Spinal Cord Contusion Causes Acute Plasma Membrane Damage

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Abstract

Spinal cord injury (SCI) launches a complex cascade of events that leads to progressive damage and loss of function. Compromise of plasma membrane integrity due to the mechanical impact is an acute event that may contribute to cellular dysfunction. Therefore, the objective of this study was to better understand the extent of acute plasma membrane damage associated with SCI as a function of injury severity and membrane defect size. Fluorescent cell-impermeant dyes were injected into the cerebrospinal fluid of adult male rats prior to contusion injury, and the anatomical location of cell bodies and axons taking up the dye within 10 min following SCI was quantified. Lucifer yellow uptake was assessed as a function of impact force (experimental groups: sham, 100 kdyn, 150 kdyn, and 200 kdyn force). In a separate group of animals, FITC-conjugated dextran molecules of various sizes (3 kDa and 10 kDa with a 1.6-nm and 2.7-nm radius, respectively) were used to approximate the size of membrane defects following moderate injury (150 kdyn force). Quantification revealed that cellular uptake of lucifer yellow was positively correlated with the force of the mechanical impact, indicating that the severity of injury is related to the degree of acute membrane failure. In addition, after moderate injury, cell bodies and axons (located up to 2 mm and 3 mm from the epicenter, respectively) took up significantly more of the 3-kDa and 10-kDa dextran permeability marker compared to sham controls. Permeable neuronal cell bodies exhibited a morphological appearance characterized by pericellular blebbing, suggesting that plasma membrane compromise is associated with pathophysiological cellular alterations. Collectively, these results enhance our understanding of acute SCI and provide targets for developing novel treatment strategies.

Key words: bleb; mechanoporation; permeability; plasma membrane; spinal cord injury

Introduction

SPINAL CORD INJURY (SCI) IS A DEBILITATING CONDITION that dramatically affects quality of life for the affected individual and the family. Clinically available treatments provide modest benefit, if any at all; therefore current research is aimed at developing more effective therapies for spinal cord repair and regeneration (Baptiste and Fehlings, 2007; Hall and Springer, 2004; Kwon et al., 2004). A greater understanding of the time course of the destructive and degenerative mechanisms involved in SCI will improve the ability to develop targeted therapeutic strategies.

In the acute phase of SCI, primary damage occurs as a direct result of trauma when structural thresholds are surpassed, leading to immediate physical and biochemical cellular alterations. These acute events initiate a complex secondary injury cascade that ensues in the following hours, weeks, and months. Secondary injury mechanisms such as apoptotic cell

death, inflammation, and formation of the glial scar are considered to be deleterious to regeneration and are commonly targeted for treatment of SCI (Dumont et al., 2001; Hagg and Oudega, 2006). Although it is well-documented that increasing the severity of loading parameters exacerbates tissue damage and neurological dysfunction (Basso et al., 1996; Beattie et al., 1997; Kloos et al., 2005; Scheff et al., 2003; Wrathall et al., 1985), relatively little is known about how the mechanical impact (primary injury) initiates secondary injury. Improving the current understanding of the characteristics of primary damage after contusion SCI can facilitate a better understanding of acute injury mechanisms and their relationship to the overall pathophysiology.

One direct result of the mechanical impact in traumatic CNS injury is the formation of non-specific breaches in the neuronal plasma membrane. Plasma membrane compromise has been observed in many models of neuronal injury and is postulated to be detrimental to post-injury outcomes (Choo

et al., 2007; Farkas et al., 2006; Geddes-Klein et al., 2006; Geddes et al., 2003; Kilinc et al., 2007; LaPlaca et al., 1997; LaPlaca et al., 2007; Povlishock and Pettus, 1996; Prado et al., 2005; Shi et al., 2000; Shi and Whitebone, 2006; Whalen et al., 2007). Under physiologic conditions, the plasma membrane serves as a barrier that maintains the structure and organization of the cell, regulates molecular transport, and preserves concentration gradients essential for cell survival and function. However, these homeostatic mechanisms are disrupted when mechanical impact to CNS tissue physically ruptures the plasma membrane. Although many permeabilized cells die immediately through necrosis, a subset are able to reseal without undergoing acute cell death or degeneration (Farkas et al., 2006; Shi, 2004; Shi et al., 2000; Whalen et al., 2007). Surviving cells, however, may experience downstream consequences of plasma membrane compromise (for review see Barbee, 2005; Farkas and Povlishock, 2007). By temporarily or permanently destroying the barrier between the cytosol and the extracellular fluid, ions and other molecules may freely enter or exit the cell. This unregulated molecular flux would be expected to negatively affect cell function and survival. For example, calcium influx down its concentration gradient could result in mitochondrial damage, aberrant enzyme activation, changes in gene expression, and apoptosis (for review see Dumont et al., 2001). In fact, increases in plasma membrane permeability after CNS injury have been associated with pathophysiological events such as protease activation (Farkas et al., 2006), conduction block (Shi and Borgens, 2000), and delayed cell death (Whalen et al., 2007). Although membrane compromise may play a critical role in the pathophysiology of SCI, little is known about the anatomical distribution of permeable cells within the injured spinal cord, or the size of the membrane defects in those cells.

