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Effects of extracellular calcium and surgical techniques on restoration of axonal continuity by PEG-fusion following complete cut- or crush-severance of rat sciatic nerves

Cameron L. Ghergherehchi¹, George D. Bittner¹, Robert Louis Hastings¹, Michelle Mikesh¹, D. Colton Riley^{1,3}, Richard C. Trevino¹, Tim Schallert², Wesley P. Thayer³, Solomon Raju Bhupanapadu Sunkesula¹, Tu-Anh N. Ha¹, Nicolas Munoz¹, Monika Pyarali¹, Aakarshita Bansal¹, Andrew D. Poon¹, Alexander T. Mazal¹, Tyler A. Smith¹, Nicole S. Wong¹, and Patrick J. Dunne¹

¹Department of Neuroscience, University of Texas at Austin, Austin, Texas 78712

²Department of Psychology, University of Texas at Austin, Austin, Texas 78712

³Department of Plastic Surgery, Vanderbilt School of Medicine, Nashville, Tennessee 37232

Abstract

Complete crush- or cut- severance of sciatic nerve axons in rats and other mammals produces immediate loss of axonal continuity. Loss of locomotor functions subserved by those axons are not restored for months, if ever, by outgrowths regenerating at ~1 mm/d from the proximal stumps of severed axonal segments. The distal stump of a severed axon typically begins to degenerate in 1–3 days. We have recently developed a PEG-fusion technology consisting of sequential exposure of severed axonal ends to hypotonic Ca²⁺-free saline, methylene blue (MB), polyethylene glycol (PEG) in distilled water, and finally to Ca²⁺-containing isotonic saline. We examined factors that affect the PEG-fusion restoration of axonal continuity within minutes as measured by conduction of action potentials and diffusion of an intracellular fluorescent dye across the lesion site of rat sciatic nerves completely cut- or crush-severed in the mid-thigh. We also examined factors that affect the longer-term PEG-fusion restoration of lost behavioral functions within days to weeks as measured by the Sciatic Functional Index. We report that exposure of cut-severed axonal ends to Ca²⁺-containing saline prior to PEG-fusion and stretch/tension of proximal or distal axonal segments of cut-severed axons decrease PEG-fusion success. Conversely, trimming cut-severed ends in Ca²⁺-free saline just prior to PEG-fusion increases PEG-fusion success. PEG-fusion prevents or retards the Wallerian degeneration of cut-severed axons as assessed by measures of

Corresponding Author: George D. Bittner, Department of Neuroscience, University of Texas at Austin, Austin, TX 78712. 512-923-3735; 512-471-9651 (FAX), bittner@austin.utexas.edu.
Current addresses. RLH: Texas A&M Institute for Neuroscience², Texas A&M University, College Station, TX 77843

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no known or potential conflicts of interest, including financial, personal or other relationships with people or organizations that could inappropriately influence, or be perceived to influence their work.

AUTHOR'S ROLES

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors participated in the writing of this paper and collaborated in obtaining and publishing data and concepts described in this paper.

axon diameter and G ratio. PEG-fusion may produce a paradigm-shift in the treatment of peripheral nerve injuries.

Graphical Abstract

Distal portions of cut-severed sciatic nerves that are repaired with micro-sutures and PEG-fusion have larger diameters compared to sciatic nerves repair with micro-sutures. That is, PEG-fusion prevents or slows Wallerian degeneration of cut-severed axons.

Keywords

axotomy; axonal regeneration; polyethylene glycol; Wallerian degeneration; nerve repair; rat sciatic nerve

INTRODUCTION

Voluntary hindlimb locomotion in mammals such as rats or humans depends on a precise sequence of action potentials in many thousands of motor (and sensory) sciatic nerve axons each having specific synaptic connections (or receptor fields) in the limb and spinal cord (Kandel et al., 2014). Cut- or crush-severance of these peripheral nerve axons (PNAs) produces severe behavioral deficits that are not restored unless motor and sensory axons re-establish appropriate peripheral and central connections. In mammals, PNAs distal to a severance site begin to morphologically degenerate within 1–3 days (Wallerian degeneration). To successfully restore lost behavioral functions, severed proximal ends must rapidly (within minutes) seal off their cut ends to survive, then contact their appropriate denervated postsynaptic target tissues via outgrowths at 1–2 mm/d (Cajal, 1928; Wolfe et al., 2010; Kandel et al., 2014). This re-innervation takes months to years depending on the site of the lesion and the size of the mammal, at which time muscles may have atrophied and/or lost the capacity to be re-innervated (Bittner et al., 2000; Burch, 2011), unless artificially stimulated (Kern et al., 2004). Furthermore, unless glial sheaths provide mechanical or chemical guidance that bridge a maximum gap of several millimeters between surviving proximal and degenerated distal axonal ends, regenerating outgrowths often do not appropriately re-innervate original target tissues. Consequently, restoration of locomotor function after PNA severance is typically rather poor, especially if large-diameter nerves, such as the sciatic, are severed several centimeters or more proximally from their postsynaptic targets.

Current procedures to increase the restoration of behavioral functions lost after PNA severance include synthetic or autologous conduits, trophic factors, stem cells, and micro-sutures, with the latter being the clinical “gold standard” (Burdick et al., 2006; Campbell et al., 2008; deRuiter et al., 2008; Isaacs, 2010; Wolfe et al., 2010). These procedures have had limited success for more proximal PNA cut-severance lesions, e.g., complete sciatic nerve severance in the thigh. Furthermore, these techniques do not **(1)** increase the rate of nerve regeneration; **(2)** decrease the time to re-establish functional connections from months to days; or **(3)** prevent Wallerian degeneration and/or atrophy of target tissues.

In contrast, we have reported that a “PEG-fusion” procedure can rapidly (within minutes) restore morphological/anatomical and physiological continuity of sciatic PNAs crush- or cut-severed in the upper thigh, and restore within 1–4 weeks many lost behavioral functions (Britt et al., 2010; Bittner et al., 2012; Riley et al., 2015). This PEG-fusion procedure uses a well specified sequence of solutions that include hypotonic Ca^{2+} -free saline to open cut axonal ends, methylene blue (MB) to reduce vesicle formation, and polyethylene glycol (PEG), a membrane fusogen, to non-specifically join the open cut axolemmal ends of sciatic axons closely apposed by micro-sutures. PEG-fusion also prevents or retards Wallerian degeneration of cut-severed sciatic axons (Sexton et al., 2012; Rodriguez-Feo et al., 2013; Riley et al., 2015).

This paper examines four variables that could interact in unexpected ways to affect successful repair of severed axons by our PEG-fusion procedures: **(1)** crush- vs cut-severance each made in **(2)** Ca^{2+} -containing vs Ca^{2+} -free salines in which **(3)** cut ends are or are not trimmed and **(4)** more vs less stretch/tension is used to closely appose cut ends as required for successful PEG-fusion.

From previously published data (Krause et al., 1994; Bittner and Fishman, 2000; Lore et al., 1999), we know that hypotonic Ca^{2+} -free saline causes crush-severed nerve ends to swell open, increases axonal length, expels existing vesicles, and reduces vesicle formation. MB also reduces vesicle formation at crush- or cut-severed axonal ends. MB application in (hypotonic Ca^{2+} -free) ddH₂O increases PEG-fusion success for axons that have received 1–2 mm long crush-severances (Britt et al., 2010; Bittner et al., 2012; Spaeth et al., 2012a,b) or cut-severances with open axonal ends closely apposed by micro-sutures (Bittner et al., 2012).

