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Plasma Membrane Damage as a Marker of Neuronal Injury

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Abstract— **Traumatic injury to neurons, initiated by high strain rates, consists of both primary and secondary damage, yet the cellular tolerances in the acute post-injury period are not well understood. The events that occur at the time of and immediately after an insult depend on the injury severity as well as inherent properties of the cell and tissue. We have analyzed neuronal plasma membrane disruption in several in vitro and in vivo injury models of traumatic injury. We found that insult severity positively correlated with the degree of membrane disruptions and that the time course of membrane breaches and subsequent repair varies. This approach provides an experimental framework to investigate injury tolerance criteria as well as mechanistically driven therapeutic strategies. It is postulated that a traumatic insult to the brain or spinal cord results in cellular membrane strain, inducing acute damage that upsets plasma membrane homeostasis. An increased understanding of the pathophysiological mechanisms involved in membrane damage is required in order to specifically target these pathways for diagnostic and treatment purposes and overcome current clinical limitations in the treatment of traumatic brain injury (TBI) and traumatic spinal cord injury (SCI).**

I. Introduction

Although the response to neural injury consists of a complex cellular series of events that can lead to chronic disability and dysfunction, identification of previously unrecognized and critical events in the neural cells of the brain may not only lead to the development of improved acute diagnosis (i.e. including point-of-care), but can also predict long-term neurological outcomes and monitor the effectiveness of a therapy. We investigated cell membrane damage in brain regions that are known to be both selectively vulnerable after injury and involved in many of the neurobehavioral deficits seen in patients. These studies represent the first step in directly characterizing the changes in cell membrane permeability following TBI and SCI in a manner that may lead to a better understanding of membrane homeostasis and lead to targeted therapies.

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Immediately following a traumatic insult to the brain, the plasma membrane of neural cells may become disrupted and potentiate detrimental pathways. Although mechanical insults can directly kill cells by surpassing structural thresholds, sub-lethal forces may cause transient increases in permeability and lead to delayed cellular injury. Due to the heterogeous material properties within the brain and region-specific cell orientation, it is likely that acute responses, such as membrane permeability alterations are also highly heterogeneous. Transient cellular uptake of normally impermeant molecules as well as release of cytosolic molecules has been observed in various models of TBI (Geddes et al., 2003; LaPlaca and Thibault, 1997; Pettus et al., 1994). The neuronal plasma membrane plays a critical role in cell function and may fail structurally if strain exceeds a threshold, thereby permitting uncontrolled ion flux into and out of the cell. Membrane disruptions caused by mechanical stimuli have been postulated to affect axonal signal conduction (Gallant, 1992; Pettus et al., 1994). In addition, transient acute permeability at the neuronal soma following trauma (Geddes et al., 2003; Singleton and Povlishock, 2004) may contribute to acute activity disturbances. Increased permeability causes shifts in transmembrane ion concentrations, possibly depleting energy stores and altering membrane potential. Altered ionic channel conductance has been shown following *in vitro* mechanical insult (Zhang et al. 1996; Goforth et al. 1999), and K+ effluxes have been shown to negatively impact neuronal survival following chemical hypoxia, excitotoxic insult, or apoptotic stimuli (Liu et al. 2003). We provide evidence that traumatic cellular injury is non-uniform and leads to profound changes in cell morphology.

II. Materials and Methods

In vitro models: Primary cortical neurons were obtained from embryonic day 18 rats and cortical astrocytes were obtained from post-natal day 1 rats (both Sprague-Dawley, Charles River, Wilmington, MA). Neuronal cultures were plated on the bottom of a 2-D cell shear stress device (Prado et al., 2005) (density = 1.25-2.0x10^3 cells/mm^2) and maintained in Neurobasal medium supplemented with B-27 (2%; Invitrogen), Glutamax (0.5 mM; Invitrogen), penicillin (1000 units/L), streptomycin (1 mg/L), and amphotericin (2.5 μg/L) (Sigma). Neural co-cultures were plated by mixing cortical neurons with cortical astrocytes (1:1 ratio) homogeneously dispersed throughout Matrigel® matrix (final concentration = 7.5 mg/mL) (density = 2.5x10^3 cells/mm^3, thickness = 500-600 μm) in a 3-D chamber for

shear strain application (LaPlaca et al., 2005) and maintained in Neurobasal medium with B-27 (2%), G-5 (1%), and L-glutamine (0.5mM) (Invitrogen). Cultures were maintained at 37°C and 5% CO_2-95% humidified air and fed 24 hours post-plating and every 2-3 days thereafter.