The objective of this study was to better characterize acute plasma membrane damage induced by *in-vivo* contusion SCI, since membrane compromise may be an initiating factor for numerous deleterious events. The spatial profile of cellular damage was assessed as a function of injury severity and the size of the plasma membrane defect in a rat contusion model. Results obtained in this study aid in defining the extent of SCI-induced primary damage, and can facilitate the discovery of pathophysiological mechanisms that may be effectively targeted in therapeutic interventions.

Methods

Surgical procedures

All procedures involving animals were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (weight 275–400 g; total $n = 35$) were anesthetized with sodium pentobarbital (50 mg/kg). Isoflurane was used (1–3%) to maintain anesthesia when necessary. The animals were placed on a stereotaxic apparatus and the atlanto-occipital membrane was surgically exposed. An incision was made in the dura, and a catheter composed of PE10 tubing was attached to the needle of a Hamilton syringe and inserted into the intrathecal space. A fluorescent dye normally unable to penetrate the cell membrane was injected slowly over 10 min (4% lucifer yellow in deionized water, 2.5% FITC-conjugated 10-kDa dextran [10dex] in saline, or 2.5% FITC-conjugated 3-kDa dextran

[3dex] in saline; 15–20 μ L total volume). Only one type of fluorescent dye was injected into the cerebrospinal fluid of each animal. All permeability markers were obtained from Invitrogen (Carlsbad, CA). The concentrations and solvents used were based on previous studies assessing plasma membrane damage in neural tissue (Choo et al., 2007; Farkas et al., 2006; Howard et al., 1999). Ten minutes after the dye injection, the catheter was removed and the muscle and skin at the surgical site were sutured closed.

The permeability dyes were allowed to diffuse throughout the CSF for 3 h prior to injury. A laminectomy was performed at the T10 vertebral level to expose the underlying spinal cord tissue. Three injury severities were used to examine the distribution of membrane damage: 100, 150, or 200 kdyn impact force (Infinite Horizons device; Precision Systems and Instrumentation, Lexington, KY). These forces were chosen because they have been well characterized in the literature, and are shown to correspond to mild, moderate, and severe injury levels, respectively, according to histological and behavioral outcome measures (Scheff et al., 2003). It should be noted that the impact force corresponds linearly with displacement in this injury model (Scheff et al., 2003). The velocity of impact was approximately 120 mm/sec for all injury severities. Sham animals were subjected to all surgical procedures including the dye injection and laminectomy, but the spinal cord was not impacted. Sample sizes ranged from 3–5 per experimental group. Animals were transcardially perfused 10 min following SCI with phosphate-buffered saline (PBS) followed by fixative (4% paraformaldehyde/0.1% glutaraldehyde in PBS). The vertebral column was subsequently removed from the rat and immersed in fixative at 4°C. After 24 h, spinal cord tissue was dissected from the vertebral column and placed in sucrose at 4°C. Tissue was cut into 2-cm segments, frozen in OCT, and then cryosectioned in the transverse plane.

Verification of cellular uptake of permeability markers

Immunohistochemistry was performed using thin sections (10 μ m) to verify that permeability marker uptake was neuronal. Primary antibodies specific for neurofilament-160 (NF-160, 1:100; Sigma NF264, St. Louis, MO), NeuN (1:100; Millipore MAB377, Billerica, MA), and glial fibrillary acidic protein (GFAP, 1:500; Millipore MAB360) were used to identify axonal projections, neuronal cell bodies, and reactive astrocytes in the tissue, respectively. Briefly, slides were rinsed in PBS, then immersed in blocking/permeabilization solution (8% goat serum and 0.4% Triton X100 in PBS) for 1 h at room temperature. Sections were then incubated in primary antibody solution (primary antibody diluted in 8% goat serum and 0.4% Triton X100 in PBS) overnight at 4°C in a humidified chamber. Slides were rinsed in PBS then incubated in AlexaFluor 546 or 568 (1:500, Invitrogen) secondary antibody solution (2% goat serum in PBS) for 2 h at room temperature in a humidified chamber. Following a final rinse in PBS, the slides were coverslipped using an aqueous mounting medium. Images were obtained at 40 $\times$ magnification using a confocal microscope (LSM 510; Carl Zeiss MicroImaging Inc., Thornwood, NY).

