MB, 500mM 3kDa PEG.

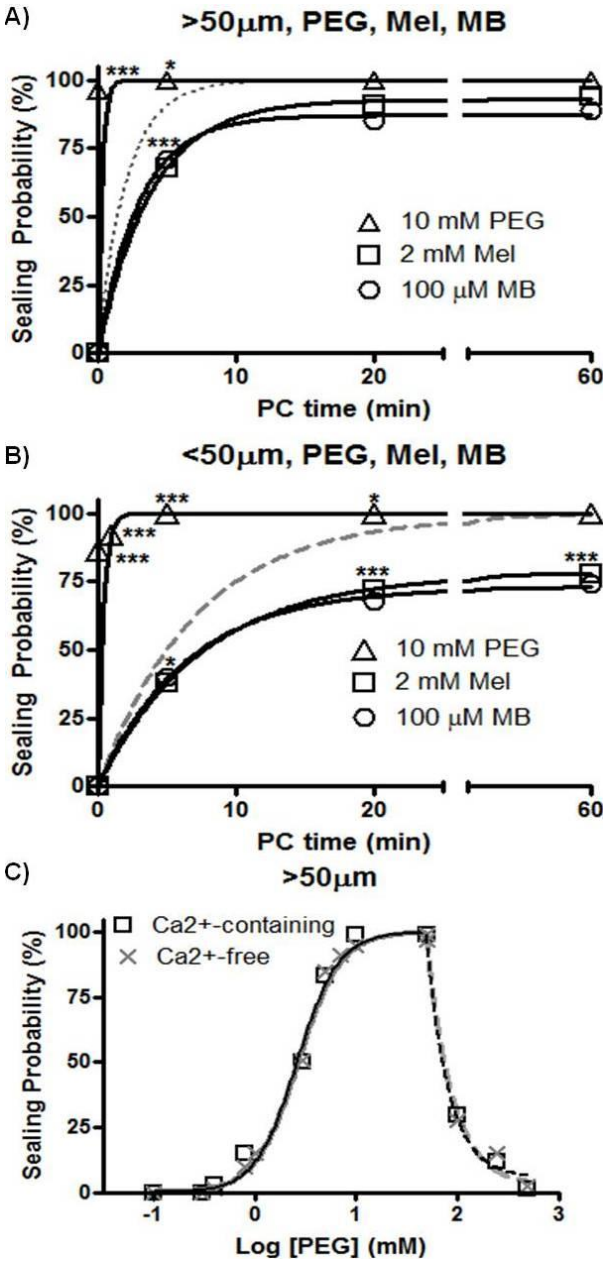
A)
>50μm, PEG, Mel, MB
Sealing Probability (%)
PC time (min)
10 mM PEG
2 mM Mel
100 μM MB
B)
<50μm, PEG, Mel, MB
Sealing Probability (%)
PC time (min)
10 mM PEG
2 mM Mel
100 μM MB
C)
>50μm
Ca2+-containing
Ca2+-free
Sealing Probability (%)
Log [PEG] (mM)

Figure 3.2: A,B.

Figure 3.2: A,B: Sealing probability (%) vs. post-calcium addition (PC) time of B104 cells with neurites transected >50µm (**A**) or <50µm (**B**) from the soma in Ca^{2+}-free saline containing 2mM MEL (open squares) or 100µM MB (open circles) before adding Ca^{2+} or 10mM PEG (open triangles) for 1min after transection and then adding Ca^{2+}. Solid lines: exponential fit to data. Asterisks: significant differences compared to control sealing (dotted, dashed lines); * = $p<0.05$, ** <0.01, *** <0.001. **C**: Sealing probability 1min after adding 2kDa PEG vs. log PEG concentration (mM) for B104 cells transected >50µM from the soma and maintained in Ca^{2+}-containing saline (open squares) or Ca^{2+}-free saline (gray X's). Solid lines: sigmoidal fit to data; Dashed lines: exponential decay fit to data.

