

TEMPERATURE DEPENDENCE OF MEMBRANE SEALING FOLLOWING TRANSECTION IN MAMMALIAN SPINAL CORD AXONS

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Abstract—Using an *in vitro* sucrose-gap recording chamber, sealing of cut axons in isolated strips of white matter from guinea pig spinal cord was measured by recording the "compound membrane potential". This functional sealing was found to correlate well with anatomical resealing, measured by a horseradish peroxidase uptake assay. Near-complete functional and anatomical recovery of the axonal membrane occurred routinely within 60 min following transection at 37°C in regular Krebs' solution. The rate of membrane potential recovery is exponential, with a time-constant of 20 ± 5 min. The sealing process at 31° C was similar to that at 37° C, and was effectively blocked at 25° C, under which condition most axons continued to take up horseradish peroxidase for more than 1 h, and failed to substantially recover their membrane potential. Seventy-five percent of the cords transected at 40° C had similar sealing behavior to those at 37° C to above 31° C significantly decreases axonal permeabilization to horseradish peroxidase (increases the sealing of transected ends) across all areas of a transverse section of spinal cord. Moreover, this enhancement of sealing exists across all axon calibers. Since severe cooling compromises membrane resealing, caution needs to be taken when hypothermic treatment (below 25° C) is applied within the first 60 min following mechanical injury.

In summary, we have found that at normal temperature (37°C), nerve fibers repair their damaged membrane following physical injury with an hour. This is similar at mildly lower (31°C) and relatively higher (40°C) temperature, although some fibers tend to collapse under this febrile temperature. Moreover, severely low temperature (25°C) hindered the repair of damaged membranes. Based on our study, caution is needed in treating spinal cord injury with low temperatures. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: axon, temperature, membrane sealing, spinal cord injury, neurotrauma, nerve repair.

There is strong experimental evidence that membrane resealing of mammalian axons is a critical step of neuronal survival from mechanical injury.^{3,38,53,62} It is therefore usually believed that the earlier the transected ends are sealed, the better chance the cell should have to survive the injury and perhaps function once again.^{8,28,40,62–64} Since axonal degeneration causes the loss of motor and sensory function seen in spinal cord injury, a better understanding of sealing in damaged axons, and the conditions which may promote it, could ultimately provide novel strategies to enhance functional recovery following CNS trauma.

The potential for the therapeutic benefit of lowered temperature on neuronal recovery following various types of injury has a long, but controversial, history.^{15,17,29} Mild to severe hypothermia has long been believed to reduce various types of nerve injury^{17,21,22,30,33,39,58} and hypothermia has been suggested to be used clinically to improve the outcome of trauma by reducing the damage from primary and secondary injuries.^{4,16} However, the observation that low temperature is capable of inhibiting membrane sealing of an invertebrate giant axon has received less attention,⁶⁴ and the potential conflict between these two phenomena has not been examined.

The present study was designed to examine the dependence of the process of membrane sealing on temperature in adult mammalian spinal cord axons isolated *in vitro*. Sucrose gap recordings were used to monitor membrane potential changes in strips of white matter isolated from guinea pig spinal cord, by techniques that have been described previously.^{49–51} Morphological examination of sealing using the uptake of horseradish peroxidase (HRP) by the injured axons provided additional information on the distribution of axonal damage within the cords, axonal damage as a function of diameter, and the time-course of resealing of the cut membrane to large molecules. The structural analysis was found to correlate well with electrophysiological findings. The membrane resealing process was found to be highly dependent upon temperature. Parts of this work have appeared in abstract form.⁴⁷

EXPERIMENTAL PROCEDURES

Isolation of spinal cord

Adult female Hartley guinea-pigs of 350-500 g body weight were used (Sasco, Omaha, NE). The experimental protocols have been reviewed and approved by the Purdue University Animal Care and Use Committee (PACUC). All efforts were made to minimize the number and suffering of animals used. The technique for isolation of the cord was described previously.^{49–51} In brief, guinea-pigs were anesthetized deeply with ketamine (80 mg/kg), xylazine (12 mg/kg) and acepromazine (0.8 mg/kg). They were then perfused through the heart with 500 ml oxygenated, cold Krebs' solution to remove blood and lower core temperature. The vertebral column was excised rapidly and a complete laminectomy was performed. The spinal cord was removed from the vertebrae and immersed in cold Krebs' solution. The cord was separated first into two halves by midline sagittal division. The ventral white matter was then obtained by separating it from the gray matter with a scalpel blade against a soft plastic block. The composition of the Krebs' solution was as follows: NaCl 124 mM; KCl 2 mM; KH₂PO₄ 1.2 mM; MgSO₄ 1.3 mM; CaCl₂ 2 mM; dextrose 20 mM; sodium ascorbate 10 mM; NaHCO₃ 26 mM, equilibrated by bubbling with 95% O_2 -5% CO_2 to produce a pH of 7.2-7.4.