Quantification of permeability marker uptake in cell bodies

Three different fluorescent dyes (lucifer yellow, 3dex, or 10dex) were used to assess membrane damage in neuronal

TABLE 1. PERMEABILITY MARKERS USED TO ASSESS PLASMA MEMBRANE DAMAGE

Permeability marker	Molecular size (Da)	Molecular radius (nm)
Lucifer yellow	457	0.6 nm
FITC-conjugated dextran	3000	1.6 nm
FITC-conjugated dextran	10,000	2.7 nm

Cellular uptake of three different fluorescent aldehyde-fixable molecules was quantified at 10 min following acute spinal cord injury. Dextran radii were approximated according to the equation $r = 0.488 \text{ MW}^{0.437}$ (to account for its chain-like configuration), whereas the radius of lucifer yellow was calculated using the equation $r = 0.421 \text{ MW}^{0.427}$ (which assumes a globular conformation). These equations are based on a previous study in which the relationship between molecular weight and size was derived from experimental data examining particle movement across biological membranes (Oliver et al., 1992). Note that dextran size is reported as an average, and actually contains a range of molecular masses.

cell bodies (Table 1). Lucifer yellow uptake was assessed after sham surgery or mild, moderate, and severe injury (100, 150, or 200 kdyn impact force) in order to investigate whether plasma membrane damage is dependent upon impact force. Additional experiments compared uptake of 3dex and 10dex after moderate injury (150 kdyn) in order to better characterize the size of SCI-induced membrane defects.

Uptake of permeability markers was quantified at 1-mm increments up to 3 mm from the injury epicenter in both the rostral and caudal directions ($n = 3-5$ rats per group). The number of permeable cells was quantified in thick sections ($35 \mu\text{m}$) to allow for visualization of large motor neurons. Cells were counted manually with the assistance of Stereo-Investigator software (MBF Bioscience, Williston, VT). Although the fluorescent dyes accumulated around blood vessels, only cells with neuronal morphology (as shown in Fig. 1) were included in the quantification. Sections at the injury epicenter were excluded from the analysis because the extensive tissue damage did not permit reliable cell counts. In order to reduce variability, two adjacent sections were counted at each 1-mm increment distance from the epicenter, and these values were averaged together to yield one data point per animal at each distance.

Quantification of axonal uptake of dextran molecules

The density of dextran-positive axons was quantified within thin sections ($10 \mu\text{m}$) using ImageJ software (National Institutes of Health, Bethesda, MD). Lucifer yellow was not observed within axonal projections, possibly due to dye leakage or rapid diffusion of the small molecule along the axon. Montage images of each section were acquired at $10\times$ magnification using NeuroLucida software (MBF Bioscience). The density of permeable axons was determined within an area of approximately $85 \mu\text{m}^2$ in the ventral, dorsal, and lateral regions. Within these areas, thresholds for fluorescence intensity (determined by a blinded observer) and size ($0.25-25 \mu\text{m}^2$) were used to eliminate autofluorescent debris from the analysis. Each section was quantified using two independent measurements in each quadrant of the section (ventral, dorsal, lateral left, and lateral right) and an average was taken.

Assessment of cellular morphology

Cellular morphology is presented in paraffin sections for display purposes ($n = 2$). The images shown are representative of the cellular permeability uptake observed in cryopreserved sections, but this methodology permits clearer visualization of cellular morphology at the microscopic level. 3dex was injected intrathecally 3 h prior to 150-kdyn spinal cord impact, and animals were perfused 10 min after SCI with PBS followed by 4% paraformaldehyde and 0.1% glutaraldehyde in PBS (as described above). Longitudinal sections ($5 \mu\text{m}$ thick) were stained with cresyl echt violet prior to imaging. Fluorescent and bright field images were obtained at $63\times$ magnification using a confocal microscope (Zeiss LSM 510; Carl Zeiss Corporation, Oberkochen, Germany).

Statistical analysis

All statistical tests were conducted using SigmaStat 3.0 software (Systat Software Inc., Richmond, CA). The statistical analysis used for each data set varied based on the hypothesis being tested, as described below. Data are presented as mean $\pm$ standard deviation for parametric data sets, whereas data that did not meet the assumptions of parametric statistical tests are presented as median $\pm$ range.