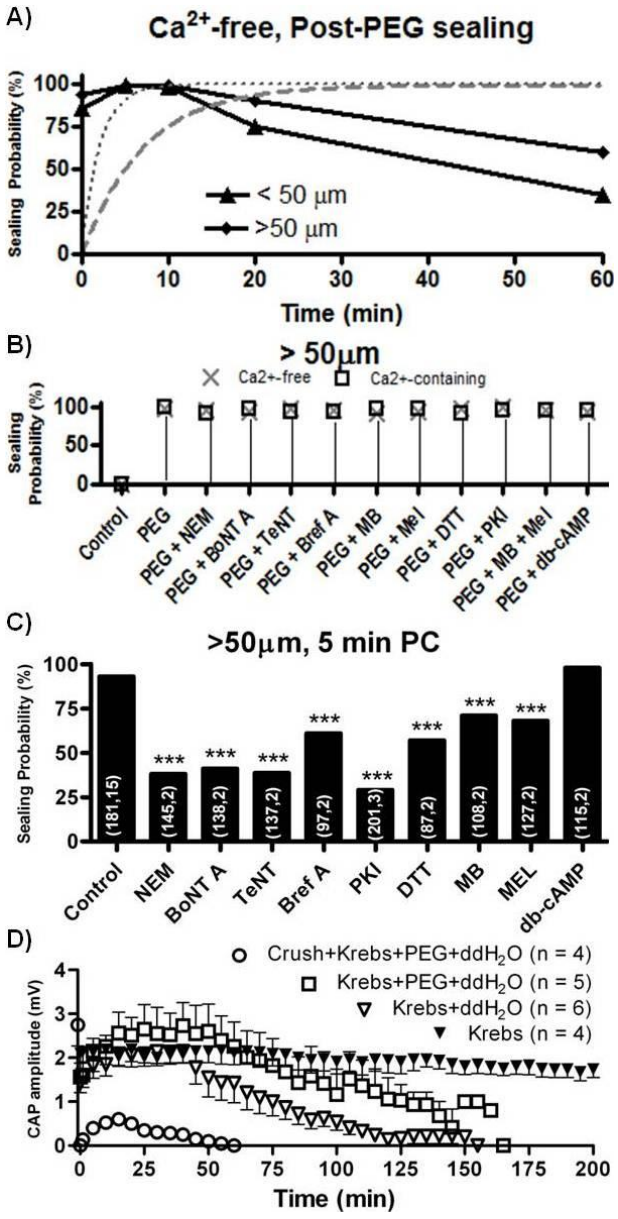
A)
Ca2+-free, Post-PEG sealing
Sealing Probability (%)
100
75
50
25
0
< 50 µm
>50 µm
0
10
20
30
40
50
60
Time (min)
B)
> 50µm
Ca2+-free
Ca2+-containing
Sealing Probability (%)
100
50
0
Control
PEG
PEG + NEM
PEG + BoNT A
PEG + TeNT
PEG + Bref A
PEG + MB
PEG + MeI
PEG + DTT
PEG + PKI
PEG + MB + MeI
PEG + db-cAMP
C)
>50µm, 5 min PC
Sealing Probability (%)
100
75
50
25
0
(181,15)
(145,2)
(138,2)
(137,2)
(97,2)
(201,3)
(87,2)
(108,2)
(127,2)
(115,2)
***
Control
NEM
BoNT A
TeNT
Bref A
PKI
DTT
MB
MEL
db-cAMP
D)
Crush+Krebs+PEG+ddH2O (n = 4)
Krebs+PEG+ddH2O (n = 5)
Krebs+ddH2O (n = 6)
Krebs (n = 4)
CAP amplitude (mV)
4
3
2
1
0
0
25
50
75
100
125
150
175
200
Time (min)

Figure 3.3A.