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Abbreviations: DMSO, dimethylsulfoxide; HRP, horseradish peroxidase.



Fig. 1. Diagram showing the recording apparatus and the transection injury method. (A) The recording arrangement. The isolated spinal cord tract is shown mounted in the apparatus, with the central well continuously perfused with oxygenated Krebs' solution. The two ends of the tract were placed in separate wells filled with isotonic KCl, divided from the central well by narrow gaps filled with flowing, isotonic sucrose solution. Electrodes were formed from silver/silver chloride wires. Action potentials were generated at the left-hand sucrose gap, conducted though the central part of the spinal cord and were recorded at the right-hand gap, using a bridge amplifier. (B) To examine the response to transection, the tissue was cut near the face of the recording gap, seen here in diagrammatic view from the front of the chamber. The tissue was also cut in the middle of the chamber, and was transferred to HRP solution at 60 min after injury, to examine the sealing of axons to HRP in different temperature.

Recording chamber

The construction of the recording chamber is illustrated in Fig. 1. A strip of isolated spinal cord white matter, approximately 35 mm in length, was supported in the central compartment and continuously superfused with oxygenated Krebs' solution (c. 2 ml/min) by means of a peristaltic pump. The free ends of the spinal cord strip were placed across the sucrose gap channels to side compartments filled with isotonic (120 mM) potassium chloride. The white matter strip was sealed on either side of the sucrose gap channels using fragments of plastic coverslip and a small amount of silicone grease to attach the coverslip to the walls of the channel. Isotonic sucrose solution was continuously run through the gap channels at a rate of 1 ml/min. The temperature of the chamber was maintained with a Peltier unit in the base, thermostatically controlled with a thermistor system (Cambion Instruments). The axons were stimulated and compound action potentials recorded at opposite ends of the strip of white matter by silver/ silver chloride wire electrodes positioned within the side chambers and the central bath. The central bath was connected to instrument ground. Stimuli were delivered through stimulus isolation units and were usually in the form of 0.1 ms constant current unipolar pulses. Recordings were made using a bridge amplifier and Neurocorder (both from Neurodata Instruments) for digital data storage on videotape. Subsequent analysis was performed using custom Labview® software (National Instruments) on a Macintosh Power PC G3 computer.

Transection

To study the response of the nerve fibers to transection, the tissue

strip was cut at the face of the recording sucrose gap, using microscissors, which were also used to cut through the tissue at the center of the chamber, so that the isolated tract could be transferred to HRP solution at different times after injury for evaluation of sealing at the cut ends (Fig. 1B).

Horseradish peroxidase histochemistry

To examine the extent of sealing of disrupted axons, white matter strips were transferred after electrophysiological recording to oxygenated Krebs' solution containing 0.015% HRP (Sigma Type VI, St Louis, MO, U.S.A.). After incubation for 1 h at room temperature, the tissue was fixed by immersion in 2.5% glutaraldehyde in phosphate buffer. Transverse sections of the tissue were cut at 30 μ m on a Vibratome and stained with a diaminobenzidine reaction to reveal the extent of HRP uptake into damaged axons. Sections near the cut and at 5 mm distance were compared with control for the presence of damaged fibers that might be unrelated to the deliberate injury. Sections were examined and photographed with a Nikon Optiphot microscope. Image analysis was performed using an Optronics video camera and NIH Image software on a Macintosh Quadra 950 computer.

Two-dimensional morphometry

The number of unsealed axons was obtained from HRP-stained Vibratome sections. The images were digitized and captured on a Macintosh Quadra 800 Computer using a Leitz Ortoplan microscope and JVC video camera. A cross-section was selected from each strip, using the $6.3 \times$ objective. Representative area samples were chosen from peak regions of dye uptake in transverse sections to quantify axonal sealing. The area was selected in such a way that it could be divided into three squares from pia to the gray matter: lateral, intermedial, and medial. The lateral square included the outer surface and the medial included the inner edge of the white matter, or the border area between the white and gray matter, where present. The size of the square varied depending on the thickness of the white matter. Then, using a $16 \times$ objective, axons were measured within the three squares. The number of unsealed axons was counted and expressed as density. Images were first color-transformed and binarized using IP Lab spectrum. Subsequently, the axon diameter was measured. The shortest distance across the axon was taken as the diameter, based on the interpretation that deviations from circularity represented axons cut obliquely. Axons sampled in two squares were assigned to the more lateral one. Counts of individual axons were normalized per unit area of each of the three subregions and expressed as a density (axons/mm²).