A linear regression was used to determine the relationship between impact force (independent variable) and lucifer yellow uptake (dependent variable). The total number of permeable cells counted at 1, 2, and 3 mm in the rostral and caudal directions from the injury epicenter was subtracted from baseline (the average number of permeable cells in the sham group). A linear regression was performed, with actual impact force as the dependent variable and the number of permeable cells as the independent variable. Note that the control mechanism of the Infinite Horizons device causes the actual impact force to be greater than or equal to the intended impact force, and varies to some degree from animal to animal.

In order to compare the overall amount of cellular uptake of 3dex and 10dex after injury and sham procedures, we first determined the total number of permeable cells within 3 mm of the rostral and caudal areas of the epicenter. Data were found to have equal variance and a normal distribution. A one-way ANOVA followed by Student-Newman-Keuls pairwise comparison was used for analysis.

We also hypothesized that the experimental groups would have differing amounts of permeability marker uptake within discrete locations relative to the injury site. This represents a multi-factorial experimental design (location relative to epicenter and injury severity or permeability marker size), with repeated measures on the location factor. In all cases, these data did not meet the assumptions of parametric statistical analyses (equal variance and/or normality), and performing a logarithmic transformation of the data did not create a parametric data set. Therefore, we analyzed the data using the Scheirer-Ray extension, as previously described (Scheirer et al., 1976; Sokal and Rohlf, 1995). Briefly, raw values were converted to a ranked data set by sorting the values from smallest to largest and then assigning a rank value to each data point. When two or more data points were the same, the average rank value was assigned to all tied data points. This ranked data was then analyzed using a two-way repeated measures ANOVA to determine whether differences existed between the groups. In all cases, identical conclusions were

FIG. 1. Permeability marker uptake following SCI. Histological assessment revealed permeability marker uptake in neuronal cell bodies and axons 10 min following SCI. Images shown here represent uptake of FITC-conjugated dextran within 1 mm caudal of the injury epicenter (3 kDa, **B** and **C**; 10 kDa, **E** and **F**). Immunohistochemistry specific for NeuN (**A** and **C**) and NF-160 (**D** and **F**) verified that dye uptake was within neuronal cell bodies and axons, respectively. Uptake was similar for both dextrans used in the study. However, lucifer yellow was only observed within cell bodies and not axonal projections, presumably due to dye leakage or rapid diffusion of the small molecule along the axon (scale bars = 50 μ m (**A**–**C**) and 100 μ m (**D**–**F**)).

made regarding differences between groups when the ranked data and the raw data were analyzed using a two-way repeated measures ANOVA, which further supports the conclusions drawn from the study and emphasizes the robustness of the ANOVA. All F-scores and *p* values reported in this article were derived from analysis of the ranked values rather than the raw scores. If differences were found between groups, the raw (unranked) data were compared using the Mann-Whitney U test, a post-hoc test for non-parametric data.

Results

Intra-axonal and intra-cellular uptake of fluorescent dyes following SCI

Immunohistochemistry confirmed that regions with intense fluorescence represented cellular uptake of the permeability markers. NeuN staining demonstrated that dye uptake within the gray matter was neuronal (Fig. 1A–C). All three permeability markers (lucifer yellow, 10dex, and 3dex) were easily visualized within cell bodies, allowing for quantification of cells that had become permeable to the fluorescent dye. Dye uptake was distributed homogeneously throughout the

gray matter, with no preferential uptake in the dorsal or ventral regions.

Axonal uptake of the permeability markers was verified using immunohistochemistry specific for NF-160. Regions with dye accumulation co-localized with NF-160 staining, confirming intra-axonal uptake of the permeability marker (Fig. 1D–F). Accumulation of 10dex and 3dex was observed within the axolemma. Although influx of the smallest dye (lucifer yellow) was expected within axons, the presence of this molecule was not observed at levels above background. The absence of intra-axonal lucifer yellow may be attributed to a low signal-to-noise ratio due to diffusion of the dye along the axon, or leakage of the molecule out of large tears in the plasma membrane. Axonal dextran uptake was evenly dispersed at the injury epicenter, with the vast majority of axons taking up the dye.

Additionally, permeability marker uptake was observed within astrocytes (mainly located adjacent to blood vessels) within the spinal cord white and gray matter, as confirmed by immunohistochemistry specific for GFAP (data not shown). This was seen in both injured and sham animals; therefore we did not interpret this to be an SCI-induced response. Perme-

ability marker uptake within cells with this morphology was not included in the quantification.

Lucifer yellow uptake within cell bodies following sham surgery or injury at mild, moderate, or severe impact forces

Lucifer yellow uptake within cell bodies was quantified at 1-mm increments up to 3 mm from the epicenter in the rostral and caudal directions after mild, moderate, or severe injury, as well as after sham surgery ($n=4-5$ per group; Fig. 2). Representative images are shown in Figure 2A–H.

