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Anatomical repair of nerve membranes in crushed mammalian spinal cord with polyethylene glycol

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Summary

Acute damage to axons is manifested as a breach in their membranes, ion exchange across the compromised region, local depolarization, and sometimes conduction block. This condition can worsen leading to axotomy. Using a novel recording chamber, we demonstrate immediate arrest of this process by application of polyethylene glycol (PEG) to a severe compression of guinea pig spinal cord. Variable magnitudes of compound actions potentials (CAPs) were rapidly restored in 100% of the PEG-treated spinal cords. Using a dye exclusion test, in which horseradish peroxidase is imbibed by damaged axons, we have shown that the physiological recovery produced by polyethylene glycol was associated with sealing of compromised axolemmas. Injured axons readily imbibe horseradish peroxidase—but not following sealing of their membranes. The density of nerve fibers taking up the marker is significantly reduced following polyethylene glycol treatment compared to a control group. We further show that all axons—independent of their caliber—are equally susceptible to the compression injury and equally susceptible to polyethylene glycol mediated repair. Thus, polyethylene glycol—induced reversal of permeabilization by rapid membrane sealing is likely the basis for physiological recovery in crushed spinal cords. We discuss the clinical importance of these findings.

Introduction

Injury to the spinal cord is associated with a partial to complete loss of volitional movement, some involuntary function, and sensation because nerve impulses are not conducted across the region of damage to white matter (Dimitrijevic, 1995; Fehlings & Tator, 1995). In clinical injuries, spinal axons are not usually transected—rather they are compressed, producing membrane "lesions" that progressively enlarge. Thus, the pathology produced by acute mechanical damage to nervous tissue worsens with time, sometimes producing axotomy and Wallerian degeneration of the distal segments of nerve fibers (Griffin *et al*., 1995; Honmou & Young, 1995; Maxwell, 1996). The earliest manifestation of such nerve damage at the cellular level is permeabilization of the membrane where ions readily run down their concentration gradients and exchange across the compromised barrier (Dimitrijevic, 1995, Maxwell, 1996). In part, the influx of Na^+ and Ca^{2+}, and efflux of K^+ not only depolarizes local regions of membrane producing conduction block, but also destabilizes the axonal cytoarchetecture leading to a delayed, progressive, dissolution of individual axons—and thus the white matter tissue as a whole (Griffin *et al*., 1995; Maxwell, 1996). It would be important to devise a means to repair damaged nerve membranes, inhibiting further permeabilization and reversing the progressive dissolution of nerve fibers.

Polyethylene glycol (PEG) is a membrane fusogen, for over twenty years known to be able to induce the fusion of cell membranes (Davidson, 1976; Ahkong *et al*., 1987; Lentz *et al*., 1996). Recently we have shown that application of this hydrophilic polymer to completely transected—and reapposed—adult guinea pig spinal cords can lead to anatomical fusion and an immediate recovery of compound action potential (CAP) propagation through the former transection plane within minutes of the treatment (Shi *et al*., 1999). The remarkable morphological reconnection of severed individual axons was demonstrated by the diffusion of intracellular fluorescent dyes through the plane of transection following chemically induced fusion (Shi *et al*., 1999). We have also shown that PEG induced a rapid recovery of CAP conduction in severely compressed spinal cords in vitro (Shi & Borgens, 1999). Physiological recovery of CAP conduction, as well as a recovery of function of the cutaneous trunchi muscle reflex (a behavioral index

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of white matter integrity; Blight *et al*., 1990) also occurs in response to a local application of PEG to severe compression injured guinea pig spinal cords *in vivo* (Shi & Borgens, 1999). We have hypothesized that the physiological repair of crushed axons by this hydrophilic polymer is mediated by an anatomical repair where immediate PEG induced sealing—and a reversal of membrane permeabilization—of the axolemma allowed the rapid recovery of action potential propagation (Shi & Borgens, 1999). Using a novel dye exclusion technique to measure anatomical sealing of damaged nerve membranes (Asano *et al*., 1995) together with a new method of computer managed morphometry (Moriarty *et al*., 1998), here we show that this hypothesis is correct.

Methods

ANIMALS, THE RECORDING CHAMBER, AND STANDARDIZED INJURY

Thirty-six adult ($\sim$300 g) guinea pigs were used in this study. The experimental protocols have been reviewed and approved by the Purdue University Animal Care and Use Committee. All efforts were made to minimize the number and suffering of animals used, and to utilize alternatives to *in vivo* techniques. Twenty-four spinal cords were removed from deeply anesthetized animals by a previously described technique (Shi & Blight, 1996, 1997; Shi & Borgens, 1999; Shi *et al*., 1999), and placed in a three compartment, double sucrose gap recording chamber. A full description of this chamber, including diagrams and details of its construction and use, has also been reported (Shi & Borgens, 1999; Shi *et al*., 1999). Briefly: a $\sim$35 mm strip of guinea pig spinal cord ventral white matter was placed in the chamber crossing all of its three large interconnected compartments. The ends of the spinal cord were immersed in isotonic KCl (120 mM), while the central region of the cord was immersed in Krebs' solution (NaCl, 124 mM; KCl, 2 mM; KH_2PO_4, 1.24 mM; $MgSO_4$, 1.3 mM; $CaCl_2$, 26 mM; sodium ascorbate, 10 mM ; dextrose, 10 mM; $NaHCO_3$, 26 mM; equilibrated with 95% O_2 - 5% CO_2). Thus the ends were maintained at approximately intracellular potential while the middle of the cord was maintained at approximately extracellular potential. Each of these three large compartments were separated by a small compartment of flowing sucrose (230 mM) helping to maintain electrical isolation of the ends of the cord and to reduce mixing of the media. Compound action potentials (CAPs) were evoked at one end by bipolar electrodes and recorded at the other end of the strip of spinal cord during each experiment. Such recordings were started after the tissue had equilibrated within the chamber, and during and after a standardized compression of the spinal cord within the middle compartment. This compression was produced by advancing a Plexiglas rod at approximately 25 μm/s using a motor controlled micromanipulator onto the cord eliminating all CAPs (described in detail by Shi & Blight, 1997 and Shi & Borgens, 1999). Note that in this study, the duration of compression was shortened from $\sim$15 seconds to $\sim$2 seconds to lessen the severity of the injury-increasing the ability to evaluate the anatomical sealing of damaged axons. Severe compression as in Shi & Borgens (1999) leads to rapid axotomy and Wallerian degeneration. The storage of real time digitized physiological data, management of this data, and the signal averaging of elicited CAP wave forms was accomplished using a custom designed Labview computer program on a Power Macintosh G-3 computer.