Statistical treatment

Throughout the paper, Student's *t*-test (unpaired, two-tailed) was used to compare electrophysiological and histological measurements between two groups in different experimental conditions. Linear correlation between electrophysiological and histological measurements was expressed by Pearson correlation coefficient (*r*). Statistical significance was attributed to values of P < 0.05. Averages were expressed as mean \pm standard error.

RESULTS

Membrane potential recovery is a function of temperature

Spinal cord strips placed in the recording chamber showed a period of stabilization of the resting membrane potential, requiring 30–60 min. During this time, the "compound resting membrane potential" recorded across the sucrose gap became more negative and the amplitude of the maximal evoked compound action potential increased. The form and quantitative characteristics of the compound action potential and of the "gap potential" or "compound resting membrane potential" all have been described previously.⁴⁹

When the white matter strip was cut within 1 mm of the face of the sucrose gap, the "gap potential" was reduced within seconds toward bath potential, then began to repolarize slowly. Full recovery of the initial resting potential was



Fig. 2. Exponential rate of recovery of the gap potential following transection of a spinal cord white matter strip in normal Krebs' solution. Averaged responses to transection in five groups of white matter strips maintained at 25° C (n = 8), 31° C (n = 8), 40° C(a) (n = 6), and 40° C(b) (n = 2), respectively, are shown here on a linear scale and (inset) on a log scale. The group of 40° C(a) represents the axons that did substantially regain the membrane potential at the end of 60 min following transection. The group of 40° C(b) represents the axons in which membrane potential collapsed following transection at 40° C. The gap potential in each case was normalized to the peak of the depolarization produced by transection. The rate of recovery of the gap potential was reduced with decreasing temperature, except the group 40° C(b). Asterisks indicate significant difference of membrane potential recovered at 31° C, 37° C and 40° C when compared with that at 25° C. *P < 0.05, **P < 0.001.

routinely achieved in approximately 45-60 min at 37°C, with an average recovery at the end of 60 min following injury being $97 \pm 3\%$ (Fig. 2). The form of the recovery curve was approximately exponential, with a time-constant of 20 min, which is consistent with our previous studies.^{48,52} At 31°C, similar results were obtained with an average recovery of $92 \pm 4\%$. However, when the cords were cut at 25°C, they failed to recover their membrane potential substantially for the duration of the experiments (60 min following transection). In this severe hypothermic condition, the average recovery of membrane potential was $70 \pm 7\%$, which is significantly less than that at $37^{\circ}C$ (P < 0.001) and $31^{\circ}C$ (P < 0.05), respectively. In addition, the rate of recovery of resting potential was slower, with a time-constant of approximately 40 min, but was still exponential to the same baseline (Fig. 2).

Since 40°C represents a fever in mammals, it would be interesting to know if this temperature affects membrane sealing following transection. In about three-fourths of the cases, exposure to 40°C enabled the transected axons to restore membrane potential to pre-injury levels by the end of 60 min (99% \pm 1%), which was similar to that at 37°C (Fig. 2). In contrast, the balance displayed a different pattern of membrane potential change. The gradual recovery of the membrane potential was reversed 10–20 min following transection, leading to a collapse of membrane potential over the course of the next 50 min (Fig. 2). This apparent catastrophic loss of sealing was confirmed by the significant HRP staining of axons when spinal cord strips were transferred to HRP for the "dye exclusion" test (Fig. 3F).

Membrane resealing revealed by horseradish peroxidase staining

HRP histochemistry showed a rate of sealing of the axonal membrane to entry of the enzyme molecule comparable to