A linear regression was performed to examine whether there was a relationship between impact force and cellular permeability to lucifer yellow, as shown in Figure 2I. Results demonstrated that there was a positive correlation between these variables ($r=0.80$, $p<0.001$). This indicates that the degree of acute biophysical membrane failure is related to the severity of the mechanical impact.

We also analyzed the data within each distance to determine if altering the injury severity causes a change in the number of permeable cells at discrete distances. Analysis of the data using the Scheirer-Ray extension of the two-way repeated measures ANOVA demonstrated a significant main effect among groups ($F=8.546$, $p=0.002$) and among distances ($F=21.56$, $p<0.001$). There was also a statistically significant interaction effect ($F=4.506$, $p<0.001$). Mann-Whitney U pairwise comparisons revealed several significant differences between groups, as depicted in Figure 2J. An impact force of 100 kdyn resulted in greater lucifer yellow uptake compared to sham injury at 2 mm and 1 mm rostral to the epicenter, as well as 2 mm caudal. Interestingly, permeability marker uptake at 2 mm rostral of the epicenter was significantly less than sham, suggesting that there may have been a “sink” effect in which permeability marker became more concentrated near the epicenter at the time of injury, and was less concentrated at locations farther away from the site. Moderate injury (150-kydyn impact) caused significantly more permeability marker uptake compared to sham at 1 mm rostral and 1, 2, and 3 mm caudal of the injury epicenter. Severe injury (200 kdyn) enhanced permeability marker uptake compared to sham injury at 1 mm rostral as well as 1, 2, and 3 mm caudal of the injury. In addition, both moderate and severe injury resulted in greater cellular permeability compared to mild injury at 2 and 3 mm rostral and caudal to the site of injury.

Plasma membrane defect size within cell bodies following SCI contusion

We compared the cellular uptake of two different permeability markers (3dex and 10dex) in order to better characterize the size of SCI-induced defects in the plasma membrane ($n=3-4$ per group; Fig. 3). We did not directly compare lucifer yellow uptake to dextran uptake because of differences in the shape of the molecules, and the finding that sham uptake of lucifer yellow was much greater than dextran. In animals receiving the dextran permeability markers, sham uptake was not significantly different for 3dex and 10dex; therefore, these sham groups were combined for analysis. Representative images are shown in Figure 3A–C.

To determine if there were overall differences between the groups, we compared the sum total of cell counts at the dis-

crete distances assessed in this study (data shown in Figure 3D). One-way ANOVA revealed differences between the groups ($F=19.537$, $p<0.001$). Student-Newman-Keuls post-hoc comparisons revealed significant differences among all three groups ($p<0.001$ for sham versus 3dex, $p=0.044$ for sham versus 10dex, and $p=0.003$ for 3dex versus 10dex). These results demonstrate that injury caused more overall permeability marker uptake compared to sham controls for both dyes, and that there was greater uptake of the smaller 3dex molecule in comparison to the larger 10dex molecule.

Additionally, we assessed differences in permeability marker uptake at discrete distances from the injury epicenter (Fig. 3E). Statistical analysis (Scheirer-Ray extension of the two-way repeated measures ANOVA) demonstrated a significant main effect among groups ($F=9.907$, $p=0.003$) and among distances ($F=13.07$, $p<0.001$), whereas the interaction effect was not statistically significant ($F=1.935$, $p=0.061$). Mann-Whitney U pairwise comparisons revealed statistically significant differences as follows. Uptake of the 3dex molecule after injury was greater than sham controls at 2 mm rostral as well as 1 and 2 mm caudal of the injury site. Cellular permeability to the 10dex marker after injury was increased compared to sham controls at 1 and 2 mm in both the rostral and caudal directions.

Plasma membrane defect size in axons following SCI contusion

Axonal uptake of 10dex and 3dex was assessed at discrete distances from the injury epicenter in order to determine the size of membrane defects ($n=3-4$ per group). Permeability marker uptake in the sham-injured 10dex and 3dex groups were not statistically different; therefore, all sham animals were combined in the analysis. Representative images are shown in Figure 4A–C.

We analyzed the density of axonal uptake of the permeability markers at discrete distances from the injury epicenter (Fig. 4D). Data analysis demonstrated a significant main effect among groups ($F=42.779$, $p<0.001$) and distances ($F=14.096$, $p<0.001$), and a significant interaction effect ($F=1.981$, $p=0.041$). Mann-Whitney U pairwise comparisons were then performed. Post-injury axonal 3dex uptake was greater than sham controls at the injury epicenter, and at 1 and 2 mm in both the rostral and caudal directions. Axonal permeability of the 10dex molecule after injury was greater than sham controls at the injury epicenter, 1 and 2 mm in the rostral direction, and 1, 2, and 3 mm in the caudal direction. There were no differences in 3dex uptake compared to 10dex uptake after injury at any distance relative to the injury epicenter.