Given the above, crushes of shorter (<3 mm) length made in hypotonic Ca^{2+} -free saline are consistently successfully PEG-fused because the vesicle-free crushed ends are in close apposition needed for successful PEG-fusion (Britt et al., 2010; Bittner et al., 2012). However, we hypothesized that crushes made in isotonic Ca^{2+} -containing saline might not be repaired by our current PEG-fusion protocol because Ca^{2+} influx into crush-severed ends would induce vesicles to seal off the severed ends and reduce PEG-fusion success (Spaeth et al., 2012a,b) or for crush severances 3 mm long. That is, previous studies (Spaeth et al., 2012b; Bittner et al., 2015) have shown that PEG applied to the cut or crush-severed ends of axons that are filled with vesicles and/or are not in close apposition causes the ends to seal off (“PEG-seal”) rather than rejoin to become a continuous axon (“PEG-fuse”).

From previously published data (Bittner et al., 2012; Rodriguez-Feo et al., 2013; Riley et al., 2015), we know when axons are cut-severed in hypotonic Ca^{2+} -free saline, the opened cut ends can be brought into close apposition with micro-sutures and successfully PEG-fused. We hypothesized that if axons were cut in, or exposed to, Ca^{2+} -containing saline prior to PEG-fusion, then the axonal ends would fill with vesicles and thereby decrease PEG-fusion success. These vesicles could be expelled by exposing the severed axonal ends to Ca^{2+} -free hypotonic saline with MB and/or other antioxidants before micro-suturing.

Finally, trimming the cut axonal ends should increase PEG-fusion success by creating better axonal apposition for PEG-fusion, as does trimming in the absence of PEG-fusion (Campbell, 2008; Green and Wolfe, 2011). Clinical studies have shown that if a gap between cut ends is greater than ~ 3 mm, the stretch-tension required for micro-suturing may cause many nerves to be irreversibly damaged and subsequent behavioral recovery is often very poor or nonexistent (Campbell, 2008; Green and Wolfe, 2011). Therefore, we hypothesized that trimming cut axonal ends might in some cases decrease PEG-fusion success if the distance needed to bring the severed nerve ends into close apposition were greater than 2 mm, thereby increasing the amount of stretch-tension on the nerve.

MATERIALS AND METHODS

Animals

Male (n=60) and female (n=75) outbred Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 300–350 g (males) and 225–275 g (females) were used in this study. All rats were housed three of the same sex per cage with a 12-hr light/dark cycle and given food and water *ad libitum*. Animals used in behavioral assessments were handled and trained for at least a week prior to surgery. The University of Texas at Austin's and Vanderbilt University Medical Center's Institutional Animal Care and Use Committees approved all procedures.

Surgical Procedures

Prior to surgery, animals were anesthetized using a small mammal isoflurane inhalation system (Handlebar Anesthesia, Austin, TX). Anesthesia was induced initially with a 4% isoflurane/oxygen mixture at a 1.5 L/min flow rate, and then maintained with a 1.5–2% mixture at 1 L/min for the duration of the surgery. The surgical area (lateral aspect of the left hindlimb) was trimmed of fur and disinfected using 10% povidone-iodine. A 2–3 cm long incision was made in the skin above the thigh muscles of the left hindlimb of each rat. The biceps femoris muscle was split parallel to the muscle fibers using dissection scissors to expose the sciatic nerve. The sciatic nerve was trimmed free of any connective tissue using micro-dissection scissors. Crush- or cut-severances were made in either hypotonic Ca^{2+} -free or isotonic Ca^{2+} -containing saline as specified for a given protocol.

We completely crush-severed 1–2 mm lengths of sciatic nerves using Dumont #5 micro-forceps placed perpendicular to the nerve by applying enough force to cause the underlying axons to separate while leaving the epineurium intact. After crush-severance, a small slit was made in the epineurium with micro-scissors to allow solutions to diffuse more readily to

axons at the injury site. Crush excision repairs were made by completely excising the crushed nerve segment with dissection scissors. This typically resulted in a 2–3 mm gap between the severed nerve ends, which were then closely re-apposed with micro-sutures similar to our cut-severance protocol (see below). For details of these and other crush-severance surgical procedures, see previous publications (Britt et al., 2010; Bittner et al., 2012).

Cut-severances were made by transecting the entire sciatic nerve with a single stroke of dissection scissors to completely sever all PNAs as well as their endo-, peri-, and epineurial sheaths. Cut axonal ends and sheaths completely separated by 1–3 mm. Bundles of axons would sometimes swell out of the epineurial sheath at the cut ends. In some animals as specified, these axonal ends and epineurial sheaths were carefully trimmed so that the cut ends formed smooth flat planes that could be closely apposed with minimal gaps or axonal protrusions. Similar axonal trimming was performed, but not always noted, in previous publications (Bittner et al., 2012; Sexton et al., 2012; Rodriguez-Feo et al., 2013; Riley et al., 2015). Cut-severed nerves were closely re-apposed with 10–0 micro-sutures (Ethicon, Somerville, NJ), leaving enough space between sutures to allow close positioning of a micropipette for diffusion of sterile solutions of PEG, antioxidants, etc., to the lesion site. Unless stated otherwise, cut axonal ends were typically re-apposed within 5–15 min after severance. For additional details of this cut severance surgical procedure, see Bittner et al., (2012).

*In this paper unless explicitly stated otherwise, the term PEG-fusion always denotes the following sequence of bio-engineered solutions applied to the lesion site of crush-severed sciatic nerves or cut-severed and micro-sutured sciatic nerves: (1) exposure of cut- or crush-severed axonal ends to hypotonic Ca^{2+} -free saline (PlasmaLyte-A: Baxter, Deerfield, IL), (2) application of 1% MB (Acros Organics, Morris Plains, NJ) dissolved in double-distilled water (ddH_2O) for 1–2 min, (3) application of 50% by weight PEG (3.35kD; Sigma Aldrich, St. Louis, MO) in sterile ddH_2O for 1–2 min, and (4) extensive rinsing with Ca^{2+} -containing isotonic saline (Lactated Ringers: Hospira, Lake Forest, IL). [See Figure 4, Bittner et al., 2015.] The term “negative control” describes the same procedures and solution applications as above, but without the addition of PEG to a crush- or cut-severed nerve. Note that the ends of cut negative controls are apposed by micro-sutures, the “gold standard” for clinical repair (Isaacs et al., 2010; Wolfe et al., 2010). As another negative control for dye diffusion experiments, PEG was applied to cut-severed nerves that were *not* micro-sutured, i.e., cut ends *not* closely apposed by micro-sutures do not PEG-fuse, but rather PEG seals off the cut ends (“PEG-sealing”. See below and Spaeth et al., 2012b). PEG-fused and negative control animals to be tested for behavioral recovery by SFI tests received 5 mg/kg subcutaneous injection of carprofen after surgery.*

Electrophysiological Testing

As previously described (Britt et al., 2010; Bittner et al., 2012; Riley et al., 2015), we first confirmed that extracellularly recorded action potentials (compound action potentials: CAPs) in intact rat sciatic nerves conducted across any potential lesion site *in vivo* using stimulating and recording tungsten hook electrodes placed on or under the sciatic nerve

(Lore et al., 1999; Britt et al., 2010; Bittner et al., 2012; Riley et al., 2015). Complete crush- or cut-severances were confirmed by an inability to record CAP conduction through the severance site. Sciatic nerves were typically stimulated within minutes after PEG-fusion to determine if CAPs conducted through the lesion site. Stimulations and recordings were made with a Powerlab 4/30 data acquisition system (ADInstruments, Colorado Springs, CO).