membrane potential recovery following transection. The quantitative analysis of HRP labeling in different temperatures was in agreement with the membrane potential recovery profiles. In order to obtain the maximal, or the background, number of axons that could be expected using this HRP uptake technique, we transected the axons and exposed them to HRP immediately. In this situation, the average density of HRP-labeled axons was 4359/mm² (Table 1) and almost all axons were shown to take up HRP, indicating that this is a very effective technique in detecting membrane permeability to large molecules (Fig. 3A). Similar to electrophysiological studies, there were a large number of axons that failed to seal when they were transected at 25°C as indicated in Fig. 3B and Table 1. The quantitative analysis revealed that there was an average of 2956 axons/mm², or 68% of all the axons, within the white matter were labeled with HRP at 25°C (Table 1). At 37°C, however, only 9% of the fibers were labeled with HRP at 1 h post-transection, a significant decrease compared with that at 25°C (P < 0.005, Fig. 3D and Table 1). This indicates a near complete sealing of the damaged axonal membranes to large molecules at this temperature. Likewise, at 31°C there was only 13% of the axons labeled with HRP, which was also significantly different than that of 25°C (P < 0.005, Fig. 3C,D, Table 1). At 40°C, three-quarters of the cords tested sealed the membranes to a degree similar to that at 37°C. The cords that did seal at 40°C had an HRP axonal labeling of 8% which was also significantly different than that at 25°C (P < 0.005, Fig. 3E, Table 1). Consistent with the electrophysiological studies, one-fourth of the cords tested at 40°C showed a reversal of recovery, leading to membrane permeabilization accompanying membrane potential loss. One of these cases is shown in Fig. 3F. Notice that the axons are somewhat swollen, perhaps indicating more profound deterioration. The quantification of HRP labeling of the cords in this group was not performed due to the poor quality of the tissue sample.

Table 1. Density of axons permeable to horseradish peroxidase

	Immediate post-transection	1 h @ 25°C	1 h @ 31°C	1 h @ 37°C	1 h @ 40°C
Axons permeable to HRP (axons/mm ²)	4359 ± 226	2956 ± 505	545 ± 97*	372 ± 54*	349 ± 82*

Data are presented as mean densities of $axons/mm^2 \pm S.E.M$. Note that approximately 4400 $axons/mm^2$ were labeled by this technique following transection and immediate labeling of the spinal cord. Three-fourths of this number of axons were labeled when the spinal cord was transected and recovered for 60 min at 25°C before HRP labeling. Raising the temperature to above 31°C resulted in a dramatic reduction in the density of labeled axons, demonstrating significant membrane resealing, i.e. the ability of the injured axon to exclude the HRP molecule. Asterisks indicate significant difference in HRP labeling when compared with that at 25°C. n = 4 for 25°C, 31°C, 37°C and 40°C. n = 12 for immediate transection group. Only those cords that sealed at 40°C were included in quantification analysis. *P < 0.05



Fig. 3. Examples of HRP labeling under different conditions. Vibratome sections were cut 1 mm from the transected end of spinal cord tracts and stained for HRP using the diaminobenzidine technique. (A) A cross-section from a transected spinal cord is shown in which the cord segments were immediately (<2 min) immersed in the HRP solution following injury. Such a method was used to establish the maximal level of HRP uptake that could be expected during the evaluation of transected injured cords. (B–F) The strips were transferred to HRP containing Krebs' solution at 60 min post-transection at 25°C (B), 31°C (C), 37°C (D), and 40°C (E, F). At 25°C, more than half of the axons stained with diaminobenzidine reaction product, showing the axons had taken up and transported HRP. At 31°C, 37°C, and some 40°C (E), almost all axons were effectively sealed to uptake HRP with reaction product only in endothelial cells and some glial processes. (F) A cord that displayed a collapse of membrane potential after initial repolarization. Note the greater extent of staining and enlarged diameter compared with E. Scale bar = 50 μ m.

Morphometry

In order to examine the possibility that location of the axons within the spinal cord cross-section and axon diameters affect the sealing of transected axons, we have performed morphometric analysis using Vibratome sections labeled with HRP. As shown in Fig. 4, axons were grouped based on their location within the cross-section. The transverse cord



Fig. 4. The density of axons permeable to HRP (axons/mm²) as a function of the temperature and position within the spinal cord segment. The five groups, 25° C (n=4), 31° C (n=4), 37° C (n=4), 40° C (n=4), and background (n=12), respectively, are grouped by location within the crosssection; M = medial (adjacent to gray matter), L = lateral (adjacent to the pial surface), and I is an intermediate position between these two locations (see Experimental Procedures). Note that in background HRP labeling, there was a decrease in HRP labeling as the regions sampled were deeper within the cord segment. The density of labeled axons was statistically significant when the locations near to the cord surface was compared with the medial regions (P < 0.05). Note also that at 25° C, 31° C and 40° C, the HRP labeling pattern in the transverse section is reversed. At 37° C, however, there is more labeling in lateral regions when compared with other regions. At 40° C, only those cords that sealed were included in quantification analysis.

sections were divided into three areas, medial (adjacent to gray matter, M), lateral (adjacent to the pial surface, L), and the intermediate position between these two locations (I) (see Experimental Procedures for details). First, increasing the temperature from 25°C to above 31°C significantly decreased axonal permeability to HRP in all three areas. It is also obvious that in background HRP labeling, there was a decrease in HRP labeling from the lateral to medial regions. The density of labeled axons in lateral regions was significantly higher than that in medial regions (P < 0.05). Interestingly, at 25°C, 31°C, and 40°C, the HRP labeling pattern in the transverse section is reversed. There was less labeling in lateral regions of the cord than its medial and intermedial region, indicating that the axons located closer to the pia surface seal more readily than those closer to gray matter (Fig. 5). At 37°C, however, there was more labeling in lateral regions.