Morphological alterations associated with plasma membrane compromise following SCI

High-magnification confocal imaging of cells within spinal cord tissue revealed abnormal cellular morphology in many cells taking up the permeability marker 3dex. In many permeable cells, we observed pericellular blebs that were not filled with the 3dex permeability marker. Blebs were visibly less dense than the cytoplasm within the cell, and varied in size from very large blister-like features that surrounded nearly the entire cell (typically accompanied by cellular shrinkage; Fig. 5D–5F), to small circular extensions on a small portion of the cell surface (Fig. 5A–C and G–I). Blebbing was

FIG. 2. Lucifer yellow uptake as a function of injury severity and distance from the epicenter. A dye exclusion assay was used to characterize plasma membrane damage 10 min following sham surgery (**A** and **E**) or contusion SCI with an impact force of either 100 kdyn (**B** and **F**), 150 kdyn (**C** and **G**), or 200 kdyn (**D** and **H**). Images shown here were taken from representative sections 2 mm caudal of the epicenter ($n=4-5$ per group). A linear regression of the total number of lucifer yellow-positive cells (with baseline sham values subtracted) revealed a positive correlation between the actual impact force imparted to the spinal cord and the number of permeable cells (**I**, mean $\pm$ standard deviation; $r=0.80$, $p<0.001$). Further analysis of the data revealed differences between the groups at discrete distances from the epicenter (**J**; median $\pm$ range; $*p<0.05$ compared to sham, $^{\dagger}p<0.05$ compared to 100 kdyn; scale bar = 500 μ m [**A-D**], 50 μ m [**E-H**]).

FIG. 3. FITC-dextran uptake in cell bodies within gray matter. The number of cells permeable to 3-kDa FITC-conjugated dextran (3dex; **B**) and 10-kDa FITC-conjugated dextran (10dex; **C**) was quantified within tissue sections taken at discrete distances from the injury epicenter and compared to sham controls (**A**) ($n = 3-4$ per group; mean $\pm$ SEM). A comparison of the sum total of the permeable cells in each group (**D**) demonstrated a significant difference between 3dex and 10dex uptake after injury compared to sham injury, and that uptake of 3dex was greater than 10dex uptake after injury (mean $\pm$ standard deviation; * $p < 0.05$ compared to sham, † $p < 0.05$ compared to 10dex). Analysis of the data at discrete distances from the epicenter (**E**) revealed significantly greater cellular uptake of both 3dex and 10dex compared to sham controls up to 2 mm from the injury site in both the rostral and caudal directions (median $\pm$ range; * $p < 0.05$). Images shown here are confocal reconstructions at 1 mm caudal of the epicenter (scale bar = 40 μ m).

observed in a very small subset of cells that did not take up 3dex (<1%) but was far more common in 3dex-positive cells. We therefore concluded that neuronal blebbing is associated with plasma membrane permeability. Although others have observed blebbing in response to chemical or mechanical cellular perturbation in other cell types (Barros et al., 2003; Keller et al., 2002; Marin-Castano et al., 2005; Mills et al., 1998; Shi et al., 2005), we are the first to our knowledge to report neuronal blebbing *in vivo* after traumatic CNS injury.

Discussion

Investigation of primary membrane permeability changes in SCI may facilitate an improved understanding of the overall pathophysiology and thus may lead to the development of treatment strategies targeted at spinal cord repair. To this end, we characterized acute plasma membrane damage in a clinically relevant contusion model by quantifying the plasma membrane defect size in both cell bodies and axons

FIG. 4. Axolemmal permeability marker uptake. Axonal uptake of 3dex (**B**) and 10dex (**C**) permeability markers after injury was compared to sham controls (**A**). The density of axons taking up the permeability marker was quantified at discrete distances from the epicenter out to 3 mm in the rostral and caudal directions (**D**). The density of dextran-positive axons was significantly greater than the sham controls up to 2 mm in both the rostral and caudal directions. No significant differences between 3dex and 10dex uptake were found, demonstrating that axonal membrane defects are at least 2.7 nm in radius ($n = 3-4$ per group; median $\pm$ range; $*p < 0.05$ compared to sham animals). Images shown here are confocal reconstructions taken from sections 1 mm caudal to the injury epicenter (scale bar = 40 μ m).