Antioxidants and sealing inhibitors

The following pharmacological agents were used to inhibit sealing after cut- or crush-severance: Protein kinase A inhibitor (PKI; 50 μ M, 1.8 kDa; Sigma Aldrich), protein kinase C isozyme η pseudosubstrate fragment (η PSF; 7 μ M, 2 kDa; EMD Chemicals, Gibbstown, NJ), protein kinase C isozyme θ pseudosubstrate fragment (θ PSF; 9 μ M, 2.3 kDa; EMD Chemicals, Gibbstown, NJ), and MB (100 μ L, 1%; Acros Organics, Morris Plains, NJ). The concentrations of each chemical have been reported to effect axonal sealing (Bittner et al., 2012; Spaeth et al., 2012; Zuzek et al., 2012).

Intracellular dye diffusion

Morphological/anatomical continuity across a cut- or crush- severance site was assessed *ex vivo* by intracellular diffusion of dye in 2–4 cm lengths of sciatic nerve excised immediately following any sham or operative procedure. In a Petri dish, the 1–2 cm long nerve segment proximal to the injury site was mounted inside a watertight ring of petroleum jelly (Vaseline) containing 50 μ L of a fluorescent dye (3 kD Texas red dextran: Molecular Probes, Eugene, OR) in hypotonic Ca^{2+} -free saline. The injury site and the 1–2 cm long segment of nerve distal to the injury site extended outside the well and were bathed in isotonic Ca^{2+} -containing saline. The nerves were maintained for 12–24 hr at 4°C to allow intra-axonal diffusion of Texas-red dextran throughout the entire length of nerve. Sciatic nerves were examined for the presence of fluorescent dye anywhere along its entire length using a Leica SP2 AOBS confocal microscope.

Behavioral analyses

Sciatic behavioral function was assessed using the Sciatic Functional Index (SFI). The SFI is a reliable and commonly used test to evaluate behavioral recovery and return of function after sciatic nerve injury (deMedinaceli et al., 1978; Wood et al., 2011). Animals to be behaviorally tested were handled and trained for at least a week prior to surgery to acclimate the rats to the testing procedures. The left hind limb was always the experimental (injured) limb. Both hind paws were dipped in blue (left) or red (right) ink. The rat was then placed on a 100 mm-wide wooden board lined with 100 mm-wide white paper strips and allowed to run back to their home cage. A trial run without any stopping or hesitation during at least three consecutive footprints of both the left and right hindlimb was considered acceptable and the paper strip retained for measurements. The normal footprint length, experimental footprint length, normal toe spread, experimental toe spread, normal intermediary toe spread and experimental intermediary toe spread were measured (in millimeters) for all footprints (Carlton and Goldberg, 1986). These six values were averaged among the three footprints for each limb. Each rat ran two trials and the average was recorded as the SFI score for that

rat for that day, as previously described (Britt et al., 2010). The SFI test was conducted by individuals blind to what procedure the rat had received. Animals were first tested at 72 hr after surgery and then on a weekly basis for six weeks, at which time they typically obtained a maximum asymptote (plateau) value that previous papers have shown does not increase for an additional 6 (Bittner et al., 2012) or 12 (Bittner et al., 2015) post-operative weeks.

Morphological procedures

Animals were deeply anesthetized with 0.8 mL pentobarbital (Euthasol®, Virbac Corp., Ft. Worth, TX), then transcardially perfused with 0.1 M sodium cacodylate buffer followed by 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate. Sciatic nerves and soleus muscles were dissected and pinned to a Sylgard coated dish and allowed to post-fix overnight in the same fixative at room temperature. After washing in 0.1 M sodium cacodylate, nerves and muscles were placed in 1% osmium tetroxide with 1% potassium ferrocyanide in 0.1 M sodium cacodylate buffer for 4–5 hr, washed thoroughly with water, stained in aqueous 1% uranyl acetate for 2 hr, then stored in water. Stained tissues were dehydrated through graded alcohols, exchanged to acetone, and embedded in Hard Plus-812 embedding resin (Electron Microscopy Sciences, Hatfield, PA). Muscle cross-sections were examined for neuromuscular junctions (NMJs), then imaged at 63x on a Zeiss Axiovert. Axonal cross sections approximately 5 μ m distal to the lesion were cut on a Leica UCT, collected on slides or Formvar coated grids, and imaged on a Zeiss Axiovert or Technai Spirit transmission electron microscope. Axons in electron micrographs were selected for morphological analysis if they lacked fixation artifacts or extensive invaginations and were individually numbered in Photoshop. The diameter of each axon and each axon plus its myelin sheath were measured in perpendicular axes with ImageJ and averaged. Data were recorded in Microsoft Excel.

Statistical analyses

All statistical analyses were performed in Graphpad Prism 6.0 (GraphPad Software, San Diego, CA) or in Microsoft Excel. A two-tailed Student's t-test was used to compare mean SFI scores between PEG-fused and negative control (no PEG-fusion) groups on a given postoperative day. Student's t-tests are reported as (t(degrees of freedom)= t-value, p value). Mixed subject ANOVA was used to test for differences in curves between PEG-fusion groups and negative control groups. ANOVA values are reported as (F(dF₁, dF₂)= F-value, p value), where dF₁=degrees of freedom between groups and dF₂=degrees of freedom within groups. Significance levels are given at p<0.05 and p<0.01.

RESULTS

No differences were observed in results between rats of different sex for any data reported herein.

Uptake and intra-axonal diffusion of fluorescent dye in crush- and cut-severed nerves

Figure 1 shows effects of extracellular calcium, PEG-fusion, and PEG on uptake and intra-axonal diffusion *ex vivo* of fluorescent dye as a measure of morphological/anatomical continuity following crush- or cut-severance of sciatic nerves. For intact sciatic nerve

controls (Fig. 1A), dye placed extracellularly diffused into opened proximal axonal ends and then intra-axonally throughout the nerve to its distal axonal ends within 24 hr (Lore et al., 1999; Britt et al., 2010; Riley et al., 2015). For nerves that were crush-severed (Fig. 1B) or cut-severed (Fig. 1C) without PEG-fusion repair *in vivo*, dye diffused *ex vivo* intra-axonally to the severance site outside the watertight ring, but did not diffuse across the severance site, confirming a disruption in axonal continuity at the lesion site. For sciatic nerves that were crush- (Fig. 1D) or cut-severed (Fig. 1E) in Ca^{2+} -free saline and then PEG-fused in hypotonic Ca^{2+} -free saline *in vivo*, dye diffused *ex vivo* intra-axonally across the lesion site and into the distal segment, confirming previous reports (Britt et al., 2010; Bittner et al., 2012; Spaeth et al., 2012b). As hypothesized in the previous section, intra-axonal dye did not diffuse *ex vivo* past the lesion site into the distal segment in nerves that were crush- (Fig. 1F) or cut-severed (Fig. 1G) in Ca^{2+} -containing saline *in vivo*, and then PEG-fused.

Figure 1H shows that dye was not able to diffuse *ex vivo* into the proximal segment of an excised, intact, sciatic nerve if PEG was applied directly to the severed open ends in Ca^{2+} -free saline before adding dye to the watertight ring. These data were consistent with previous reports that this procedure causes open axonal ends to seal off (“PEG-sealing”), prevents dye uptake (Spaeth et al., 2012b) and reduces PEG-fusion success (Krause and Bittner, 1990; Lore et al., 1999; Bittner et al., 2012).