Figures 5 and 6 show the sealing in different axon caliber groups, expressed in density and percentage, respectively. The number of HRP-labeled axons was significantly decreased in all five axon caliber groups when temperature was raised from 25°C to above 31°C (P < 0.005, Fig. 5). When expressed as a percentage of the population, it shows that the axon populations of different caliber respond differently to temperature-mediated resealing. Based on Fig. 6, the percentage of HRP-labeled axons with a diameter of less than



Fig. 5. The density of injured axons as a function of temperature and axon caliber. The five groups, 25° C (n=4), 31° C (n=4), 37° C (n=4), 40° C (n=4), and background (n=12), respectively, are grouped by their range in axons diameter in micrometers. Since the most numerous axons were between 3 and 4 μ m in diameter, this group showed most intense labeling following transection. Note that at 31° C or higher temperatures, the number of axons permeable to HRP at each caliber group was significantly decreased compared with that at 25° C (P < 0.005). At 40° C, only those cords that sealed were included in quantification analysis.

2.5 μ m decreased significantly (*P* < 0.05) when the temperature was changed from 25°C to 37°C. A similar phenomenon was also seen in axons with a diameter of 2.5–3 μ m (*P* < 0.05). In contrast, in groups with axons >3 μ m in diameter, warming from 25°C to 37°C caused HRP-labeling to either increase significantly (*P* < 0.05) or remain relatively similar (*P* > 0.05).

DISCUSSION

If axons are disconnected from the cell body by transection or severe compression, the proximal segment may survive and regenerate.^{2,6,7,9-11,13,14,19,32,35,38,54} In order to survive, however, an injured axon must seal its breached membrane to reduce disruptive changes in the internal concentration of ions and macromolecules.^{40,62} This sealing of cut processes and the consequences for cell survival have been mainly examined previously in large, isolated, invertebrate axons, 34,64 and in cultured vertebrate cells.40,53,62 In addition to its established calcium-dependency, ^{23,62,64} the process of axonal sealing itself was also shown to be temperature dependent in cockroach giant axons.⁶⁴ However, this temperaturedependent phenomenon has received little attention and has not been tested in the mammalian spinal cord, where it is of key clinical relevance. The potential significance of these mechanisms is highlighted by the fact that cooling is considered one of the effective acute treatments for brain and spinal cord injury.^{4,17} The present experiments were designed to use a novel dynamic measure of sealing in mammalian spinal axons to determine whether the normal, mild and severe cool temperatures seen in spinal cord trauma acute treatment might affect membrane resealing. In addition, a



Fig. 6. The percentage of population of each axon caliber group as a function of temperature. Experimental condition and numbers are similar to Fig. 5. Each caliber group is expressed as a percentage of the total axons evaluated within its own group: background, 25°C, 31°C, 37°C and 40°C. Likewise, the sum of the percentages of labeling following transection would equal 100% for each of the five temperature groups. Therefore, if axons of the different caliber groups were equally susceptible to temperaturemediated repair, there would not be any statistical difference between the percentage of axons among different experimental groups. This, however, does not hold true for any of five caliber groups. Note that in the groups of axons with small diameters (<2.5 and 2.5–3 μm), the percentage of HRP labeled axons decreased significantly (P < 0.05) when the cords were warmed from 25°C to 37°C. Similar comparisons in axons with large diameters (>4-5 μ m and >5-6 μ m) are not significant (P>0.05). Interestingly, the axons with medium-sized diameter ($>3-4 \mu m$) showed a significant increase on HRP labeling upon warming (P < 0.05). At 40°C,

only those cords that sealed were included in quantification analysis.

relatively high temperature seen in fever conditions has also been tested for its effect on sealing.