PEG APPLICATION

After $\sim$1 hour incubation time, physiological recordings of CAPs were obtained prior to and after lesioning of the spinal cord. A solution of PEG (1800 MW; 50% by weight in distilled water) was applied by pressure injection through a micropipette directly to one side of the lesion and removed simultaneously by aspiration on the other side using a second micropipette. This produced a stream of the PEG solution $\sim$0.5 mm wide washing across the cord lesion for $\sim$2 min. Immediately following the PEG application the oxygenated Krebs' solution in the central compartment was maintained in a continuous flow. The physiological properties of the treated spinal cord strip were monitored continuously during this procedure and for $\sim$1 more hour. This procedure is identical to a previously reported one, which includes further details (Shi & Borgens, 1999).

DYE EXCLUSION TEST

Approximately 15 minutes following compression of the spinal cord, crush-injured (PEG-treated) and crush injured (no PEG) control spinal cords were immersed in an aqueous solution of 0.015% HRP (type VI, Sigma) for 1 hour. Subsequently the tissues were fixed in 2.5% glutaraldehyde for 1 hour and stored in buffer, later to be cross-sectioned at 30 μm on a Vibratome. Cross sections of the spinal cord strips were then floated in buffer in laboratory fabricated wells (fashioned from polypropylene centrifuge tubes) (Fig. 1), and later developed by conventional methods using DAB (Borgens *et al*., 1986). Stained sections were dehydrated for 3 minutes for each step in ascending concentrations of acetone (50, 70, 90% $\times$ 1, to 100% $\times$ 3). Dehydrated sections were subsequently immersed in 100% xylene, removed to microscope slides using a fine bristle brush, and mounted with Permount. Using this technique, crushed axons readily take up the intracellular marker HRP, while sealed axons do not (Asano *et al*., 1995). In order to determine the upper limit of HRP uptake by injured axons, a second control procedure was utilized. In this, we transected spinal cords at their midpoint within the central Krebs' containing compartment in 12 additional spinal cord strips—and immediately (30 sec–1 min.) immersed the two segments in the HRP solution. These strips of white matter were then treated identically to crush-injured (PEG-treated) and crush-injured (no PEG) spinal cords. Viewing and computer acquisition of digitized images of the histology used an Olympus Van OX microscope, and final plates were printed using an Epson Stylus or Techtronics Phaser 400 Dye sublimation printer.

SAMPLING, COMPUTER-GRAPHICS, AND MORPHOMETRY

For morphometry, images were digitized and captured to a Macintosh Quadra 800 Computer using a Leitz Orthoplan

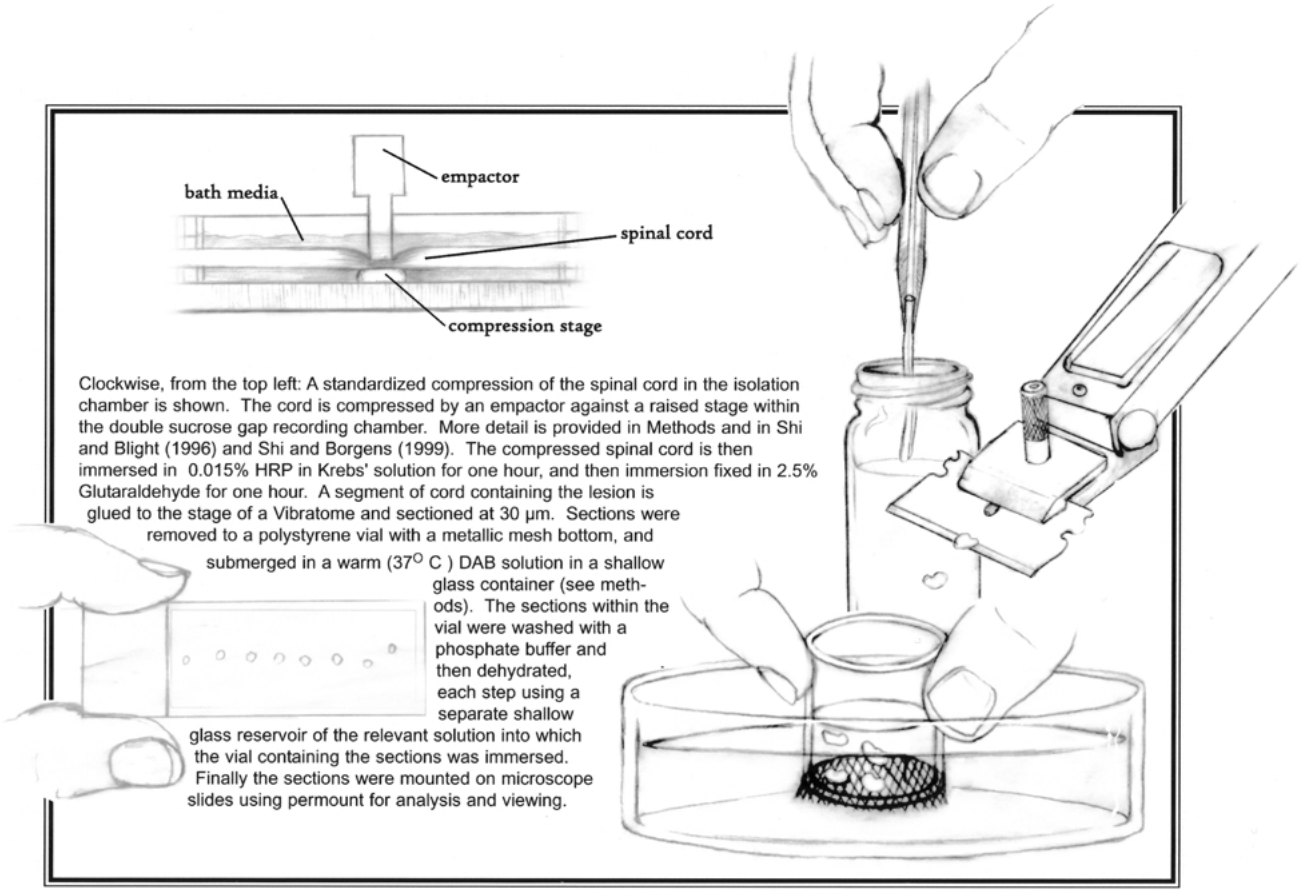

Fig. 1. Experimental Procedures.