Restoration of CAP through-conduction in crush- and cut-severed nerves

All lesions (crush or cut) were made between a proximal stimulating and a distal recording electrode (see Methods). CAP recordings were always taken in intact sham nerves or before any intended injury in other experimental groups (plotted as “Initial” Fig. 2) to confirm that the sciatic nerve was viable as assessed by recording a CAP at the distal electrode placed 1–3 cm from the proximal stimulating electrode. A CAP recording was again taken immediately following each injury to confirm that PNAs were completely severed (plotted as “Severance” in Fig. 2), i.e., that no CAP through conduction was recorded before any repair or other procedure was attempted, consistent with complete disruption of axolemmal continuity in a crush- or cut-severed nerve (Lore et al., 1999; Riley et al., 2015). CAPs were again assessed after PEG-fusion repair or other experimental procedure to assess whether conduction of action potentials across the lesion site was again restored (plotted as “Post-Repair” in Fig. 2). Peak CAP amplitudes were recorded after multiple (at least three acceptable) recordings for each experimental protocol. Sham operations were performed by exposing the sciatic nerve and taking CAP recordings without severing the nerve. Sham nerves were bathed in Ca^{2+} -free saline (Fig. 2 A,C) or in Ca^{2+} -containing saline (Fig. 2B).

MB and other antioxidants have been reported to have neuroprotective effects including improving behavioral recovery after nerve damage (Zhang et al., 2006; Rodogna et al., 2009; Rojas et al., 2009), decreasing the ability of severed sciatic or B104 cell axons to seal (Spaeth et al., 2010, 2012a,b,c), and increasing the success of PEG-fusion if applied before applying PEG to closely apposed cut axonal ends (Britt et al., 2010). PKI, η PSF, and θ PSF also decrease the sealing probability of cut axonal ends of B104 cells *in vitro* (Spaeth et al., 2010, 2012a,b,c; Zuzek et al., 2012).

Given these previously published data, we examined whether CAP through-conduction in sciatic PNAs *in vivo* was restored by PEG-fusion after crush-severance (Fig. 2A,B) or cut-severance without axon trimming (Fig. 2C) in Ca^{2+} -free (Fig. 2A,C) or Ca^{2+} -containing (Fig. 2B,C) salines. We also tested whether various substances that *decrease* vesicle formation and plasmalemmal sealing described above (50 μM of 1.8kDa PKI, 7 μM of 2kDa ηPSF , 9 μM of 2.3kDa θPSF , 100 μL of 1% MB) could help restore CAP through-conduction if applied after crush-severance (Fig. 2A,B), but before PEG-fusion. The key to the right of each panel in Figure 2 describes the sequence of steps in each protocol. Note that in some protocols, PEG-fusion is not attempted (labeled with “#” Fig. 2A–C), and that only cut-severance lesions receive micro-sutures (Fig. 2C).

Figure 2A shows whether CAP conduction was restored in Ca^{2+} -free saline for each experimental protocol given in the key. CAPs were always detected after a sham operation in which the sciatic nerve was exposed and washed with Ca^{2+} -free saline and no other solutions applied before CAPs were recorded (Sham; $n=3$ sciatic nerves). CAP conduction across a crush-severance made *in vivo* in Ca^{2+} -free saline was restored within minutes in sciatic nerves that were treated with PEG without addition of any antioxidant substances (Crush+PEG; $n=3$) or if the antioxidant MB was added before adding PEG (Crush+MB+PEG; $n=10$). Similarly, CAP conduction was always restored across the crush-severance site when other substances that inhibit axonal sealing were added before adding PEG, such as (1) PKI (Crush+PKI+PEG; $n=2$); (2) ηPSF (Crush+ ηPSF +PEG; $n=6$); (3) ηPSF and MB (Crush+ ηPSF +MB+PEG; $n=6$); (4) θPSF (Crush+ θPSF +PEG; $n=2$); (5) ηPSF and θPSF (Crush+ ηPSF + θPSF +PEG; $n=2$); and, (6) ηPSF , θPSF and PKI (Crush+ ηPSF + θPSF +PKI+PEG; $n=3$). CAPs were not restored if all sciatic PNAs were crush-severed *in vivo*, but not subsequently PEG-fused for two experimental protocols (Crush; $n=5$ and Crush+ ηPSF + θPSF +PKI; $n=3$). Both protocols are labeled with “#” in the key to Fig. 2A.

Figure 2B shows CAP conduction through the crush-severance site *in vivo* for the same experimental protocols shown in Figure 2A, except that the crush-severance was made in Ca^{2+} -containing saline. Except for the sham protocol (Sham; $n=2$), CAP conduction across the crush-severance site was **not** restored for any PEG protocol (all labeled with “##” in the key), including PEG protocols that combined a set of inhibitors of plasmalemmal sealing that would be expected to enhance PEG-fusion success (Crush+ ηPSF + θPSF +PKI+PEG; $n=4$). As for Figure 2A, protocols in which crush-severance were not followed by application of PEG did not restore CAP through-conduction (Crush; $n=4$ and Crush+ ηPSF + θPSF +PKI; $n=4$). Both protocols labeled with “#” in the key to Figure 2B.

Figure 2C shows CAP conduction through a cut-severance made in Ca^{2+} -free or Ca^{2+} -containing salines *in vivo* **without** careful trimming of cut axonal ends. CAPs were always observed in shams that received no cut-severance (Sham; $n=2$). CAP conduction through the lesion site was not restored if PEG-fusion was not attempted (Cut in Ca^{2+} -free, ms, MB; $n=4$, labeled with “#” in the key). After cut-severance in Ca^{2+} -free saline, CAP conduction was restored in nerves that were micro-sutured and then PEG-fused (Cut in Ca^{2+} free, ms, PEG-fusion; $n=8$). [Note that the standard PEG-fusion protocol exposes the nerve to a set of Ca^{2+} -free, hypotonic salines when micro-sutured. See Methods.] If the nerve was cut-severed and then immediately repaired with micro-sutures in Ca^{2+} -containing saline and

treated with MB and PEG, CAP conduction was not restored (Cut in Ca^{2+} -containing, immediately ms, MB, PEG in Ca^{2+} -containing; $n=6$). In contrast, if the nerve was cut-severed and maintained in Ca^{2+} -containing saline for 30 min, but then repaired by rinsing the ends in hypotonic Ca^{2+} -free saline before micro-suturing, then PEG-fused, CAP conduction was restored (Cut in Ca^{2+} -containing, maintain 30 min, ms in Ca^{2+} -free, PEG-fusion; $n=4$). If an initial cut- or crush-severance was made and maintained for 30 min in Ca^{2+} -containing saline the ends could be re-cut (if initially cut) or the crush site excised (if initially crushed) in Ca^{2+} -free saline, micro-sutured and then PEG-fused to restore CAP conduction (Cut in Ca^{2+} -containing, maintain 30 min, re-cut ends in Ca^{2+} -free, ms, PEG-fusion; $n=4$ and Crush in Ca^{2+} -containing, maintain 30 min, excise crush ends in Ca^{2+} -free, ms, PEG-fusion; $n=3$).

Restoration of behavioral function after crush-severance

The SFI test was used to assess behavioral recovery after complete crush-severance of the entire left sciatic nerve, as confirmed by visual observation and inability to record CAPs across the lesion site (see Methods). Baseline scores were obtained for each animal before any operative procedures, and animals were tested post-operatively at 72 hr and then weekly thereafter for up to six weeks. SFI baseline scores for individual animals typically ranged from -10 to $+10$, averaging 1.1 for 75 rats, and did not differ significantly ($p>0.05$, Student's t -test) between any treatment or control group. Figure 3 shows $\text{SFI} \pm \text{SE}$ scores for 6 wks post-crush-severance for negative control animals that received MB, but were not PEG-fused (No PEG; black dotted line) or PEG-fused animals (PEG: blue solid line).