Figure 3.3A: Sealing probability (%) vs. Post-PEG time (min) for B104 cells with neurites transected >50µm or <50µm from soma in Ca^{2+}-free saline, treated with 10mM PEG for 1min, and maintained in Ca^{2+}-free saline (Solid lines). Dotted,dashed lines: Control sealing probability vs. PC time for cells transected >50µm or <50µm from the soma in Ca^{2+}-free saline and maintained in Ca^{2+}-containing saline. **B:** Sealing probability (%) of B104 cells transected in Ca2+-free saline at >50µm from the soma in the presence of substances that inhibit sealing, then treated with 10mM PEG in Ca^{2+}-free saline, then bathed in Ca^{2+}-containing (open squares) or Ca^{2+}-free (X) saline and immediately assessed for sealing. **C**: Sealing probability (%) of B104 cells transected >50µm from the soma in Ca^{2+}-free saline containing an inhibitor of sealing, and assessed for sealing 5min after Ca^{2+} addition, i.e., at 5min PC. **D**: CAP amplitudes (mV) vs. time (min) recorded *ex vivo* (SI Fig. S1) from intact or crush-severed sciatic nerves exposed to isotonic saline (Krebs), hypotonic saline (Krebs+ ddH_20) or 2kDa PEG (500mM) dissolved in double-distilled water (ddH_20). Data points: mean+/-SEM in one direction.

GENERAL DISCUSSION

While it has been known for over a century that mammalian peripheral nervous system axons are capable of regenerating following axotomy, it is also widely recognized by physicians and researchers that prognosis is often poor following traumatic nerve injury. After severed distal axonal segments degenerate, proximal axonal ends grow towards their former targets at the rate of 1-2 mm/day. In larger mammals, such as humans, this rate of regeneration is slow enough that axons often never reach their targets due to changes in a complex extracellular environment following injury and inflammation. Such changes may disrupt gradients of growth factors and cell adhesion molecules axonal outgrowths require to reach their appropriate destinations. When regenerating axons are able to reach their targets after an extended period of time, those targets are often no longer receptive to innervation. It will be important to provide neuroprotection treatments to prevent the loss of target neurons (Schallert & Lindner, 1990).

Much of the research exploring potential treatments for traumatic nerve injury has centered around improving guidance for slow axonal outgrowth. Many such studies focus on silencing cell adhesion molecules that inhibit axonal outgrowth, or manipulating the extracellular environment with nerve growth conduits.

PEG-fusion offers a rapid and effective repair strategy for crushed or severed nerves. Fusing the severed proximal and distal axonal segments

together allows us to bypass both Wallerian degeneration and subsequent regeneration by axonal outgrowth. This therapeutic strategy results in rapid recovery of lost behavioral function. This advantage is critical in treating larger mammals (such as humans) because the many months or years it takes to regenerate axons to distal targets frequently results in those targets often no longer able to accept innervation. Therefore, rapid repair by PEG-fusion not only increases the rate of behavioral recovery, but also results in a better overall prognosis than regeneration alone.

In chapter one of this dissertation, we illustrate how PEG-fusion may be complemented by the administration of antioxidants following nerve injury. Oxidative stress is known as a component of secondary injury damage in axonal segments that have been crushed or severed. By applying the antioxidant melatonin *in vitro* and *in vivo* to severed mammalian PNS axons, we demonstrated improved axonal viability following injury (as measured by CAP amplitude). Additionally, in this section we were able to show that the application of melatonin enhances the success of PEG-fusion in severed mammalian PNS axons.

Chapter two introduces evidence that PEG-fusion not only immediately restores axonal continuity, but also allows for rapid recovery of long-lasting behavioral function. In this section we describe the use of two common behavioral tests for assessing hindlimb motor function in the rat. Our results indicate that the FF test is particularly sensitive to differences in recovery

occurring in the first few weeks following injury, while the SFI appears to be more sensitive to subtle, persisting deficits in later weeks of recovery. Through a series of experiments we attempt to show that PEG-fusion is a useful strategy for repairing crush-severed axons, providing lasting behavioral recovery following traumatic nerve injury.