microscope and JVC TK-1070U color video camera at 16× and 6.3× magnification using RasterOps Media Grabber 2.3 software. Two images at 16× (640 × 480 pixels each) were captured and pasted together to form one large image using Colorit 2.3 software. These images were chosen as a representative sample from peak regions of dye uptake in Transected, Control, and PEG-treated spinal cords. Prior to counting dye labeled axon profiles, the montages were manipulated to increase their contrast and the intensity of the brown HRP chromagen against background staining (Fig. 2). Each montage of white matter was then divided into three equal regions: a lateral (L), intermediate (I), and medial region (M) with respect to an axis extending from the central canal to the pial surface. The low power images captured at 6.3× magnification were useful in determining this precise orientation. Images were first color transformed, and binarized using IP Lab spectrum software as in Moriarty *et al*. (1998) (Fig. 2A–C). Subsequently, the diameter in pixels of the binarized axons was determined with NIH Image 1.6.1 software. This was accomplished by computing the minor elliptical cross sectional axis of filled axons and this pixel count converted to μm, and organized into categories based on a range of axons calibers (2.5–3, >3–4, >4–5, >5–6 μms). These data were analyzed using Excel 4.0 software. Counts of the individual axon diameters were normalized per unit area in each of the three subregions (I, L, and M) of the montage and expressed as a density (axon counts per mm^2). The management of all steps in the acquisition of these data was performed by a technician who was unaware of the experimental status of any spinal cord.

STATISTICS

Since axon profiles fit a normal distribution, the density of axons was compared between groups using unpaired, two tailed, Students *t* test. Linear regression analysis and the *t* tests were performed on Instat^{TM} software.

Results

A rapid but variable physiological recovery of CAPs was observed in all crushed spinal cords following the administration of PEG for 2 minutes directly to the standardized lesion within the double sucrose gap chamber (Fig. 3). This physiological recovery was defined by a statistically greater magnitude of CAP amplitude in the crush-injured and PEG treated group of 11 cords relative to the 13 crush-injured but untreated control spinal

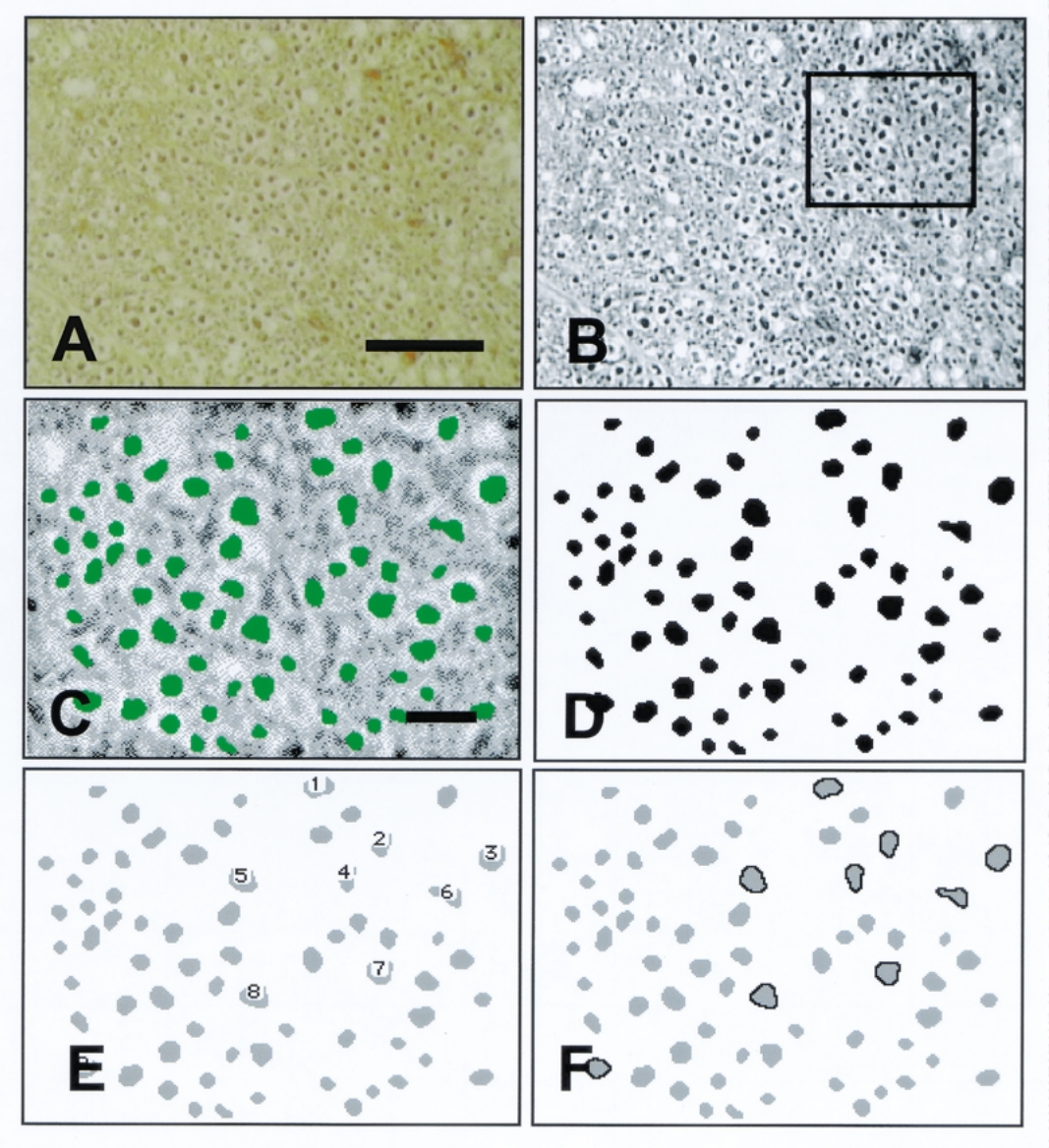

Fig. 2. The dye exclusion test: 2-D morphometry in a transected spinal cord. A) A cross sectional field of view of a spinal cord in which numerous transected axons have taken up the HRP label. This image is typical, unadulterated, and is as it appeared on the computer monitor during acquisition of the image. B) Identical field of view with image converted to grayscale with contrast enhancement to better visualize labeled axons. The boxed area is shown in C) at higher magnification. C) The range of pixel values delineating the HRP labeled axons has been converted to a single pixel value (green) in preparation for binarization and computer assisted counting. Note that this color transformation excludes all anatomical structures except the labeled axons to be measured and counted. D.) Same field as C) in which color transformed axons are now binarized in preparation for measurement of their diameters. E) and F) show stages of measurement in the counting of axons and measurements of their diameters. In E) axons of a single specific predetermined caliber of four separate categories (2.5–3.0; >3.0–4.0; >4.0–5.0; >5.0–6.0 μm diameter) are labeled by the computer (only one group shown in E), while F) reveals these same axons without the number label, their borders highlighted. Using these methods HRP labeled axons of different calibers could be individually counted, their diameters measured, and categorized (see Methods). The scale bar in A = 50 μm for A and B; in C = 10 μm for C–F.

cord strips. This finding corroborates earlier measurements using a similar injury technique, PEG application technique, and the same method of physiological recording (Shi & Borgens, 1999). Moreover these recordings were required to confirm that the group of PEG treated cords evaluated anatomically in this study responded to PEG in a manner similar to that previously reported.