The correlation of membrane potential recovery and horseradish peroxidase labeling

In previous studies, membrane resealing after neurite or axon transection was monitored most frequently by measuring changes in cellular resting membrane potential and input resistance with an intracellular microelectrode. 34,40,42,63-65 In our opinion, electrophysiological measurements alone have not been shown to unambiguously assess the extent of axonal sealing. This is because membrane potential is, to some degree, influenced by many factors in addition to the extent of sealing. For example, membrane potential measurement of the injury site depends on the distance between the site of the measurement and the transected axonal end and on the length constant of nerve tissue. This distance cannot be accurately determined due to the nature of microelectrode impalement^{34,64} and axonal constriction.²³ Hence, a combination of electrophysiological and anatomical measurements allows a more definitive assessment of the time-course and extent of sealing. Moreover, directly compared to anatomical



Fig. 7. The correlation of membrane repolarization and HRP labeling. The normalized membrane repolarization of transected axons is plotted against the density of axons labeled with HRP. The data are assigned to six groups, background, 25°C, 31°C, 37°C, 40°C and uninjured. The data points plotted in these linear regressions are taken from Fig. 2 and Table 1, except the uninjured group. The repolarization value of background labeling was taken at 2 min following transection. The linear regression line is drawn by the computer. Evidently, the correlation coefficient (r) is 0.93, and P = 0.006, which suggests that there is a significant correlation between membrane repolarization and HRP labeling. This evaluation demonstrates that compound membrane potential can be used as an indicator of the recovery of membrane integrity at various temperatures.

assessment, the electrophysiological measurement can be examined for its reliability.

It is clear that in our study, membrane potential and HRP labeling correlates well (Fig. 7), indicating that the membrane potential measured using our sucrose gap chamber can be used as an indicator of the recovery of membrane integrity. In addition, morphological study allows us to examine the influence of anatomical characteristics on membrane sealing, such as axon diameters and locations within the spinal cords.

The completeness of membrane sealing following transection injury

It has been two decades since Borgens and colleagues demonstrated that the cut faces of severed lamprey axons remained "leaky" for several days, indicating the incompleteness of membrane sealing following transection.⁸ Since then, scenarios for both complete and incomplete sealing of the cut ends of severed axons have been reported, even in the same preparation. For example, Xie and Barrett have demonstrated a complete exclusion of fluorescent dye 30 min after neurite transection in cultured rat septal neurons.⁶² Likewise, the current study shows a full recovery of axonal membrane potential and concomitant exclusion of HRP in an isolated guinea pig spinal cord white matter strip 60 min after transection. In contrast, Krause and colleagues noted that squid giant axons exhibited a persistent inward current in the cut ends and an accompanying failure of substantial recovery of membrane potential within 2.5 h after transection.³⁴ Interestingly, Meiri and co-workers reported that an injury current remained for days in the cut end of a severed cockroach giant axon,⁴² while Yawo and Kuno observed a completed recovery of membrane potential and input resistance within 30 min using the same preparation and similar injury method.⁶⁴

These sometimes contrary observations suggest that neuronal membrane sealing is not a uniform phenomenon and these variations should be considered to be rules rather

than exceptions. It is increasingly clear that the underlying mechanism of such variable efficacy in the re-establishment of an effective barrier to extracellular ions and macromolecules probably lies in the intrinsic properties of the neuronal process, such as membrane fluidity and mobility. Physical and biochemical factors that could affect sealing are those that usually also influence membrane fluidity and mobility either directly or indirectly by changing the interaction of the membrane with the cytoskeleton and extracellular matrix.^{26,31,47,48,62} The manipulations that enhance membrane fluidity and mobility usually encourage membrane sealing, while those that decrease membrane fluidity and mobility inhibit sealing. For example, when the membrane is heated above the transition temperature, it will adopt a more fluid liquid state.²⁴ Therefore, raising the temperature increases membrane fluidity.

Additionally, application of calpain and dimethylsulfoxide (DMSO) have been shown to partially disconnect the membrane from the rest of the cytokeleton^{18,25,46,59} and therefore increase membrane mobility. This is accomplished by disrupting the components of the subcortical network that lends structural support to the plasma membrane. Calpain has been shown to proteolyse fodrin and DMSO is reported to disrupt actin, 18,46,59 both of which are major constituents of the subcortical cytoskeleton network.41,43 Both raising temperature and applying calpain and DMSO have been shown to enhance sealing.^{26,47,64} In contrast, the treatments that tend to decrease membrane fluidity and mobility and stabilize microtubules and cytoskeleton components inhibit sealing, such as the inhibitors of calpain and phospholipase A₂.^{26,31,48,62,64} To further support this, Lucas has shown that membrane sealing is a function of neurite retraction.³⁸ Therefore, it can be concluded that neurite mobility affects sealing. This could also explain why different sealing outcomes were observed by different groups using the same preparations. Yawo and Kuno pretreated cockroach nerve cords to free the individual giant axons from attaching to the extracellular matrix.⁶⁴ Consequently, this led to an increase in the mobility of the axonal membrane. As a result, they observed a rapid recovery of membrane potential and input resistance following a complete transection. In contrast, Meiri and colleagues did not separate the individual giant axons and failed to see such recovery with similar transection.⁴² Therefore, the difference in sealing observed in these two groups may be explained by the fact that adhesion of the axonal membrane to the extracellular environment may hinder the neurite mobility.