as a function of insult severity and anatomical location. Our investigation of the effect of injury severity demonstrated that impact force and acute cellular permeability are positively correlated, as increasing the impact force caused a corresponding increase in permeability marker uptake. This is supported by previous studies that have examined the relationship between injury biomechanics and plasma membrane permeability (Choo et al., 2007; Geddes-Klein et al., 2006; Geddes et al., 2003; Shi and Whitebone, 2006). *In-vitro* work has demonstrated that membrane permeability increases with increasing strain rate and magnitude in axons as well as cell bodies (Geddes et al., 2003; Shi and Whitebone, 2006). In addition to a dependence upon injury parameters, the extent of primary damage has been shown to be a function of the mode of injury: a recent *in-vivo* study demonstrated that distraction and dislocation injury results in more extensive plasma membrane damage in comparison to contusion SCI (Choo et al., 2007). Our data provide further supporting evidence in an *in-vivo* model to suggest that the parameters of the mechanical impact dictate acute biophysical plasma membrane failure.

We also evaluated the size of the membrane defect by quantifying uptake of 10dex and 3dex molecules after moder-

ate contusion SCI. Examination of both permeability markers confirmed that spinal cord impact causes plasma membrane damage in axons and cell bodies. Comparison of overall 3dex and 10dex uptake revealed differences in permeability marker uptake within the cell bodies located in the gray matter. Therefore, a subset of cells allowed entry of the smaller 3dex molecule while excluding the larger 10dex. Although no significant differences were found between post-injury 3dex and 10dex uptake within a particular anatomical location, this may be due to the relatively small sample size in this experiment (sample size calculation showed that $n = 5$ per group would be necessary to achieve 80% power at 1 mm caudal of the injury site, but our study used only 3-4 per group). Overall, these results reveal at least a partial size range of plasma membrane defects occurring after *in-vivo* SCI, and support a previously published *in-vitro* study demonstrating that membrane defect size in neuronal cell bodies varies according to the degree of mechanical damage (Geddes et al., 2003).

Quantification of permeability marker uptake in axons showed no significant difference between SCI-induced uptake of 10dex and 3dex; therefore, we concluded that axolemmal plasma membrane defects are at least 2.7 nm in

FIG. 5. Membrane blebbing is associated with plasma membrane permeability. High-magnification confocal imaging (63 $\times$) of 3dex-filled cells within histological sections fixed 10 min after SCI revealed that membrane permeability is associated with SCI-induced cellular blebbing. Images shown here are representative phase (A, D, G, and J), fluorescence (B, E, H, and K), and phase overlaid with fluorescence (C, F, I, and L). A subset of cells near the epicenter (A–C) displayed a necrotic appearance (stars), but did not take up the fluorescent dye, whereas other neighboring cells became 3dex-positive. Most permeable cells were comprised of a dense, dextran-filled cytoplasm surrounded by a less dense layer of fluid that did not fill with the fluorescent dextran molecule (arrows in A–I). The majority of dextran-impermeable cells did not exhibit blebbing (G–L). These results indicate that plasma membrane damage is associated with pathophysiological alterations in cellular morphology.

radius. Previous studies have shown axonal uptake of molecules as large as 44 kDa (horseradish peroxidase) (Pettus et al., 1994; Pettus and Povlishock, 1996; Shi et al., 2000; Shi and Whitebone, 2006). Taken together, these data suggest that contusion injury causes larger membrane defects in axons compared to cell bodies. In comparison to cell bodies, axons may be more vulnerable to mechanical failure because of their elongated morphology or anatomical location on the periphery of the spinal cord. In addition, *in-vitro* evidence suggests that axons require much longer for resealing (on the order of 1 h) (Eddleman et al., 1997; Shi et al., 2000; Yoo et al., 2003), whereas neuronal cell bodies are able to reseal within minutes (Geddes et al., 2003; Prado et al., 2005). Although it is difficult to directly compare these studies due to variations in cell type/species, mechanical insult parameters, and the size of permeability markers, the results collectively suggest that cell bodies and axons differ in their structural thresholds and subsequent repair response. It should also be noted that the time required for resealing appears to be much longer *in vivo* in comparison to *in vitro* (Farkas et al., 2006; Howard et al., 1999; Shi, 2004). Future work assessing the time course and mechanisms involved in plasma membrane damage in both cell bodies and axons may elucidate the reasons for this discrepancy and provide the foundations for developing treatment strategies aimed at membrane repair.