The dye exclusion test (Asano *et al.*, 1995) is dependent on the uptake of HRP from the bathing media by

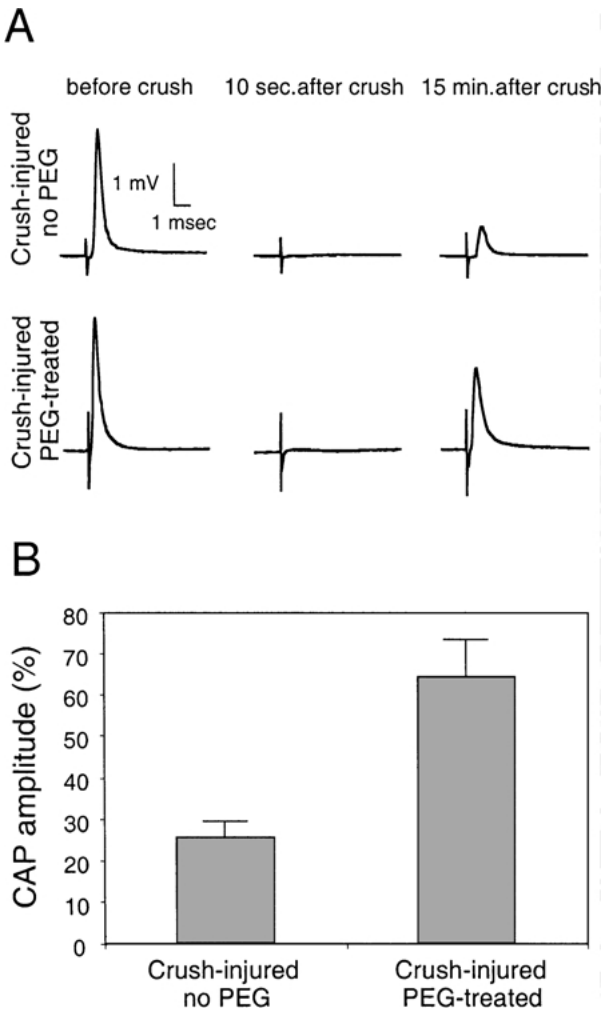

Fig. 3. Physiological recovery of crushed (no PEG) and crushed (PEG–treated) spinal cords. In A) a typical compound action potential (CAP) is shown in the upper electrical record. This CAP was elicited by stimulating the spinal cord at one end of the double sucrose gap chamber and recording it at the other end. 10 seconds following a crush to that cord, the CAP was completely eliminated, though by 15 minutes a small CAP had spontaneously recovered. In the lower electrical record, a typical CAP is shown as well as its complete elimination following the standardized compression, and the recovering CAP, 15 minutes after PEG treatment (see Methods). Note the increased amplitude of the recovering PEG-treated CAP. The Bar Graph compares the mean amplitude and standard error of the CAPs for 13 crushed (no PEG) and 11 PEG-treated spinal cords 15 min. after the crush. Note the statistically significantly higher CAP amplitudes following PEG treatment ($P = 0.0005$, Student's t two tailed t test).

injured axons, that is HRP gains access to the cytoplasm through a breach(or breaches) in the axolemma. In this study, such labeling was especially intense in the group of transected (and immediately labeled) spinal cords as well as crush-injured (no PEG) spinal cords. The intensity of labeling allowed easy measurement of axon number and axon diameter from cross-sectioned axon profiles by a custom designed computer script following image acquisition (Figs. 2 and 4). We note here, and discuss later, that this technique is carried out at relatively low magnification (16×), and thus does not discriminate axons whose diameters are less than 2.5 μm. Thus, axons with diameters smaller than this were not counted.

Prior to discussing the character of PEG treated and control labeling, we provide detail and rationale for the inclusion of data obtained from the group of severed spinal cords which were immediately placed in the HRP solution following transection. This comparator group helped determine the sensitivity of the dye exclusion technique by providing data on the maximum number of axons that could be discriminated, counted, and grouped by caliber spectra using these methods. Furthermore, this allows one to define "axon damage" following compression as a function of the maximal number of labeled axons possible following the more severe transection injury. One would expect that fewer HRP containing axons would result from a compression injury to white matter in which the crushed cord was immersed in label 15 min. following compression than immediately labeled transected axons. This hypothesis was not entirely evident in inspecting such individual cross sections (compare Fig. 4A and B) due to robust uptake of HRP in both situations—but became clearly evident when inspecting the counts of axon profiles in each of the three groups of spinal cords. The average count of labeled axons in the transected and immediately labeled group was statistically significant compared to the crushed (no PEG) control group (555 axons, $n = 12$, vs 199 axons, $n = 13$, respectively; $p \leq 0.001$, student t test, unpaired two tailed). When normalized for the unit area of the sample where counts were made, this result was identical. The density of axons labeled in transected cords was statistically greater (by more than a factor of two) than that counted in the crush-injured (no PEG) control group (Fig. 5; $P < 0.0001$, students t test, unpaired two tailed). Data obtained from the transection group was also organized by location within the spinal cord strip (Fig. 6). Each strip of white matter was divided into three zones: an intermediate zone, a lateral zone (adjacent the pial surface) and a medial zone (adjacent gray matter prior to dissection). More intense labeling occurred in the transected group in the lateral region compared to the opposite face of the white matter strip (zone M; Fig. 6, $P < 0.02$; Student t test, unpaired two tailed test).