Chapter three introduces a detailed approach to treat nerve severance injuries using microsutures, the antioxidant methylene blue, and PEG-fusion. Cut-severed PNS axons typically regenerate more poorly than crush-severed axons, presumably due to lack of support and guidance of the ECM. PEG-fusion alone typically provides inadequate repair because mechanical strength at the repair site is poor. With microsutures we are able to bring proximal and distal severed axonal segments in close apposition. Subsequent local application methylene blue in hypotonic, calcium-free saline leaves axonal ends open and vesicle-free. We then demonstrate that PEG can fuse adjacent axonal ends and rapidly restore behavioral function *in vivo.* Experiments in this section also suggest that PEG fuses and seals damaged membranes by a mechanism that is independent of Ca^{2+}-mediated sealing.

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 compensatory 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. However, the numbers and connection specificities of completely cut or crushed sciatic axons that are PEG-fused are sufficient to produce within 1-3 days dramatic recovery of behaviors mediated by this nerve – and behavioral tests assessing such behaviors are the best measures of functional recovery, as opposed to electromyograms, counts of axonal numbers, extent of myelin sheathing, etc. It is worth mentioning that I used epineural sheath landmarks in attempt to align severed proximal and distal nerve ends during microsuturing procedures in the experiments described in chapter three. Another surgeon may not use such landmarks and it is unclear what effect, if any, this may have in the efficacy of PEG-fusion and subsequent behavioral recovery. Furthermore, while it is known that Schwann cells align and aid in axonal regeneration of peripheral axons, it is possible such supportive cells may align and facilitate appropriate fusion of myelinated axons, as it is known that only six percent of sciatic axons are myelinated motor axons (Schmalbruch, 1986). 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. Longer-term behavioral recovery might also be improved by applying trophic agents, nerve growth guides, or other techniques that moderately enhance the extent or specificity of regeneration at 1-2mm/day by axons that are not PEG-fused. Various exercise or training paradigms might also improve longer-term behavioral recovery.

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 & Schallert 1988). When injured rats walk on a grid surface, the impaired hindlimb frequently slips through the openings. In the absence of a platform underlying the grid surface, rats typically learn motor strategies that reduce the number of slips, which obscure adequate evaluation of the degree of deficit. Previous reports have indicated such compensation occurs, often involving a shift in the burden of weight support and locomotion to the uninjured limbs (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.

Neuroprotective effects of MB and MEL might enhance survival of severed axons, 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 1-2mm/day. These slowly regenerating outgrowths combined with relearning to use PEG-fused axons might account for the slow important increase in SFI and/or FF behavioral recovery after the second post-operative week.

Future studies should consider using electromyographs to compare changes in electrophysiology with changes in behavior and function at various post-operative time points. This would potentially provide useful insight into how fused vs. regenerating fibers may effect behavioral recovery in PEG-fused subjects. Cutting the nerve after the subject has shown significant behavioral recovery is also an important control that should be addressed in future studies, to ensure that improvements in behavioral function did indeed stem from descending neural control, and not some unknown factor following PEG-fusion. Additionally, future experiments with severed nerves could include treatment

groups in which the proximal and distal nerve segments are rotated 90 or 180 degrees, assessing whether or not such intentional misalignment has any effect on the success of PEG-fusion and subsequent behavioral outcomes.

It is hoped that prognosis for traumatic nerve injuries will be improved in the years to come. The work described in this dissertation offers evidence that alternatives to regeneration by slow axonal outgrowth are possible. Repair of damaged axons by PEG-fusion may contribute in the treatment of traumatic nerve injury giving rise to treatments that encourage a focus on rapid repair in addition to target-oriented regeneration strategies.