It is likely that there are other factors that affect the reestablishment of an effective barrier to the influx of extracellular ions. For example, using the same set of techniques, Krause and colleagues³⁴ found that earthworm axons seal their cut ends, but squid giant axons do not, indicating that a speciesspecific difference also affects the ability to form such barrier. In the case of anoxia, the membrane barrier may also be compromised through different mechanisms, which are more subtle and mainly based on the increased permeability to damaging ions. For instance, Stys, Waxman and colleagues^{36,37,55,56,61} have shown evidence of a persistent Na⁺ leakage through non-inactivating Na⁺ channels following 1 h of anoxia using mammalian optic nerve. They have demonstrated that such Na⁺ leakage could induce secondary damage by causing the influx of Ca⁺ through reverse operation of the Na⁺/Ca²⁺ exchanger. To support this notion, many

studies have shown that reducing Na⁺ entry after anoxic and physical injury can significantly reduce tissue damage and enhance functional recovery in a wide range of tissue preparations.^{1,5,27,44,45,56,57}

Gap potential as a functional measure of sealing

In our protocol, there was concern that our final mechanical injury might be conditioned by the injuries the spinal cord received during extraction. This is unlikely, however, due to the following reasons. During the process of removing the spinal cord from the animal, the cord was first transected for the appropriate length and the white matter was then separated from the gray matter. Following these two transections, at least 2 h, (minimum of 1 h at 20°C to 25°C and 1 h at 37°C), was given to allow the cord to recover, both in terms of metabolism and to allow for complete membrane sealing. We have found that, although the cut ends of the spinal cord in 25°C do not reseal within 60 min, an additional 60 min in 37°C results in complete sealing (unpublished observations). This complete sealing is achieved both anatomically (the establishment of axonal membrane integrity) and electrophysiologically (the complete recovery of membrane potential). Since the damage caused by the first two transections appears to be completely recovered 2 h after surgery, the response of the third, experimental injury can be studied in temporal isolation. Furthermore, the experimental injury is made at least 10 mm away from the end of the strip where the isolating transverse cuts were made. This length is greater than the length constant of spinal cord axons. Thus, the experimental injury is spatially, as well as temporally, separated from the injuries created during extraction.

We have also considered the reliability of the gap potential in reflecting membrane integrity. Although membrane integrity is a necessary factor supporting normal membrane potential, it is not the only factor. For example, membrane depolarization (i.e. loss of membrane potential) could also be caused by an increase in membrane permeability between the cut end and the recording gap. In our sucrose gap model, however, membrane integrity should be the dominant factor determining membrane potential. This is because (i) the gap potential and HRP exclusion test correlated well quantitatively (Fig. 7), and (ii) the transections were made very close to the gap (less than 1 mm). Moreover, all cords that showed complete anatomical sealing never failed to substantially recover their gap potential 60 min post-transection. The gap potential would not be expected to recover completely if the membrane depolarized due to increased permeability to extracellular ions regardless of sealing.

However, at earlier stages post-transection, membrane depolarization caused by an increase in permeability may exist. For example, the initial phase of potential recovery occurred consistently, even in the absence of sealing to HRP or of subsequent return to the original baseline resting membrane potential. The initial phase of recovery of the gap potential therefore seems to be based not on sealing of a proportion of the nerve fibers in the tract, but perhaps on a decrease in the core conductance near the cut end of the fibers. This would be consistent with the kind of constriction that has been seen in morphological studies of giant axons³⁴ and which has been shown to be unaffected by replacement of extracellular Ca²⁺ with Mg²⁺.²³ Another possibility is an increase in membrane permeability immediately following

transection along the axolemma between the cut end and the recording gap. However, this depolarization recovers gradually after transection, even though the transected ends are still open.