PEG treatment significantly reduced the amount of labeling in crushed spinal cords compared to a similar number of crushed (no PEG) control cords labeled at the same time (15 min post injury) by more than a factor of 3 (Fig. 5; $P < 0.0001$, students t, unpaired and two tailed test). Fig. 6 shows that this statistically significant reduction in HRP uptake in PEG-treated cords occurred in all of the three locations defined above. Moreover,

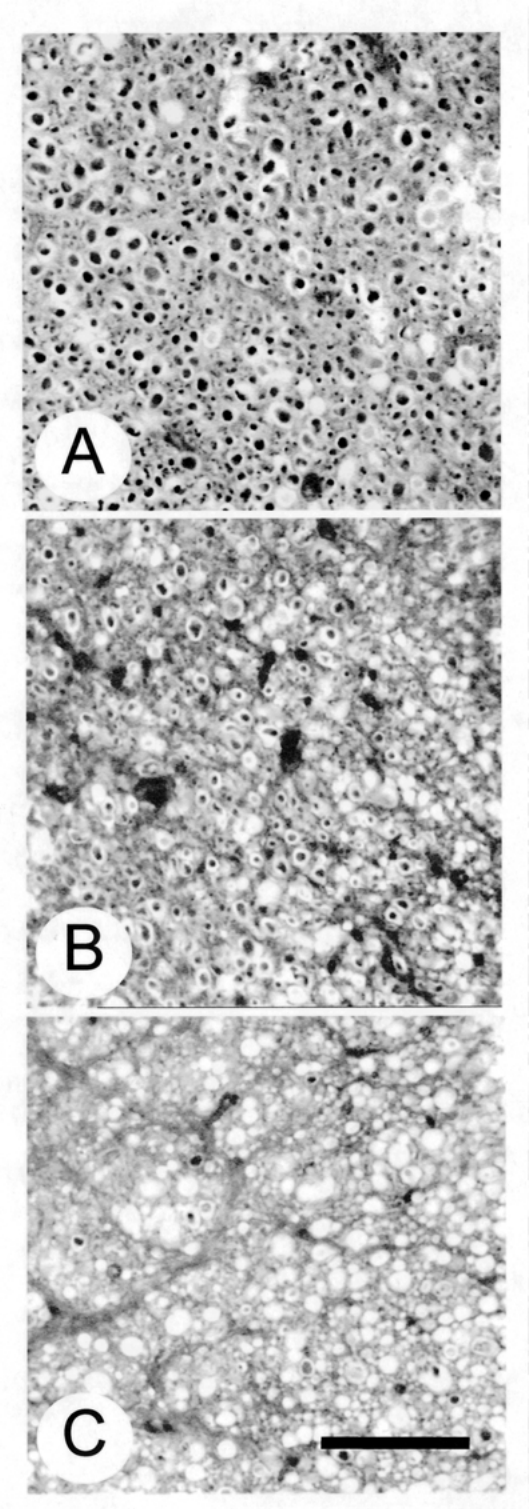

Fig. 6 also reveals that the Intermediate region of white matter was more susceptible to injury than the outer surfaces. Recall that PEG was applied to the lateral face of each spinal cord strip while the medical face was furthest from the application. (Though tangential to the specific aims of this report, it is worth mentioning that statistical comparison of labeled axons in all three regions showed the Intermediate region to be most easily damaged- providing further support for the hypothesis of a central focus of damage in "cylindrical" strips of spinal cord under compression).

We have also evaluated the susceptibility of PEG mediated repair of axons based on their caliber spectra; grouping labeled axons into four categories: 2.5–3, >3–4, >4–5, >5–6 μm in diameter (Fig. 7). PEG was effective in repairing axons in all four groups, reducing HRP uptake significantly in every category. Moreover this PEG mediated reduction in labeling was not significantly different between categories of axon diameters (Fig. 7B). Said another way, the diameter of injured axons did not affect their susceptibility to repair by PEG.

Discussion

These studies were designed to determine if a hypothesized anatomical sealing of compromised nerve membrane might be responsible for the recovery of physiological functioning following immediate repair by PEG. Mechanical compression of these axolemmas was produced by a standardized crush of adult guinea pig spinal cord white matter. As we discuss in more detail below, we have determined that PEG indeed immediately "repairs" the membranes of nerve fibers injured by mechanical damage. This repair is defined as the sealing of membrane sufficient to exclude the uptake of a large MW intracellular marker (HRP). PEG is well known to induce fusion of closely abutted cell membranes, and has been used for many varied utilities such as a means to exchange genetic material between cells, and as a means to model endogenous membrane and vesicular fusion in eukaryotic cells (Davidson *et al.*, 1976; Ahkong *et al.*, 1987; Lentz *et al.*, 1994; Lee & Lentz, 1997).

Our hypothesis was tested using an HRP dye exclusion test (Asano *et al.*, 1995). HRP is commonly used as an intracellular label and can be injected or endocytosed

Fig. 4. Examples of HRP labeling under different conditions. In A.) a cross section from a transected spinal cord is shown. In this spinal cord, the segments were immersed in the HRP solution immediately (< 2 min.) following injury. Such transected and immediately labeled spinal cords were used to establish the maximal level of HRP uptake that could be expected during the evaluation of compression injured cords. In B.) a typical cross section in which the crushed cord was immersed in the label 15 min. after a standardized compression (see Methods). This spinal cord was not treated with PEG. Note the significant labeling of axons when compared to that occurring after complete transection in A. In C.) a crushed but PEG-treated spinal cord section is shown, also immersed in the label 15 min. after the treatment. Note the infrequent occurrence of HRP uptake in PEG repaired and sealed axons. Note also that in the absence of HRP uptake, the axon's diameter and myelin stand in relief to background. The scale bar in C also applies to A and B and = 50 μm.

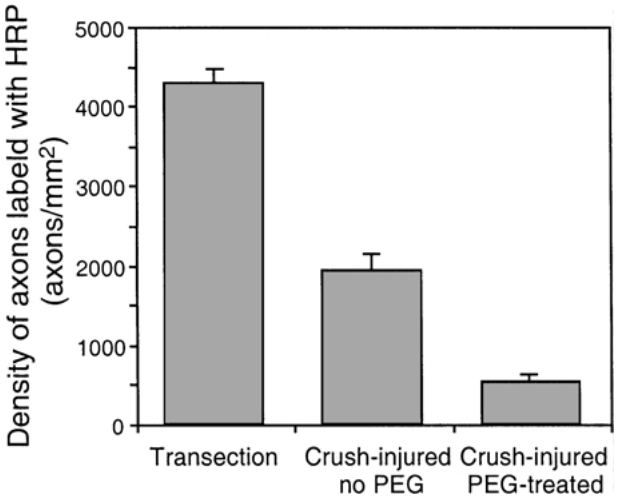

Fig. 5. Axon density (axons/mm^2) as a function of treatment. Note that approximately 4300 axons/mm^2 were labeled following transection of the spinal cord. Less than half of this density was labeled when the spinal cord was crushed by the standardized procedure. PEG treatment resulted in a further reduction in the density of labeled axons by over 70%, demonstrating significant sealing, i.e., the ability of the injured axon to exclude the HRP label. $N = 12$, transected group; $N = 13$, crushed (no PEG) control group; $N = 11$, crushed and PEG-treated group. Error bars = SEM.