Appendix Figures

PEG-fusion Protocol for Crush-Severance Repair

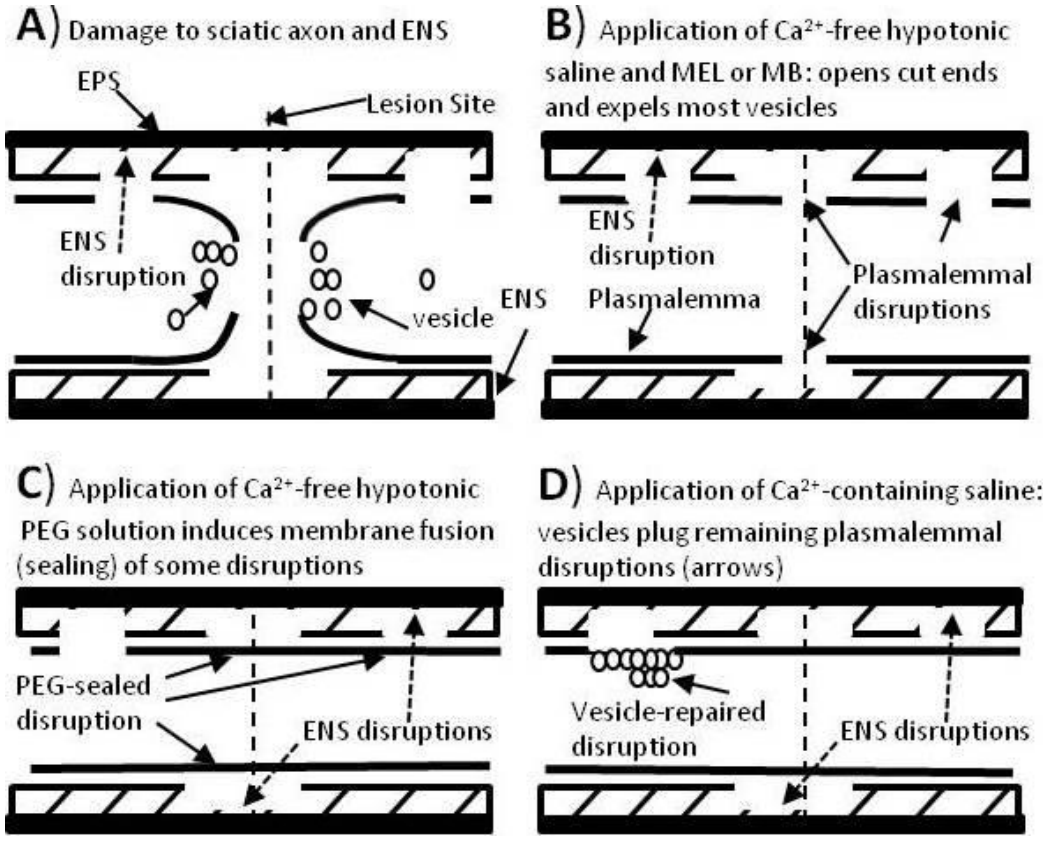

Figure A1: Hypothesized effects of each solution used in sequence to produce PEG-fusion of crush-severed axons. Crush-severance completely disrupts the axolemma and the endoneural sheath (ENS) at the lesion site, but leaves the epineural sheath (EPS) mostly intact, insuring that crush-severed ends remain in close apposition. Consequently, PEG-fusion perhaps of the same axonal halves may occur more frequently for mammalian crush-severed axons in nerve bundles that are closely apposed by hypotonic salines within their original endoneural sheaths than for cut-severed axons closely apposed by microsutures, but whose cut ends cannot be precisely re-aligned. MEL: melatonin, MB: methylene blue, PEG: polyethylene glycol. Concentrations: 2mM MEL, 100µM MB, 500mM 2kDa PEG.