Figure 3A shows no significant ($p>0.05$, Student's t -test) difference in SFI recovery between negative control ($n=5$) and PEG-fused ($n=4$) groups of rats at any post-operative time for nerves crush-severed in Ca^{2+} -free saline. Similarly, Figure 3B shows no significant difference between negative control ($n=6$) and PEG-fused ($n=5$) groups at any post-operative time for sciatic nerves crush-severed in Ca^{2+} -containing saline. For 1–2 mm long crushes in Ca^{2+} -free or Ca^{2+} -containing saline, all SFI scores recovered to near baseline levels at 6 wks with no significant difference in recovery between PEG-fused and negative control groups.

Figure 3C shows post-operative SFI scores for animals with crush-severance made in Ca^{2+} -containing saline, then excising the lesion site and re-apposing untrimmed cut ends using micro-sutures in Ca^{2+} -free hypotonic saline 30 min after crush-severance for negative control animals ($n=3$) and for animals whose sciatic nerves were PEG-fused ($n=7$). Rats having such PEG-fused nerves following excision showed significantly greater SFI recovery at 4 wks ($t(8)=2.32$, $p<0.05$) and 6 wks ($t(8)=2.31$, $p<0.05$) post-severance compared with the negative controls. However, as assessed by SFI scores, behavioral recovery after excising 1 mm crush injuries (with or without PEG-fusion in Fig. 3C) was less successful than recovery for nerves with a 1 mm crush injury that was not excised (Fig. 3A,B).

Behavioral function before and after cut-severance

Panels A and B of Figures 4 and 5 show plots of mean $\text{SFI} \pm \text{SE}$ scores for rats whose left sciatic nerve was cut, exposed to MB and micro-sutured (negative control group labeled “No

PEG-Fusion”: black dotted line) versus plots of SFI scores for rats with PEG-fused sciatic nerves (PEG: colored solid lines). Hence, we are comparing PEG-fusion repair with the best, most widely used method of clinical repair (Isaacs, 2010). The specific PEG-fusion cut-severance protocols shown in Figure 4 are as follows: (1) Panels A,C: all sciatic PNAs cut in *Ca²⁺-free saline without* careful trimming of cut ends, and (2) Panels B,D: all sciatic PNAs cut in *Ca²⁺-free saline with* careful trimming of cut ends. The specific PEG-fusion cut-severance protocols shown in Figure 5 are as follows: (1) Panels A,C: all sciatic PNAs cut in *Ca²⁺-containing saline without* careful trimming of cut ends, and (2) Panels B,D: all sciatic PNAs cut in *Ca²⁺-containing saline with* careful trimming of cut ends.

The three ways of plotting the animals in the PEG-fusion group are (1) SFI data from all rats in that group (All PEG-Fusion: solid blue lines, solid blue squares); (2) SFI data from those rats that showed significant ($p < 0.003$, see following paragraph for details) behavioral recovery having SFI scores better than -59 by 6 wks post-operatively (PEG-Fusion -59 Recovery: green solid lines, green triangles); and, (3) SFI data from those rats that did not show significant behavioral recovery to an SFI score of -59 by 6 wks post-operatively (PEG-Fusion No -59 Recovery: red solid lines, red x). Note that in Figure 4B, only the PEG-fusion curve is shown since all animals in this treatment group recovered to an SFI score of -59 or better by 6 wks. Panels C and D in Figures 4 and 5 show individual SFI scores of all PEG-fused animals whose mean $SFI \pm SE$ scores (blue solid lines) are plotted in panels A and B, respectively. Colored lines and symbols in panels C,D show each animal in that group with significant SFI recovery to -59 SFI or better within 6 wks post-operatively and black lines plot those PEG-fused nerves from rats showing no SFI recovery to -59.

Our rationale for subdividing the group of “All PEG-Fused animals” into two groups of “PEG-Fusion -59 Recovery” versus “PEG-Fusion No -59 Recovery” was as follows. The mean $SFI \pm SD$ score of 21 negative control rats was -89 ± 10 at 6 wks post-operatively and the probability of any rat having a score of -59 was $p < 0.003$. In fact, no negative control rat in this study attained an SFI score better than -66 at 6 wks post-operatively. In contrast, some rats with micro-sutured PEG-fused sciatic nerves did obtain an SFI score of -59 or better within 6 wks post-operatively in this and in a previous study (Bittner et al., 2012). Similar observations were made at 6 wks for rats having PEG-fused allografts (Riley et al., 2015), i.e., for each rat, PEG-fusion can fail or succeed in repairing a cut-severed sciatic nerve to an SFI score of -59 or better. Note that an SFI score of -59 is a very conservative measure of behavioral recovery. If we had chosen to use a significance level of $p < 0.05$, rats attaining an SFI score of -67 would be considered significant recovery, and 1 out of 21 negative control animals attained that score at 6 wks. **None** of the 21 tested negative control rats in this study reached an SFI score of -59 at 6 wks post-operatively or at any other tested time point.

Behavioral function before and after cut-severance in Ca^{2+} -free saline

Figure 4A shows that after sciatic nerve cut-severance in Ca^{2+} -free saline without trimmed ends, the mean $\pm SE$ SFI scores of all PEG-fused animals (All PEG-Fusion; $n=8$) were not significantly different from that of the negative control animals (No PEG-Fusion; $n=4$). Figure 4A also shows that “PEG-Fusion -59 Recovery” animals ($n=3/8$ total PEG animals)

showed significant recovery at 5 wks ($t(5)=3.39$, $p<0.05$) and at 6 wks ($t(5)=6.12$, $p<0.01$), compared to the SFIs of the negative control group. Figure 4C shows the individual SFI scores of all eight animals in the PEG-fused group of which three rats (colored lines) recovered to SFI scores of -59 or better by 6 wks. There were no significant differences ($p>0.05$) between any of the three PEG-fusion curves (All PEG-Fusion, PEG-Fusion -59 Recovery, PEG-Fusion No -59 Recovery) and the negative control curve according to an ANOVA.

Figure 4B shows that after cut-severance in Ca^{2+} -free saline with trimmed ends, the mean $\pm$ SE SFI scores of all PEG-fused animals ($n=5$) were significantly ($F[1,12]=12.95$, $p<0.01$) different from the negative control animals ($n=5$). Figure 4B also shows that “All PEG-Fusion” animals showed significant recovery at 2 wks ($t(8)=2.40$, $p<0.05$), 3 wks ($t(8)=4.09$, $p<0.01$), 4 wks ($t(8)=3.64$, $p<0.01$), 5 wks ($t(8)=5.30$, $p<0.01$), and 6 wks ($t(8)=8.35$, $p<0.01$) post cut-severance, compared to the SFIs of the negative control group. Figure 4D shows the individual SFI scores of all five PEG-fused animals of which all five (colored lines) recovered to SFI scores of -59 or better by 6 wks. There was a significant difference ($F[1,12]=12.95$, $p<0.01$) between the “All PEG-fusion” curve and the negative control curve according to an ANOVA (indicated by “++” to the right of each curve).

Behavioral function before and after cut-severance in calcium-containing saline

Figure 5A shows that after cut-severance in Ca^{2+} -containing saline *without* trimmed ends, the mean $\pm$ SE SFI scores of all animals in the PEG-fused group ($n=9$) were *not* significantly different from that of the negative control animals ($n=4$). Figure 5A also shows that “PEG-Fusion -59 Recovery” animals (2/9) showed significant recovery at 4 wks ($t(4)=3.02$, $p<0.05$), 5 wks ($t(4)=2.94$, $p<0.05$), and 6 wks ($t(4)=4.75$, $p<0.01$.) compared to the SFIs of the negative control group. Figure 5C shows the individual SFI scores of all nine animals in the PEG-fused group of which 2 (colored lines) recovered to SFI scores of -59 or better by 6 wks. There were no significant differences between any of the three PEG-fusion curves and the negative control curve according to an ANOVA.