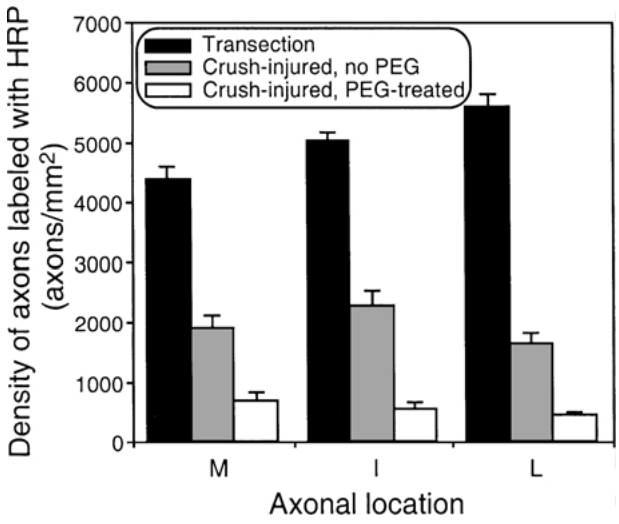

Fig. 6. Axon density as a function of the treatment and position within the spinal cord segment. Bar graphs along the abscissa are grouped by location within the cross section; M = most medial (adjacent gray matter), L = lateral, adjacent the pial surface, and I = an intermediate position between these two locations (see Methods). Note that in transected spinal cords, there was a decrease in HRP labeling as the regions sampled were located deeper within the cord segment. The density of labeled axons was statistically higher when the locations near to the cord surface were compared to the medial regions ($P < 0.02$; Student's t, two tailed t test). Note also that compression induced more significant labeling in the intermediate region of the spinal cord than at its lateral or medial face recalling that each strip of ventral white matter was placed into the sucrose gap chamber, and that these two latter positions represent the outermost faces of this spinal cord strip. This apparent increased level of labeling in the center (intermediate region) of the cord strip (region I) was confirmed statistically (Positions L vs. I, $P < 0.05$; Students t, two tailed t test). Note that PEG treatment induced significant sealing and repair of axons at all locations within the spinal cord (All treated groups vs. control groups at all locations, $P = 0.0001$; Students t, two tailed t test. In each bar graph, mean and SEM are given for 12 spinal cord strips).

into intact cells, or it can be imbibed into damaged cell processes (particularly in nerves) where it spreads throughout the cell by axoplasmic transport (Malmgren & Olsson, 1977; Borgens *et al.*, 1986). The latter characteristic makes it ideal to test how well sealed nerve processes may be. Severe, acute compromise to the axolemma would allow access of the ca. 40,000 MW marker to the axoplasm. Severe injuries to cells that seal by natural mechanisms of repair would reduce or prevent uptake of this label. This is why the ends of the white matter strip—cut during dissection many hours previously—do not readily take up the label. They are also nearly 2 cm distant from the injury site in the center of each spinal strip. Limited HRP uptake at the ends of the spinal cord strip travels only about 1.5 mm towards the center of the 35–38 mm strip within the incubation time of 1 hour. Thus the uptake at the site of damage stands apart from limited uptake of the marker at the distant ends of the spinal cord strip.

PEG MEDIATED FUSION AND REPAIR OF GUINEA PIG SPINAL CORD

We have previously shown that similar strips of guinea pig spinal cord as that used here could be completely transected within the double sucrose gap chamber, reabutted, and the two segments immediately reconnected with PEG (Shi *et al.*, 1999). PEG solutions of a similar MW and briefly applied directly to the transection plane induced a physiological recovery of CAP propagation within 5–15 min of axonal fusion. Immediate axonal fusion was not only confirmed by physiological recording, but as well by labeling studies with two flourescently decorated intracellular markers, FITC and rhodamine decorated dextrans. We further explored the transection/fusion plane using plastic embedded semithin sections stained with toluidine blue. These longitudinal sections revealed expanses of myelinated axons, denuded of myelin on either side of the cut, completely fused and reconnected (Shi *et al.*, 1999).

We have also reported that a $\sim$2 min application of PEG was able to rapidly restore CAP conduction to spinal cord strips injured by a standardized compression technique as used here (Shi & Borgens, 1999). We reported many aspects of this physiological recovery