Natural Mechanisms of Plasmalemmal Repair (Sealing)

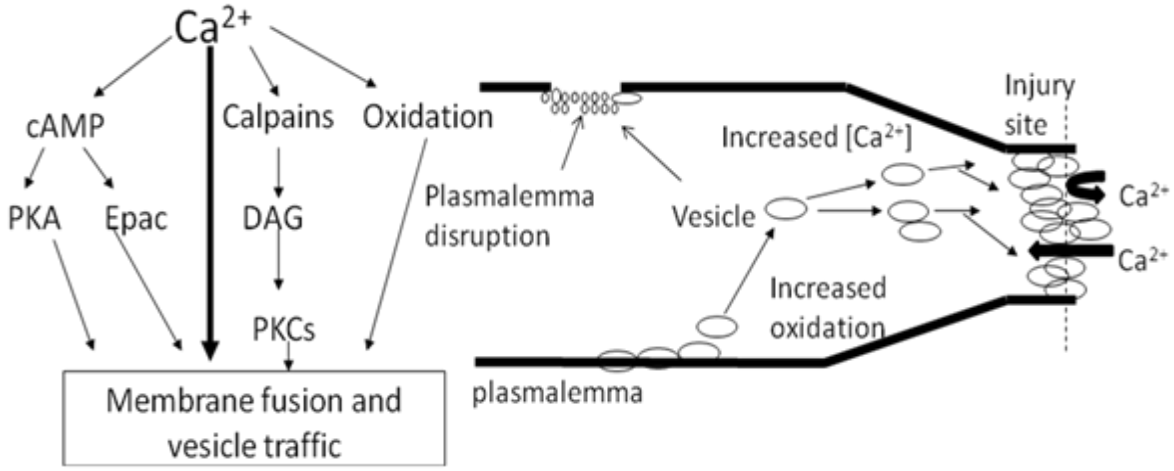

Figure A2.

Figure A2: Model for endogenous, Ca^{2+}-dependent, vesicle-mediated sealing, in which vesicles form a plug at a *partly constricted* transection site and site of minor plasmalemmal damage. Endogenous sealing in all eukaryotic cells likely occurs by this mechanism. PKA, PKC: phosphokinase A or C, Epac: exchange protein activated by cAMP, DAG: diacylglycerol.

Data from invertebrate giant axons, sea urchin eggs, mammalian axons and non-neuronal mammalian cells consistently show that endogenous, naturally-occurring plasmalemmal sealing requires various proteins, many of whose isomers are Ca^{2+}-dependent and involved in membrane fusion in synapses or the Golgi apparatus such as synaptobrevin, syntaxin, synaptophilin, synaptotagmin, calpains, TRIM proteins, PKA, and PKC (1-12). Increasing cAMP concentration, or specifically activating PKA or Epac via target-specific cAMP analogs, all produce similar increases in plasmalemmal sealing. Plasmalemmal sealing is decreased by an anti-oxidant (melatonin). That is, plasmalemmal sealing involves redundant, parallel pathways initiated by Ca^{2+} influx. Furthermore, data from many different preparations from many diverse phyla show that isomers of the same proteins, pathways and vesicle interactions are likely involved in plasmalemmal sealing of all eukaryotic cells, indicating that such proteins in eukaryotes evolved to repair plasmalemmal damage.

Immediately after plasmalemmal damage, extracellular Ca^{2+} flows in through the lesion site and activates sealing pathways (1-12; Fig. S2). This increase in internal free Ca^{2+} concentration ($[Ca^{2+}]_i$) also initiates processes that lead to axonal degeneration or cell death, often via apoptosis (1-4,6-9,12,13). Those neurons that do not quickly seal plasmalemmal damage do not survive or regenerate axonal processes (7,12,14), i.e., axolemmal sealing is often neuroprotective. B104 cells transected < 50 µm (nearer) from the soma seal at a slower rate compared to cells transected > 50 µm (farther) from the soma (3). However, axolemmal sealing does not *guarantee* neuronal survival after injury. Neurons that seal plasmalemmal damage at sites nearer to the soma are less likely to survive than neurons that seal plasmalemmal damage at sites further from the soma (3,7,12,14).