Figure 5B shows that after cut-severance in Ca^{2+} -containing saline *with* trimmed ends, the mean $\pm$ SE SFI scores of all PEG-fused animals ($n=5$) were significantly different from that of the negative control animals ($n=5$) at 72 hr ($t(8)=2.63$, $p<0.05$), 3 wks ($t(8)=2.31$, $p<0.05$), 5 wks ($t(8)=2.58$, $p<0.05$) and at 2 wks ($t(8)=3.78$, $p<0.01$) and 6 wks ($t(8)=3.36$, $p<0.01$) post-severance. Figure 5B also shows that “PEG-Fusion -59 Recovery” animals (3/5) showed significant recovery at 1 wk ($t(6)=3.05$, $p<0.05$), 2 wks ($t(6)=3.17$, $p<0.05$), 5 wks ($t(6)=2.67$, $p<0.05$) and 6 wks ($t(6)=6.89$, $p<0.01$) compared to the SFIs of the negative control group. Figure 5D shows the individual SFI scores of all five PEG-fused animals of which three (colored lines) recovered to SFI scores of -59 or better by 6 wks. There were significant differences between the curves for all three PEG-fusion groups (All PEG-Fusion ($F[1,12]=32.18$, $p<0.01$), PEG-Fusion -59 Recovery ($F[1,12]=19.58$, $p<0.01$), PEG-Fusion No -59 Recovery ($F[1,12]=38.81$, $p<0.01$)) and the negative control curve according to an ANOVA.

Taken together, the results of Figures 4 and 5 for cut-severed sciatic nerves show that (1) surgical technique e.g., trimming cut ends after an axotomy significantly improves PEG-

fusion success, and (2) the Ca^{2+} environment during cut-severance can greatly affect the rate of PEG-fusion success, e.g., severing in a Ca^{2+} -free environment produces significantly better results than severing in a Ca^{2+} -containing environment.

Axonal, NMJ and muscle morphology following cut-severance

The images in Figure 6 show representative micrographs of (A) an unoperated intact sciatic nerve, (B) a successfully PEG-fused cut-severed nerve at 6 wks post-operatively and (C) a negative control cut severed sciatic nerve at 6 wks post-operatively. Plots of G ratio (axon diameter/axon+myelin diameter) versus axonal diameter for each sciatic nerve are presented directly below the micrograph of that nerve. Each red point denotes axons located inside the sciatic epineural sheath, while a black point denotes an axon located outside the epineural sheath. No axons are found outside the sheath in the unoperated sciatic nerve (Fig. 6A); axons inside the sheath had diameters of $4.63 \pm 1.71 \mu\text{m}$ (219 axons). No axons are found outside the epineural sheath in the sciatic nerve from a rat with a successfully PEG-fused sciatic nerve (Fig. 6B) as defined by its SFI score of -7 ; sciatic axons inside the epineurium distal to the lesion site had a mean diameter of $3.26 \pm 1.23 \mu\text{m}$ (238 axons) at 6 wks after being cut in Ca^{2+} -free saline, micro-sutured, and PEG-fused (Fig. 6B). Axons are found outside the epineural sheath in the sciatic nerve from a rat with a negative control sciatic nerve (Fig. 6C) that had an SFI score of -74 ; The small diameter axons outside the sheath may be axons regenerating by outgrowth that have not reached an appropriate target. At 6 wks post-operatively, negative control axons (cut and micro-sutured: Fig. 6C) had a mean diameter of $1.73 \pm 0.76 \mu\text{m}$ (288 axons). An ANOVA of axonal diameter data followed by post-hoc analysis showed that each group of axonal diameters differed significantly from each other group ($F[2,760]=352.26$, $p<0.01$). That is, mean axonal diameters in unoperated (intact) sciatic nerves in the mid-thigh of rats are significantly greater than axonal diameters in rats with PEG-fused cut sciatic nerves distal to the lesion site at 6 wks post-operatively that are, in turn, significantly greater than axonal diameters in unoperated controls.

Figure 7 shows light micrographs of cross-sectioned soleus muscles taken 5–6 cm distal to the lesion site of (A) successfully PEG-fused or (B) negative control sciatic nerves. Rather normal NMJs with prominent secondary folds at the synaptic cleft (black arrow) and distinct nerve terminals well-capped by synaptic glia (asterisk) were noted in muscles from three rats with successfully PEG-fused sciatic nerves. The image shown in Figure 7A was from a rat with a successfully PEG-fused sciatic nerve as evidenced by an SFI score of -7 at 6 wks postoperatively. In contrast, NMJs in three negative control rats had soleus muscle fibers with rather normal accumulations of myonuclei and mitochondria characteristic of synaptic regions, but did not have secondary folds or nerve terminals beneath Schwann cells (white arrow). The image shown in Figure 7B was from a rat with a negative control sciatic nerve (cut, micro-sutured, but not PEG-fused) with an SFI score of -74 at 6 wks post-operatively.

DISCUSSION

We report that PEG-fusion rapidly (within minutes) restores axonal continuity in crush- or cut-severed nerves whose open, closely apposed ends are PEG-fused. PEG-fusion also significantly enhances the extent of recovery for behavioral functions at 6 wks post-

operative (sometimes sooner). PEG-fusion retards or prevents Wallerian degeneration as assessed by axonal diameter and maintenance of NMJs. We have examined singly and in combination the effect of the following variables on PEG fusion success: crush or cut lesions made in Ca^{2+} -free or Ca^{2+} -containing salines with or without trimming of cut axonal ends with or without the addition of various inhibitors of plasmalemmal sealing. Our data show that exposure of cut-severed axonal ends to Ca^{2+} -containing saline prior to PEG-fusion and suggest that stretch/tension of proximal or distal axonal halves of cut-severed axons decrease PEG-fusion success. Conversely, trimming cut-severed ends increases PEG-fusion success.

Repair of crush- or cut-severed sciatic PNAs in Ca^{2+} -free salines

Our data presented herein confirm that fluorescent dye placed extracellularly (Fig. 1) is taken up by axonal ends opened by hypotonic Ca^{2+} -free saline, and diffuses intra-axonally throughout the length of intact sciatic PNAs or crush- or cut-severed PNAs if and only if the severed axons have been successfully PEG-fused (Fig. 1D; Lore et al., 1999; Britt et al., 2010; Bittner et al., 2012; Spaeth et al., 2012b; Riley et al., 2015). Fluorescent dye is not taken up at cut-severed axonal ends if PEG is applied to the cut-severed ends in the presence of hypotonic Ca^{2+} -free salines (Fig. 1H). Such data (Spaeth et al., 2012b) strongly suggest that if axonal ends are not closely apposed, the application of PEG rapidly seals their cut ends (PEG-sealing) rather than fusing (joining) portions of their axolemmas (PEG-fusion).

CAP through-conduction data shown in Figure 2A,C also confirm (Lore et al., 1999; Britt et al., 2010; Bittner et al., 2012) that the axolemma of axons crush- or cut-severed in hypotonic Ca^{2+} -free salines can be repaired within minutes using PEG or PEG+MB, but not if PEG is omitted (# in Fig. 2). We now report that if axons are crushed (Fig. 2A) or cut (Fig. 2C) in hypotonic Ca^{2+} -free salines containing one or more of various types of inhibitors of axolemmal sealing (Zuzek et al., 2012: MB, PKI, etc.), then our standard PEG-fusion protocol immediately restores CAP conduction through the lesion site.