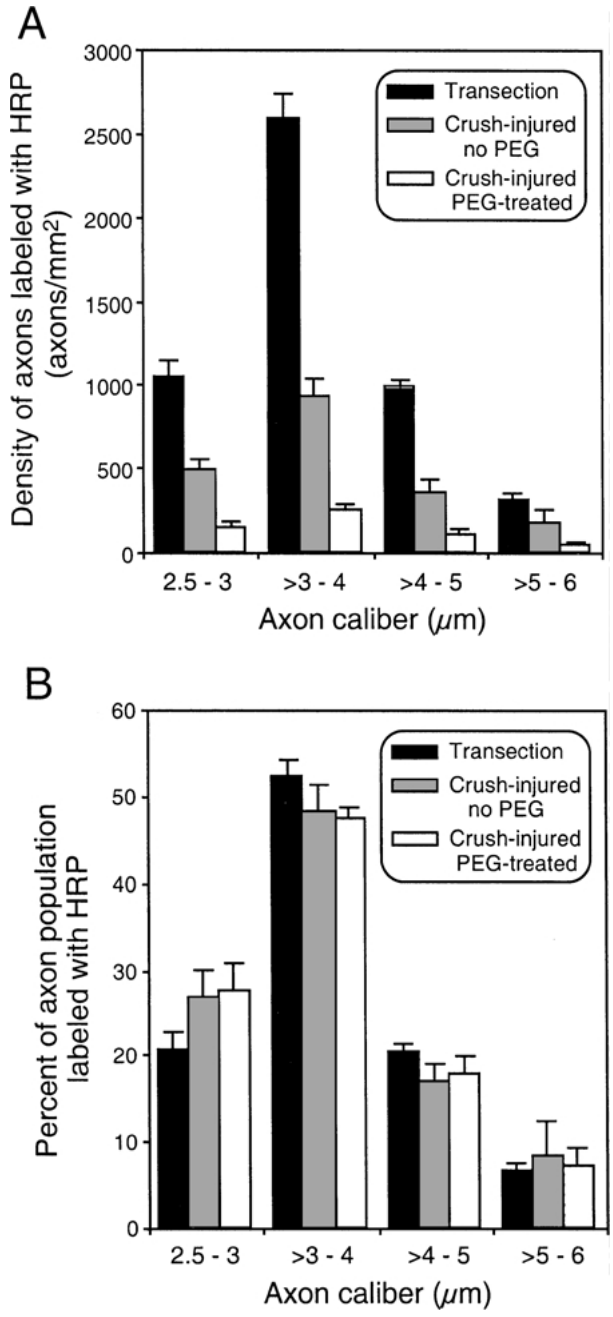

Fig. 7. The density of injured axons as a function of treatment and axon caliber. In A) the three groups: transected, crushed (no PEG), and crushed (PEG-treated) spinal cords) are grouped by range in axon diameter (given in μm). Since the most numerous axons in ventral white matter were 3–4 μm in diameter, this group showed the most intense labeling following transection. Significantly fewer axons were labeled following the compression injury across all caliber spectra (P values ranged from 0.018 to <0.0001; Students t, two tailed t test). PEG significantly repaired axons (inhibiting HRP uptake) across all caliber spectra (P values ranged from 0.016 to <0.0001; Students t, two tailed t test). In B), each group is expressed as a percent of the total axons evaluated. Note that if axons of the different caliber groups were equally susceptible to PEG repair, there would not be any statistical difference between the % axons damaged and those damaged but repaired. This relationship would also hold true for comparisons between transected and crushed axons-demonstrating that the injury does not favor one range of axon diameters over another and does not favor one caliber spectra more susceptible to PEG treatment. ($P > 0.05$ for all comparisons; Students t, two tailed t test).

including an enhancement of PEG mediated CAP recovery by potassium channel blockade, and an analysis of the susceptibility for response to PEG based on the diameter of the treated axons (Shi & Borgens, 1999). We did not however provide details of the anatomical basis of repair, the subject of this separate investigation.

THE ANATOMICAL BASIS OF PEG MEDIATED REPAIR OF COMPRESSION INJURIES TO SPINAL CORD

These data show that in PEG repaired spinal cord strips, lesions to axolemmas produced by mechanical compression sufficient to allow the uptake of the molecular marker HRP were largely sealed. In crush injured but untreated spinal cord strips, a robust labeling of axons was observed that was only about half that seen when cords were completely transected and then immediately immersed in the HRP marker. Clearly the standardized compression caused gross injury to axons within the spinal cord strips. A brief treatment with PEG significantly reduced such labeling, demonstrating that PEG-treated cords were markedly well sealed. This difference in label uptake was so apparent that it did not require quantification of axon density after the experiment was unblinded. Even a quick comparison of Fig. 4B and C, which are typical of the complete data set, reveals the remarkable level of membrane repair produced by the treatment-only reinforced by the actual counting of labeled axons.

One of the methodological limitations of this technique is that it favored the counting of larger axons (>2.5 μm in diameter). Given this fact, it is reasonable to assume the method also favored an evaluation of myelinated axons (which tend to be of larger diameters). However, we know of no reason to assume that PEG would only repair these categories of axons in white matter. In fact quite to the contrary, it is likely that both myelinated and unmyelinated axons of all calibers are equally susceptible to PEG mediated repair. We base this on the wide range of axons calibers fused following transection (Shi *et al.*, 1999), and on yet unpublished evaluation of the fusion plane using transmission electron microscopy. Additionally, we have previously reported linear regression analysis of CAP recovery in response to PEG treatment showing that there was not a selection of large over small diameter axons by PEG treatment (Shi & Borgens, 1999). In this study, a regression analysis of HRP labeling data (transected

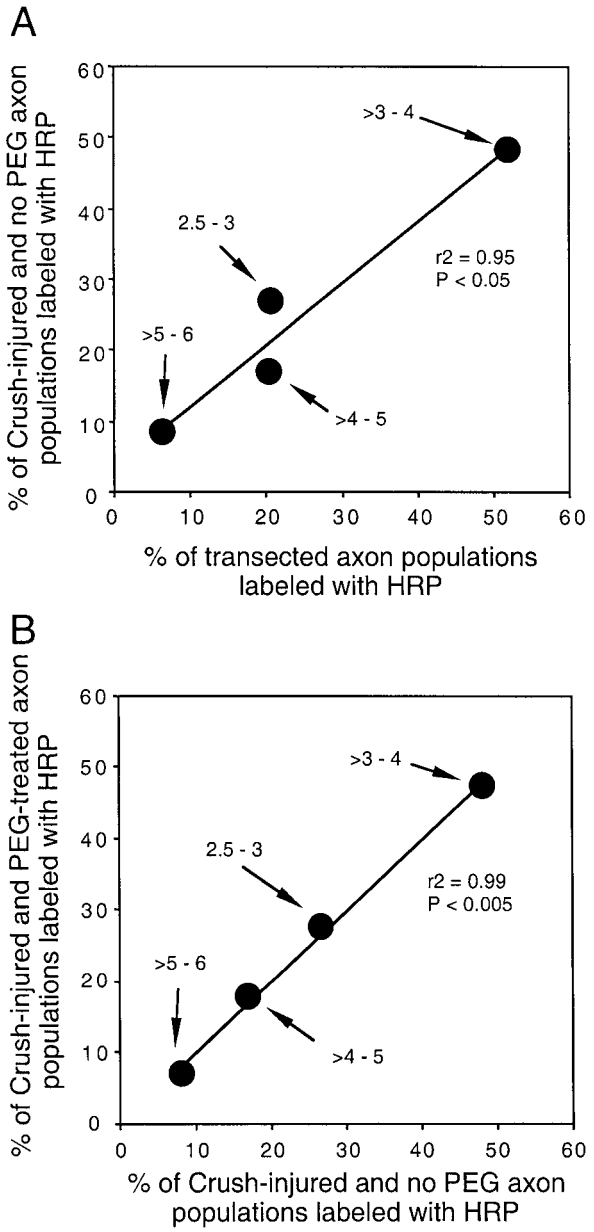

Fig. 8. A) Correlation of the numbers of HRP labeled axons (compression vs. transection) grouped by axon diameter (in μm) B) correlation of compressed but untreated axons vs. PEG-treated axons grouped by axon diameter (μm). The data points plotted in these linear regressions are taken from Fig. 7. Note that in A and B, the regression line is not significantly different from 1 to 1 linearity. That is, the slope of the regression does not vary as a function of either of the two groups compared. This evaluation demonstrates that all axon caliber spectra are equally susceptible to injury (revealed in A), and all injured axons are equally susceptible to repair by PEG (revealed in B).

group versus the crush injured (no PEG)) control group also revealed that all axons were susceptible to the standardized compression injury independent of their diameter. Note that all axon diameter means fall close to the regression line in Fig. 8A. Furthermore, axons with different diameters were equally susceptible to PEG repair (Fig. 8B). Thus, we do not shrink from referring to sealed axons in this study as "repaired" since the facilitated recovery of CAP conduction is associated with rapid sealing of the axolemma in only the crush-injured (PEG-treated) group. *It is likely that this repair is not mediated by an enhancement of the natural mechanisms of sealing* (Yawo & Kuno, 1985; Xie & Barrett, 1991). The process of axonal sealing is time dependent, and occurs over periods on the order of 1 hour (Shi *et al.*, 1999; 1997), favoring the sealing of small axons over large diameter ones. PEG treatment produced extremely rapid closure of membrane breaches, and with no bias for axon caliber.