The cut or crushed ends of severed distal axonal segments in invertebrates also seal by this same mechanism (1,2,4) – as do the severed distal ends of mammalian axons. That is, Wallerian distal stump degeneration in 24-72 hours in mammals is not due to an inability of the distal stump to seal, but rather to activation of endogenous proteases or phagocytosis by glia or other non-neuronal cells (1,2).

PEG-fusion Protocol for Cut-severance Repair

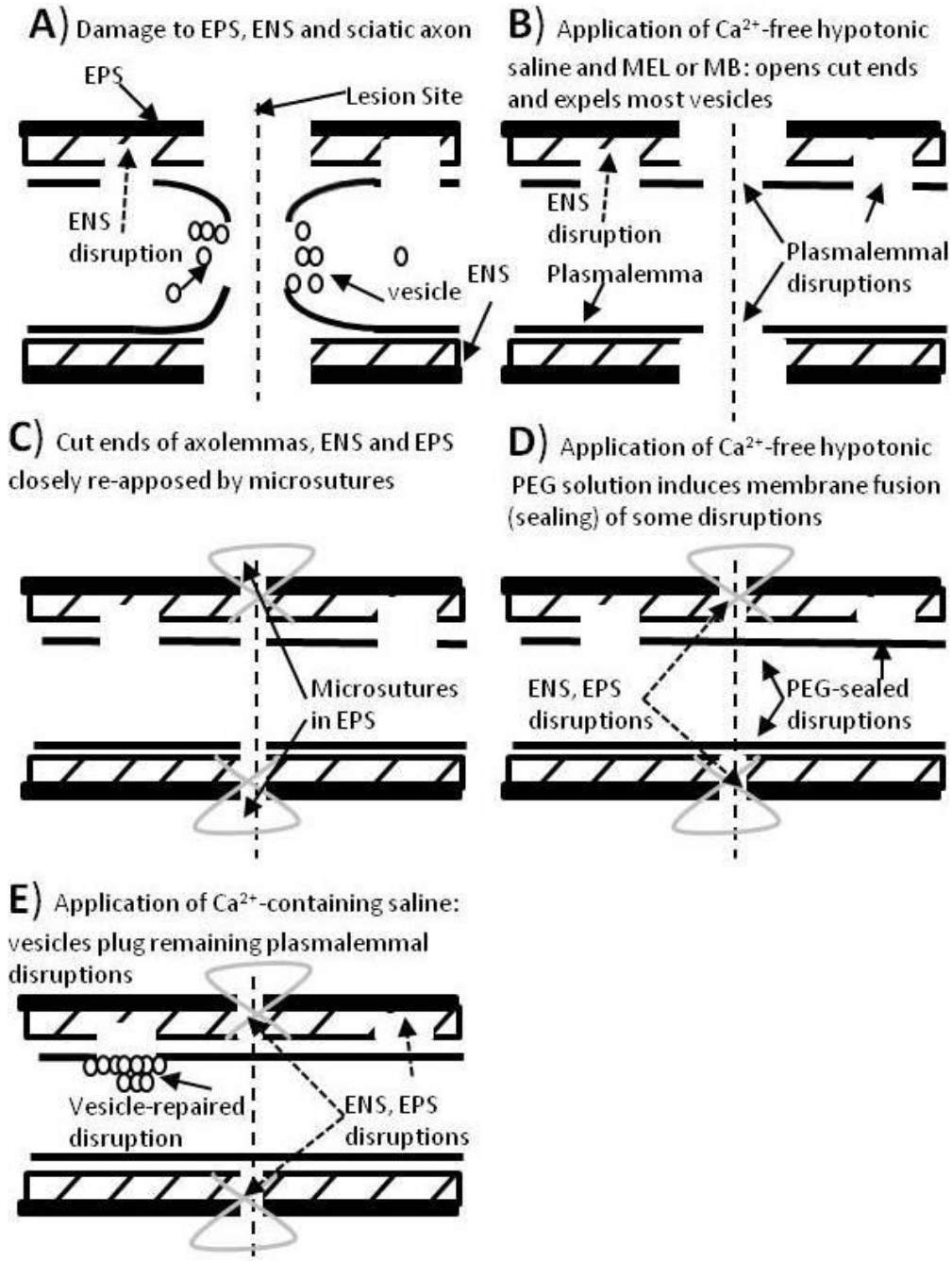

Figure A3.