We have developed several in vitro models of traumatic brain injury that utilize different configurations of neural cultures. In vitro systems offer many advantages over animal studies, including the ability to apply a uniform bulk insult to large populations of cells and monitor responses immediately following the insult. Neuronal cultures in a 2-D configuration were subjected to a fluid shear stress insult using a custom-built cone-and-plate viscometer coupled to a computer-controlled servo-motor that precisely controls acceleration, velocity, and pulse duration (Prado et al., 2005). The insult parameters for 2-D cultures were 140 dynes/cm^2 over 300 ms. The rise times, defined to be the length of time for the cone to reach maximum velocity, were either 20 or 150 ms, which were categorized as moderate and mild injury levels, respectively. Neural cultures in a 3-D configuration were mechanically loaded using a custom-built electromechanical device capable of quantifiably imparting variable rate shear deformation to 3-D cell-containing matrices (LaPlaca, et al., 2005). The mechanical action of the device was driven by a linear-actuator (BEI Kimco; San Marcos, CA) governed by a proportional-integral-derivative controller with closed-loop motion feedback from an optical position sensor (Renishaw; New Mills, UK). A trapezoidal input was provided by code written in LabVIEW® (National Instruments; Austin, TX). Neural cultures in 3-D were deformed (strain 0.50 at strain rates of 1 s^{-1}, 10 s^{-1}, or 30 s^{-1}; corresponding to rise times of 500 ms, 50 ms, or 16.6 ms, respectively) or placed into the device with the top plate (static control).

In order to quantify membrane permeability changes in injured cultures, calcein (a normally cell-impermeant molecule; 629 Da; 3.2x10^{-4} M; Sigma) was added to cultures before the insult (0 minute), 1 or 10 min post-insult and was left in contact with the cultures for 10 minutes following the insult (n = 5 each, moderate and mild injury levels). The cells were then trypsinized (2.5 g/L+1 mM EDTA) and centrifuged (1000g, 10 min). Pellets were resuspended in PBS and assayed in a Beckton Dickinson LSR Flow Cytometer to determine the amount of green fluorescence in each cell, indicating the presence of calcein. Using WinMDI flow cytometry quantification software (Scripps, San Diego, CA), a threshold was assigned based on the histogram profile of uninjured controls. Cells with a fluorescence level above the threshold were deemed positive. In the 3-D cultures, calcein was added prior to the insult and the number of calcein-positive cells was quantified throughout the full thickness of the cultures using confocal microscopy following static control (n = 4) or variable strain rate deformation (n = 4 per rate).

In vivo models: We utilized Lucifer yellow as an indicator of acute biophysical membrane failure after controlled cortical impact (TBI) and spinal contusion (SCI). Lucifer yellow is normally membrane-impermeable; therefore, cellular presence of this molecule can be used to detect plasma membrane compromise. In these experiments, Lucifer yellow was injected intrathecally three hours prior to brain or spinal cord contusion, and animals were sacrificed 10 minutes after injury. Histological evidence demonstrated heterogeneous uptake of the permeability marker in various anatomical locations, both proximal and distal to the insult site, indicating that the distribution of mechanical loading in CNS tissue is complex and not well-understood and likely tied to cell orientation and inherent cell properties.

III. RESULTS

Shear Stress in 2-D Cultures Causes Membrane Disruption

Neuronal plasma membranes were disturbed following the mechanical trauma as evidenced by a heterogeneous calcein uptake into the cytosol when cultures were injured with the marker present. The temporal profile of neuronal membrane permeability to calcein was obtained using flow cytometry and indicated that resealing occurs rapidly following this insult. The percentage of cells positive for calcein was reduced fourfold within the first minute. A smaller portion of the cell population, however, remained permeable to calcein at that time point. By ten minutes the percentage of positive cells was not statistically different than uninjured controls ($p>0.05$). In addition, the moderate injury level produced a significantly higher percentage of positive cells compared to the mild injury level at the time of injury ($p<0.05$).

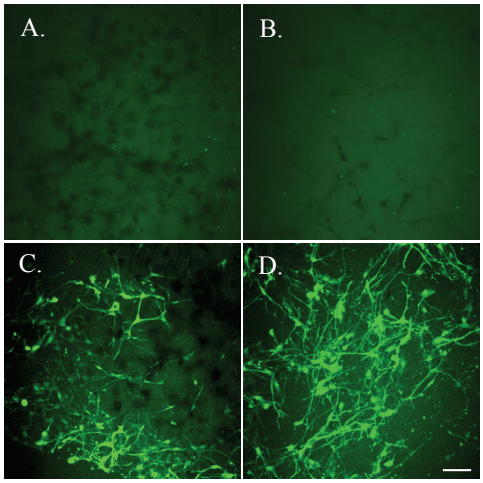

Figure 1. Acute permeability alterations in 3-D neural cultures. Cells were subjected to static control conditions (A) or 0.50 shear strain at strain rates of 1 s^{-1} (B), 10 s^{-1} (C), or 30 s^{-1} (D). There was modest calcein uptake (which is normally cell impermeant) following quasi-static deformation; however, there was robust uptake following moderate and high rate deformation. Photomicrographs are confocal reconstructions of 50 μm thick z-stacks (scale bar = 50 μm).