PEG REPAIR AS A FUNCTION OF AXON LOCATION

While the axon's diameter may not influence the character of PEG mediated repair, the location of axons within the spinal cord relative to the application site does. In clinical injuries it is well known that compression leads to "central hemorrhagic necrosis" a term denoting the fact that damage is less severe in subpial regions than at the spinal cord's center. This is due to many factors including the increased vasculature of gray matter, and the consequences of damage to more central spinal cord tissue by compromise of its blood supply, swelling, and inflammation. It is also likely that a greater susceptibility for damage to occur in central regions is based upon the simple physics of the behavior of sol/gels (i.e., spinal parenchyma) within cylinders when compressed from the outside. Greater displacement of the material within the cylinder occurs near the center compared to the material at the periphery. In the present case, the white matter strip is studied independent of those pathologies associated with hemorrhage and gray matter destruction (Shi & Blight, 1996). Furthermore these data may be the first to clearly indicate that damage to axons is still greatest in the medial regions of the spinal cord independent of other pathology such as vascular insult.

The spinal cord strip was arranged within the double sucrose gap chamber in each case such that PEG was applied to the lateral face of each strip. The medial face was not only farthest from the site of the crush injury, but also furthest from the site of PEG application. This fact helps provide some rationale for the smaller reduction in the density of repaired fibers in the medial face compared to the lateral face. We hypothesize this may be due to a reduction in penetration of the spinal cord strip by PEG. We have previously reviewed the mechanisms of action of PEG in fusing or repairing axolemmas (Shi & Borgens, 1999; Shi *et al.*, 1999; Borgens & Shi, 2000), and emphasize here that an acute dehydration of cell membranes is probably the primary action of PEG in this regard. Dehydration permits structural components of the membrane to resolve into each other, and rehydration probably initiates a spontaneous reassembly of the membrane and its structural components. Thus

water-hungry, high molecular weight, molecules such as PEG would be expected to show characteristics of "action at a distance" where physical contact of the cut or damaged membranes with the polymer might not be necessary to induce membrane fusion or repair. In some cases, PEG may act as a detergent or a molecular surfactant in reversing cell permeabilization (Lentz, 1994; Borgens & Shi, 2000). Recoveries of function in response to surfactants such as Poloxamines and Poloxamers have been shown for testicular reperfusion injury (Palmer *et al*., 1998), radiation damage (Hannig *et al*., 1999), and heat trauma (Padanlam *et al*., 1994) among others. In these cases, contact of the polymer with the damaged membranes is likely to be required. Still, one would expect a gradient of the reparative response to PEG to be inversely proportional to the distance from the site of application. In this study the density of repaired (i.e. well sealed) axons within the medial region of the strip of spinal cord was less than within the lateral region where PEG was applied.

These data clearly establish a need to explore means to improve penetration of nervous tissue by PEG. One way would be to reduce the molecular weight of PEG in solution-which also reduces the viscosity of the solution. We have pursued this line of investigation in an *in vivo* guinea pig spinal cord injury model comparing 400 MW PEG (55% by weight in water; see Davidson *et al*., 1976) to our standard 1800 MW solution (Shi & Borgens, 1999; Shi *et al*., 1999). Following a standardized compression injury to the guinea pig thoracic spinal cord, PEG induced an immediate recovery of CAP conduction through the lesion, which was associated with a recovery of behavioral function which was stable for at least 1 month post injury. This recovery *in vivo* was independent of the MW of PEG used (Borgens & Shi, 2000). At this time we do not see any improvement in these functional deficits when using the lower viscosity PEG solution when compared to our standard solution.

THE CLINICAL IMPORTANCE OF PEG MEDIATED REPAIR

The fundamental basis of all behavioral deficits resulting from trauma to the nervous system is the failure of nerve fibers to conduct action potentials. This can be due to the physical separation of the nerve fiber from its target or a alteration in the membrane of the fiber vitiating action potential propagation by "conduction block". In the latter case, the severity of the membrane defect producing nerve fibers that are anatomically intact but physiologically nonfunctional may progress to such an extent that the axon eventually separates into two segments (Maxwell, 1996; Shi & Borgens, 1999). Denervation of target cells or tissues usually results in their atrophy or degeneration (Guth, 1969). For example, irreversible atrophy of muscle tissue can result from prolonged denervation. Swift application of PEG directly to the traumatized region of CNS or PNS tissue is very likely to improve the clinical outcome from neurotrauma. First, we have demonstrated that physiological recovery and anatomical repair of severely crushed nerve fibers is an immediate result of PEG application (Shi & Borgens, 1999). Those crushed fibers that would ordinarily progress to axotomy would probably be rescued by PEG, resulting in the additional benefit of preserving the status of downstream target cells and tissues in the conduction pathway. This has prompted us to begin such preclinical tests of the utility of PEG in crushed peripheral nerve as well as in clinical cases of naturally produced paraplegia in dogs (see Borgens *et al*., 1993, 1999). We have also shown that PEG can indeed functionally fuse completely transected spinal cord axons (Shi *et al*., 1999). Development of PEG fusion techniques for clinical use in transection injuries is complicated by; 1.) the requirement for proper mechanical alignment and abutment of proximal and distal axon segments that does not produce further injury, but not so loose as to inhibit membrane fusion (Shi *et al*., 1999). 2.) subsequent mechanical stabilization of the repaired region of nervous tissue; 3.) the natural retraction of the proximal and distal axonal segment away from the transection plane of nervous tissue parenchyma following the release of tension on the axon or by retrograde degeneration of the axon tip and 4.) a yet unknown requirement to align and fuse axons of a similar conduction pathway. We believe these problems are more tractable in transections of peripheral nerve than spinal cord and frame those areas of important future research to permit full potential of PEG application when applied clinically.

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