Similarly, we confirm that that 1–2 mm long crushes of sciatic nerves made in hypotonic Ca^{2+} -free salines in the mid-thigh recover to near-normal SFI scores of ± 20 with or without PEG-fusion. PEG-fusion did not significantly increase the rate of behavioral recovery at 1–3 wks, as previously reported (Britt et al., 2010; Bittner et al., 2012). However, the number of experimental and control animals in each group in the present study ($n=3-8$) was much smaller than in these previous studies ($n=20$), and therefore a small but significant effect might not be detected in the present study.

After cut-severance in Ca^{2+} -free hypotonic salines, PEG-fusion significantly increased behavioral recovery at 5–6 wks postoperatively for those PEG-fused animals that recovered to an SFI score of better than -59 (3/8 animals) compared to negative control animals, if cut axonal ends were not trimmed (Fig. 4A). If axonal ends cut in in Ca^{2+} -free hypotonic salines were trimmed, all PEG-fused animals showed behavioral recovery to at least -59 SFI, and recovered significantly better from 2 wks ($p<0.05$) through 6 wks ($p<0.01$) post-operatively compared to negative control animals. That is, trimming cut ends enhances PEG-fusion success. [Cut ends were usually carefully trimmed in a previous study of sciatic nerves cut-severed in hypotonic Ca^{2+} -free saline (Bittner et al., 2012)]. Without PEG-fusion, but with

micro-suture of cut ends, negative control animals showed very little behavioral recovery, having mean SFI scores of -80 (Fig. 4A) or -90 (Fig. 4B).

PEG-fusion of crush- or cut-severed sciatic PNAs in Ca^{2+} -containing salines

Traumatic injuries to PNAs in sciatic or other nerves in mammals rarely occur in situations in which the severed axons are bathed and maintained in hypotonic Ca^{2+} -free saline, but rather occur in Ca^{2+} -containing isotonic salines, e.g., extracellular fluids. Our data presented herein confirm that sciatic PNAs that are crush- or cut-severed and then maintained in Ca^{2+} -containing isotonic salines exhibit through conduction of CAPs if they are PEG-fused as described in Methods (Fig. 2C; Bittner et al., 2012; Rodriguez-Feo et al., 2012).

In contrast, if exposed to PEG without exposure to hypotonic Ca^{2+} -free salines to open cut ends during PEG-fusion, crush-severed sciatic PNAs did not exhibit immediate restoration of CAP conduction through the lesion site (Fig. 2C, “##”). Such PEG-repair that does not include all steps of the PEG-fusion procedure (see Methods) for axons crush-severed in Ca^{2+} -containing isotonic salines would be expected to be problematic because: (1) Ca^{2+} -containing isotonic salines induce the formation of vesicles that interact to form a plug to seal plasmalemmal damage consisting of small holes or complete transections (Krause et al., 1994; Bittner and Fishman, 2000; Spaeth et al., 2010; 2012a,b,c); (2) Vesicles fill the axoplasm of a 1–2 mm long crush-damaged region of axolemma in invertebrate giant axons (unpublished data) (3) The presence of vesicles at the time of PEG application decreases PEG-fusion success and hence Ca^{2+} -free hypotonic salines are used in combination with other substances to open cut ends, expel vesicles and inhibit their formation and interactions (Krause and Bittner, 1990; Lore et al., 1999; Britt et al., 2010; Bittner et al., 2012).

Our data (Fig. 3B) on behavioral recovery after crush-or cut-severance of sciatic nerve PNAs in Ca^{2+} -containing isotonic salines are consistent with previous reports (Hare et al., 1992; Wood et al., 2011) that 1–2 mm long crushes of sciatic nerves in the mid-thigh recover to near-normal SFI scores of ± 20 without PEG-fusion. For nerves cut-severed in Ca^{2+} -containing isotonic salines, negative control animals do not recover to mean SFI scores of better than -90 , even with micro-sutures with or without trimming of cut ends. In contrast, rats with PEG-fused sciatic nerves cut in Ca^{2+} -containing isotonic salines recover significantly better SFI scores after 72 hr and persist for 6 wks if cut ends were carefully trimmed (Fig. 5B). Trimming cut ends significantly increases behavioral recovery as measured by mean SFI scores for all animals or by noting the relative number of animals that attain an SFI score of -59 or better by 6 wks postoperatively. We and others (Hare et al., 1992; Wood et al., 2011) find that without PEG-fusion, but with micro-suturing cut ends, negative control animals recover to SFI scores averaging about -90 by 6 wks post-operatively.

Variables affecting PEG-fusion repair after crush- or cut-severance or excision of PNAs

Behavioral recovery is equally good after 1–2 mm long crushes in Ca^{2+} -free or Ca^{2+} -containing saline, and PEG-fusion does little to improve recovery for such lesions that rarely occur naturally. Careful trimming of axonal ends of sciatic PNAs cut-severed in Ca^{2+} -free or Ca^{2+} -containing saline enhances their close apposition by micro-sutures, but increases the

separation between cut ends. Increased separation between cut ends results in stretching/ applying tension to nerves, a clinical factor often-noted to greatly decrease recovery of behavioral function after nerve severance (Birch et al., 1998; Allan, 2000; Campbell, 2008; Williams et al., 2008; Wolfe et al., 2010; Burch, 2011). Our previous data (Riley et al., 2014) showed that sciatic PNAs with 0.5–1 cm ablated segments could be repaired with no nerve stretch, sometimes with dramatically rapid and near-complete behavioral recovery, by inserting a sciatic nerve allograft from a donor rat and PEG-fusing all trimmed ends (Riley et al., 2014). For PEG-fused allografts that had their protruding axons trimmed during repair, mean SFI scores at 72 hr post-surgery were –70 to –50, and quickly recovered to –40 to –30 within 2 wks postoperatively (Fig. 3A; Riley et al., 2014). In contrast, single cut repairs with trimmed axons in the present study had SFI scores of –100 to –90 at 72 hr and did not recover to –40 to –30 until 6 wks post-operatively. Even though apposed cut axons need be PEG-fused at both the proximal and distal edge of the allograft, we suspect that the better behavioral recovery in PEG-fused allografts is likely due to little or no nerve stretching. More stretch on the nerve during micro-suturing to close a 1–3 mm gap almost-certainly results in more nerve ischemia or tearing of tissues held together by sutures (suture failure: Clark et al., 1992).

Retardation of Wallerian degeneration by successful PEG-fusion

Compared to negative controls, successful PEG-fusion retards Wallerian degeneration distal to a cut-severance (Rodriguez-Feo et al., 2013) or an ablated segment replaced by an autograft (Sexton et al., 2012) or allograft (Riley et al., 2015) as measured by counts of total axonal number and/or number of surviving sensory or motor axons. Our measures (Fig. 6) of axonal diameters and G ratios (a measure of myelination) show that successful PEG fusion retards or prevents decrease in axon diameter at 6 wks post-operatively. However, successful PEG-fusion does not prevent decreases in thickness of the myelin sheath. Our observations of intact NMJs on soleus muscle fibers distal to the cut are also consistent with retardation of Wallerian degeneration of sciatic motor axons following successful PEG-fusion.