In this study we also examined cellular morphology in permeable and non-permeable cells at 10 min post-injury. Blebbing was observed in cells within the injured spinal cord, and was almost exclusively found in the cells that took up the (3dex) permeability marker. No blebs were seen in cells located distally from the injury epicenter, suggesting that the blebbing phenomenon is specific to cells affected by the mechanical impact. Blebbing has been observed in other cell types in a process by which a decrease in membrane tension initiates a bulk flow of lipids and separation of the plasma membrane from the underlying actin cytoskeleton (Charras et al., 2007; Charras et al., 2006; Keller and Egli, 1998; Keller et al., 2002). Although mechanoporation induced by the mechanical insult may be one initiating factor for membrane blebbing (by directly decreasing membrane tension through the formation of holes/tears), subsequent events such as oxidative stress, increased intracellular pressure, cytoskeletal alterations, and sodium influx may also cause post-SCI blebbing as observed in other cell types (Cunningham, 1995; Marin-Castano et al., 2005; Rentsch and Keller, 2000; Shi et al., 2005). Plasma membrane blebbing has been associated with apoptosis and necrosis (Barros et al., 2003; Laster and Mackenzie, 1996; Mills et al., 1998; Tomiyoshi et al., 2004), but bleb formation does not necessarily lead to cell death (Hiruma et al., 2007; Marin-Castano et al., 2005; Shi et al., 2005). Future work should be aimed at a better understanding of the causes and effects of membrane blebbing and its relationship to alterations in plasma membrane permeability.

Alternatively, the morphological appearance of the permeable cells may be explained by the "patch hypothesis," a resealing mechanism that has been described in other cell types in experimental models of membrane permeability (McNeil and Terasaki, 2001; McNeil et al., 2000; Terasaki et al., 1997; Togo et al., 1999). According to the patch hypothesis, calcium entry through a breach in the membrane causes vesicles from the cytoplasm to fuse with each other to form a large intracellular vesicle. This enlarged vesicle then fuses with the

plasma membrane in order to "patch" the membrane defect. Influx of calcium and subsequent depolymerization of the actin cytoskeleton is required for the membrane fusion event (McNeil, 2002; Miyake et al., 2001). Although not explicitly evaluated in this study, this mechanism may explain the absence of 3dex in the pericellular bleb, since the cytoplasmic vesicles would be expected to exclude the permeability marker (assuming that this endomembrane component has not been breached). *In-vitro* experiments have shown that neurons are able to reseal the ruptured membrane after a traumatic injury through vesicle fusion events (Detrait et al., 2000; Eddleman et al., 1997). Therefore, membrane patching may be an endogenous mechanism by which cells are able to respond and repair the damaged membrane after a mechanical insult to the spinal cord, and should be explored in subsequent experiments.

Although plasma membrane damage has been observed in many models of traumatic CNS injury (Choo et al., 2007; Farkas et al., 2006; Geddes-Klein et al., 2006; Geddes et al., 2003; Kilinc et al., 2007; LaPlaca et al., 1997; LaPlaca et al., 2007; Povlishock and Pettus, 1996; Prado et al., 2005; Shi et al., 2000; Shi and Whitebone, 2006; Whalen et al., 2007), the fate of cells experiencing membrane rupture remains unclear. Although plasma membrane failure may be indicative of necrotic cell death, permeable cells do not necessarily die at acute time points (Geddes-Klein et al., 2006; Guzman et al., 2001; Prado et al., 2005; Stroetz et al., 2001). Some cell types have been shown to survive much larger tears in the plasma membrane, most notably up to $1 \mu\text{m}^2$ in sea urchin eggs, with no evidence of cellular dysfunction (McNeil et al., 2000). Although the fate of cells experiencing membrane damage after CNS trauma remains elusive, previous studies have correlated axonal membrane permeability marker uptake after CNS injury with irregular morphology (Kilinc et al., 2007; Stone et al., 2004) and abnormal electrophysiology (Prado et al., 2005; Shi and Whitebone, 2006). In neuronal somata, acute plasma membrane permeability after traumatic brain injury has been associated with cell death after cortical contusion (Whalen et al., 2007), but other reports utilizing a model of diffuse brain injury have found no strong correlations between dye uptake and morphological changes, fluorojade staining, or calpain activity (Farkas et al., 2006; Singleton and Povlishock, 2004). It is therefore likely that plasma membrane defects can lead to a variety of cellular consequences, depending on the magnitude and duration of damage.

In conclusion, this study investigated the anatomical distribution of plasma membrane permeability changes occurring acutely after SCI. Our results demonstrate that non-specific plasma membrane tears/defects occur as a result of mechanical impact. Disruptions in the cell membrane were large enough to allow cellular and axonal entry of permeability markers as large as 2.7 nm in radius. Furthermore, plasma membrane compromise was shown to be a function of biomechanical impact parameters, with greater impact force resulting in more cellular permeability. These results provide an enhanced foundation for understanding acute SCI pathophysiology, and may offer insights for developing more mechanistically-driven treatment strategies.

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