Membrane Disruption Induced by Shear Strain in 3-D Cultures

Immediately following bulk shear strain loading, cellular uptake of the normally cell-impermeant molecule calcein was visualized using confocal microscopy (Figure 1). The percentages of calcein-positive cells were significantly increased versus static controls for all loading rates evaluated (Figure 2). Loading at strain rates of 10 s^{-1} or 30 s^{-1} produced enhanced calcein uptake versus quasi-static loading, signifying a rate dependence of acute membrane permeability increases. These results show heterogeneous acute structural alterations that may have longer-term implications on neural cell survival.

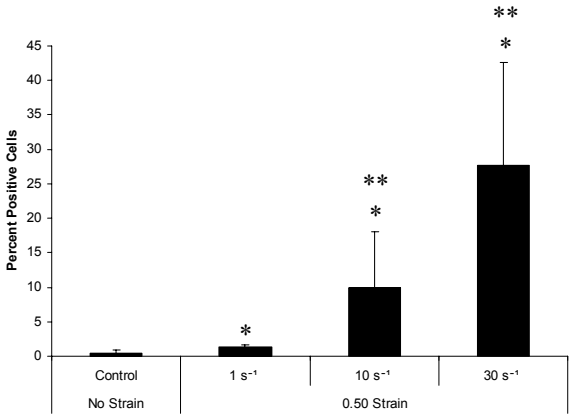

Figure 2. The percentage of calcein-positive cells following static control or variable rate deformation. All strain rates had a significant increase in the percentage of calcein-positive cells compared to controls (*, $p < 0.05$). Furthermore, the moderate and high rate deformation regimes were significantly higher than the quasi-static deformation, signifying a rate dependence in the acute permeability response (**, $p < 0.05$).

After TBI, membrane disruptions occurred in a heterogeneous fashion with the cortex (Figure 3A) and the hippocampus. Because there is a correlation between injury severity and membrane compromise, permeability markers can therefore be used as an indicator of the extent of local cellular loading parameters. More extensive permeability marker uptake occurs in specific hippocampal regions after contusion injury, suggesting that local cellular loading is more severe in certain anatomical locations. These data may explain the preferential cell death seen in these regions in the sub-acute and chronic phases, as mechanical damage during the initial impact in these regions may make the cells more susceptible to death and/or dysfunction during the secondary phase of injury. SCI produced membrane permeability in cell bodies (Figure 3B) and axons, which was evident several millimeters from the insult epicenter. Both *in vivo* trauma models showed persistent damage and morphological evidence of membrane restructuring (data not shown).

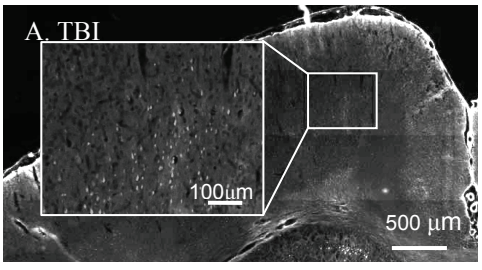

Figure 3: Acute neuronal permeability following TBI and SCI. In the acute phase of traumatic injury, the plasma membrane becomes damaged due to local cellular strains that exceed structural thresholds. Lucifer yellow uptake in the injured brain (A) and spinal cord (B) demonstrates a heterogeneous distribution of membrane failure.

IV. Discussion

An acute increase in membrane permeability due to injury-induced disruptions may play an important role in the initiation of deleterious cascades following brain injury. In order to fully understand the complex mechanisms triggered as a result of a traumatic insult to the brain, we must elucidate the acute events. It has been observed that selective regions in the brain are more affected by the mechanical insult than others. This heterogeneous response may be due to differences in strain manifestation at the cellular level. For example, we previously showed that bulk shear strain applied to 3-D cultures results in a heterogeneous strain response consisting of shear, compressive, and tensile strain components (LaPlaca et al, 2005). Membrane damage has often been postulated to be an immediate response to the initial traumatic insult, but the time course of damage and subsequent repair attempts has not been well described. Identification of critical events that occur during and acutely following the insult and their relationship to cellular outcome may lead to the development of improved protective systems and targeted clinical therapies. Furthermore, elucidating acute mechanisms may also lead to identification of risk factors for increased susceptibility to neurodegeneration and repeated insults to the brain.

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