Conclusions

PEG-fusion repair of opened ends of severed axons retards Wallerian degeneration and improves behavioral recovery (Bittner et al., 2012; Spaeth et al., 2012b). However, we do not know how PEG-fusion produces greatly enhanced behavioral recovery at the systems level. We recognize that PEG-fusion is not selectively reconnecting distal and proximal portions of severed motor or sensory axons, much less individually identifiable axons (Bittner et al., 2012; Spaeth et al., 2012b). [These are also unanswered questions when considering regeneration by outgrowth from severed proximal ends.] Whatever the molecular, cellular, and systems-level mechanisms, PEG-fusion procedures might produce a paradigm shift in the clinical treatment of traumatic injuries to peripheral nerves, perhaps rapidly because the technique employs a unique combination of currently used devices (micro-sutures) and chemicals (PEG, MB, hypotonic and isotonic salines).

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Significance Statement

Successful PEG-fusion of sciatic nerves completely severed at mid-thigh can restore axonal continuity through the lesion site within minutes and lost behavioral functions within days to weeks. Exposure of cut-severed axonal ends to Ca^{2+} -containing saline prior to PEG-fusion and stretch/tension of proximal or distal axonal halves of cut-severed axons decrease PEG-fusion success. Conversely, trimming cut-severed ends in Ca^{2+} -free saline increases PEG-fusion success. PEG-fusion prevents or retards the Wallerian degeneration of cut-severed axons as assessed by measures of axon diameter and G ratio. PEG-fusion may produce a paradigm-shift in the treatment of peripheral nerve injuries.

Figure 1. Intra-axonal diffusion of Texas-red fluorescent dye across lesion site

(A–G) Texas-red fluorescent dye taken up at proximal open-axonal ends of excised sciatic nerves bathed in Ca²⁺-free hypotonic saline *ex vivo* (see Methods) diffuses intra-cellularly throughout entire sciatic nerve if (A) intact (uncut) or (B,C) up to the lesion site if all PNAs are (B) crush- or (C) cut-severed in Ca²⁺-free hypotonic saline *in vivo* or across the lesion site if crush- or (D) or cut-severed (E) PNAs are PEG-fused *in vivo*. Intra-axonal dye diffuses up to the lesion site if sciatic PNAs are crush-severed (F) or cut-severed (G) in Ca²⁺-containing saline and then PEG-fused in Ca²⁺-free hypotonic saline *in vivo*. (H) If PEG is applied to the proximal cut end of the sciatic nerve in Ca²⁺-free hypotonic saline *ex vivo*, dye is not taken up by proximal axonal ends. All sciatic nerves oriented so that the proximal end that was bathed in Texas red is at the top of each panel. Solid white arrows indicate the severance site (B–G); dashed white arrow (H) indicates the proximal end of the excised sciatic nerve.

Figure 2. Electrophysiological assessment of CAP conduction through the lesion site
CAP conduction through lesion site before severance (initial), immediately after severance (severance), and following any attempted repair (post-repair). Severance consisted of 1mm crush injury of the entire sciatic nerve in (A) Ca^{2+} free or (B) Ca^{2+} containing saline, or after cut-severance (without trimming ends) in (C) Ca^{2+} -free or Ca^{2+} -containing saline. The key to each protocol is given to the right of each graph (see Results: “Restoration of CAP through-conduction in crush- and cut-severed nerves” for complete explanation of each protocol and definition of each abbreviation) where n= number of sciatic nerves tested and ms=micro-suture in this and other figures. For (A) crush- and (C) cut-severance protocols “#” labels any conditions in which CAPs do not conduct through the lesion site without PEG-fusion; “##” labels any conditions in which CAPs do not conduct through the lesion site with PEG-fusion.

Figure 3. Mean SFI±SE after crush-severance

(A–C) Mean±SE SFI scores 3d–6wks post-severance for PEG-fusion groups (PEG: blue, solid lines) and negative control groups (No PEG: black, dotted lines). SFI scores for 1mm crush injuries in (A) Ca^{2+} -free, (B) Ca^{2+} -containing saline, or (C) crush-severed in Ca^{2+} -containing with the crush-severance site excised and re-apposed with micro-sutures (ms) in Ca^{2+} -free saline without trimming of cut ends. (A–C) Data points from PEG-fusion curves that significantly differ from no-PEG negative control curves are indicated by “**” ($p < 0.05$, Student’s t-test) above each pair of data points.

Figure 4. SFI scores after cut-severance in calcium-free solutions with or without trimming axonal ends
(A,B) Mean±SE SFI scores or **(C,D)** individual SFI scores 3d-6wks after cut-severance in Ca^{2+} -free saline. **(A–B)** All PEG-fused animals (All PEG-Fusion: blue, solid lines), **(A)** PEG-fused rats with significant behavioral recovery to a -59 SFI score (PEG-Fusion -59 Recovery: green, solid lines), **(A)** PEG-fused rats without behavioral recovery to a -59 SFI score (PEG-Fusion No -59 Recovery: red, solid lines) and **(A,B)** all rats in the negative control groups (No PEG-Fusion: black, dotted lines) for cut-severance in Ca^{2+} -free saline **(A)** without trimmed ends, or **(B)** with trimmed ends. **(C,D)** Individual animals that showed behavioral recovery after PEG-fusion (colored, solid lines) and animals that showed no recovery after PEG-fusion (black, solid lines). **(A,B)** Data points from PEG-fusion curves that are significantly different from negative control curves are indicated by “*” and “**” ($p < 0.05$ and $p < 0.01$ respectively using Student’s t-test) above each pair of data points. Curves for PEG-fusion groups that differ significantly from the negative control curve according to ANOVA ($p < 0.01$) are indicated by “++” symbol to the right of each curve.

Figure 5. SFI scores after cut-severance in calcium-containing solutions with or without trimming axonal ends
(A,B) Mean±SE SFI scores or **(C,D)** individual SFI scores 3d-6wks after cut-severance in Ca^{2+} -containing saline. **(A–B)** All PEG-fused animals (All PEG-Fusion: blue, solid lines), PEG-fused rats with significant behavioral recovery to a -59 SFI score (PEG-Fusion -59 Recovery: green, solid lines), PEG-fused rats without behavioral recovery to a -59 SFI score (PEG-Fusion No -59 Recovery: red, solid lines) and all rats in the negative control groups (No PEG-Fusion: black, dotted lines) for cut-severance in Ca^{2+} -containing saline **(A)** without trimmed ends, or **(B)** with trimmed ends. **(C,D)** SFI scores for individual animals that showed behavioral recovery after PEG-fusion (colored, solid lines) and animals that showed no recovery after PEG-fusion (black, solid lines). **(A,B)** Data points from PEG-fusion curves that are significantly different from negative control curves are indicated by “*” and “**” ($p<0.05$ and $p<0.01$ respectively, using Student’s t-test) above each pair of data points. Curves for PEG-fusion groups that differ significantly from the negative control curve according to ANOVA ($p<0.01$) are indicated by “++” symbol to the right of each curve.

Figure 6. Axonal Morphology

Cross-section EM images with representative scatterplots of G-ratio versus axon diameter for (A) un-operated, (B) single cut rat sciatic nerves micro-sutured with PEG-fusion repair and (C) micro-sutured without PEG-fusion repair 6 wks postoperatively. Red data points represent axons measured inside the sciatic epineural sheath; black data points represent axons measured outside the sciatic epineural sheath.

Figure 7. NMJ Morphology

Light micrographs of cross-sectioned soleus muscle fibers taken distal to cut rat sciatic nerves (**A**) successfully micro-sutured and successfully PEG-fused (SFI score = -7 after 6 wks) or (**B**) micro-sutured and not PEG-fused (negative control, SFI score = -74 after 6 wks). Asterisks: synaptic glia. Arrows: synaptic cleft.