Figure A3: 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), peri-neural and epineural (EPS) sheaths at the lesion site so that the cut ends typically separate by > 1mm. 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. Concentrations: 2mM MEL, 100µM MB, 500mM 2kDa PEG.

PEG-sealing Protocol

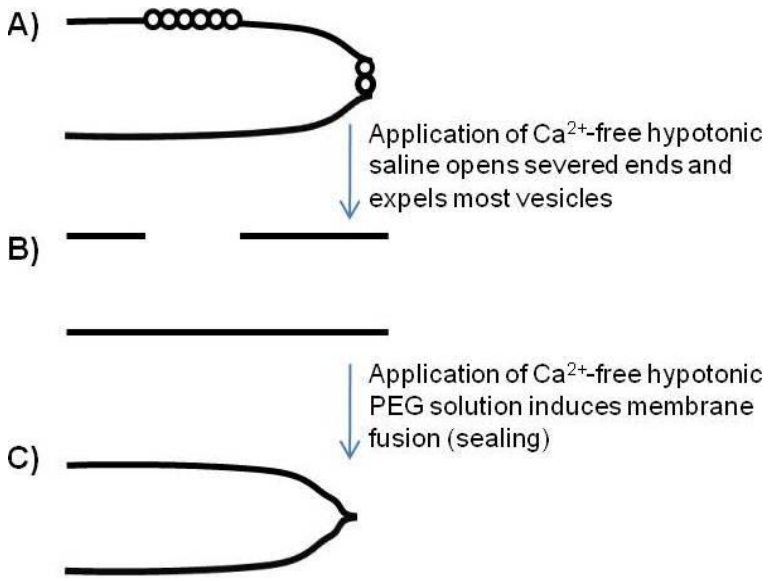

Figure S4 legend: PEG probably removes waters of hydration from membrane bound proteins at or near the damage site and decreases the activation energy required for plasmalemmal leaflets to fuse (1,2,15,16). Consequently, by removing bound water, especially in the absence of open, closely-apposed axonal segments (**Fig. S5A,B**), PEG causes damaged proximal axolemmal leaflets at severed axonal ends to collapse and fuse (i.e., PEG-seal, **Fig. S5C**) within seconds for rat sciatic nerves *ex vivo* and rat hippocampal B104 cells *in vitro* (**Figs. 1A,B,2,3**; **Tables S1-S4, S9-S13**). Small axolemmal holes are sealed by this removal of bound water, causing plasmalemmal lipids to flow into each other (1,2,15,16). Prior to 1994, transected axons were assumed to seal by collapse and fusion of plasmalemmal leaflets at cut ends and small holes to seal by spread of membrane lipids (4), as illustrated in **Figure S5C**. All such repair/sealing of complete plasmalemmal transections or small plasmalemmal holes is now known to occur by formation of vesicular plugs. (For reviews, see 1-3,17,18)

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Vita

Joshua Martin Britt was born in Beaumont, Texas and raised by his mother and father. He is told that as an infant he never drooled (something of which his father is quite proud), never cried, and rarely slept. He spent the next 18 years growing up in Southeast Texas, after which he attended the University of Texas at Austin from 1998 to 2003, earning a B.A. in biology and psychology. Deciding to simultaneously pursue careers in music and research, he stayed at the University of Texas at Austin for graduate school. He currently resides in Beaumont, Texas and works full-time as a researcher at Helena Laboratories. He has a dog, Honey Butter Chicken Biscuit (also known as “HBCB,” “Honey Bear,” or simply “Bearz”), though they are currently separated. Fan mail may be directed to the address listed below.

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