Temperature dependence of membrane sealing following transection

Resealing of axons, recorded by either electrophysiological or histological means, appears to depend on bath temperature. At 25°C, sealing does not take place within the duration of the experiment, and for up to 2 h post-transection (unpublished observations). At the mildly hypothermic and normal physiological temperatures of 31°C and 37°C, respectively, axons routinely seal their transected ends within 60 min. In addition, raising the bath temperature from 25°C to 37°C 60 min after transection can still induce sealing (unpublished observations). The mechanism of temperaturedependent sealing is not entirely clear at this point. Since temperature can directly influence membrane fluidity, it is likely that the temperature dependency of sealing reflects the dependence of sealing on membrane fluidity. However, as Yawu and Kuno pointed out, the action of certain membrane enzymes that are thought to be involved in the sealing process may also be affected by temperature.⁶⁴ In fact, Wang and colleagues have shown that lowering the temperature from 37°C to 22°C decreases the phospholipase A₂ activity in cultured endothelial cells.⁶⁰ Therefore, the failure of membrane sealing at low temperature due to the membrane's lower-mobility state could be caused by low temperature and/or a lack of enzyme actions that are essential for membrane sealing.

Interestingly, when axons were transected at 40°C, a human fever condition, 75% of the spinal cords sealed. However, most of the spinal cords that did seal at 40°C were observed, electrophysiologically and histologically, to seal slightly better than spinal cords sealing at 37°C. One obvious explanation for a slightly better sealing at 40°C is that the membrane may be more fluid at this higher temperature. However, it is also possible that the membrane is unstable at higher temperatures due to excessive membrane fluidity and mobility and overactivation of certain enzymes following transection. These factors may act synergistically to cause membrane breakdown through unknown mechanisms.

Our data also indicate that when cords were warmed from 25° C to 37° C, small axons sealed better (Fig. 6). A similar observation was made by Howard and colleagues³¹ using rat dorsal root axons. They noted that myelinated axons with small diameters resealed faster than those with larger diameters following transection, when bathed in physiological salines. This phenomenon may be explained again by the fact that the cut end of the small-diameter axons would be more easily closed by axolemmal constriction. However, the exact mechanism underlying this finding is not clear at this time. It is likely, however, that the difference in the rate of sealing may still lie in the mechanical mobility of the membrane in axons with various calibers.

Clinical implications

According to our findings, hypothermic treatment (below 25°C) following mechanical injury compromises the acute repair of broken membranes. However, the moderate hypothermic condition has been suggested to be used clinically to improve the outcome of trauma by reducing the damage from primary and secondary injuries.¹⁶ This has raised a question on the strategy of how and when to employ hypothermic treatment to mechanically injured spinal cord, if at all. It is possible, and testable, that the initial normal temperature mediating these resealing responses may need to change with time in order to achieve better recovery following nerve injury. It is also possible that a mild cooling temperature should be chosen which is low enough to protect tissue from ischemic injury and high enough to promote resealing. Based on present and previous studies, a temperature around 31°C is most appropriate to apply in mechanically injured spinal cord. At 31°C, the sealing is not significantly different than that of 37°C, while significantly better recovery from hypoxia can be achieved at this temperature in in vivo studies.^{17,4,20} Therefore, it seems to indicate that in whole animal spinal cord where the mechanical injury is usually coupled with ischemic injury, the hypothermia should be either controlled at around 31°C, or applied after 60 min if the temperature is to be below 25°C. To support this, Povlishock and colleagues have recently demonstrated that a moderate hypothermia of 32°C applied in the acute postinjury period drastically reduced the number of damaged axons in a rat following traumatic brain injury.¹²

However, caution needs to be taken in applying hypothermic treatment to different situations. For example, temperature dependence of membrane fluidity may be different in various animals, including human. Furthermore, there is an ongoing tissue deterioration caused by various secondary insults, such as oxygen free radicals and ischemia, in addition to the physical injury inflicted by primary impact. This deterioration may be more significant and consequential in a more diffusive scale than local sealing events in certain preparations and experimental conditions. An example of this is demonstrated by Lucas and colleagues.³⁹ They have shown that cooling mammalian spinal cord neurons lesioned close to their perikarya immediately after cell surgery to 17°C led to a significant increase in survival but more moderate cooling (27°C) did not. This seems to indicate that the slowing of axonal resealing could be offset by a cooling-induced slowing down of the deterioration process that enhanced overall cell survival.

Acknowledgements—We thank Drs Andrew R. Blight and Richard B. Borgens for their support and encouragement, and Melissa Peasley and Jason Renschler for their invaluable assistance with the analysis of these experiments. We also thank Dr Brad Duerstock for his critical reading of the manuscript. This study was supported by funding from Purdue University and a grant from NIH-NINDS. Part of this study was conducted at the Division of Neurosurgery, University of North Carolina.

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R. Shi and J. D. Pryor

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(Accepted 22 February 2000)