

High Rate Shear Insult Delivered to Cortical Neurons Produces Heterogeneous Membrane Permeability Alterations

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Abstract— Traumatic brain injury (TBI) occurs when brain tissue is subjected to stresses and strains at high rates and magnitudes, yet the mechanisms of injury and cellular thresholds are not well understood. The events that occur at the time of and immediately after an insult are hypothesized to initiate cell dysfunction or death following a critical cell strain and strain rate. We analyzed neuronal plasma membrane disruption in two *in vitro* injury models— fluid shear stress delivered to planar cultures and shear strain induction of 3-D neural cultures. We found that insult severity positively correlated with the degree of membrane disruptions in a heterogeneous fashion in both cell configurations. Furthermore, increased membrane permeability led to increases in electrophysiological disturbance. Specifically, cells that exhibited increased membrane permeability did not fire random action potentials, in contrast to neighboring cells that had intact plasma membranes. This approach provides an experimental framework to investigate injury tolerance criteria as well as mechanistically driven therapeutic strategies.

I. INTRODUCTION

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 heterogeneous 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 across a culture, regardless of the configuration and that increased permeability (i.e. the opening of non-specific pores/tears in the membrane) correlates to changes in firing ability.

II. MATERIALS AND METHODS

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 = $1.25\text{-}2.0 \times 10^3$ cells/mm²) 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 = 2.5×10^3 cells/mm³, 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% CO₂-95% 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

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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/cm² 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 addition, using separate cultures subjected to shear stress, whole-cell patch clamp recordings were made in cortical neurons with or without permeability marker (n = 8 each). All experiments were performed at 10-24 days *in vitro*.

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 (Figure 1). 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).

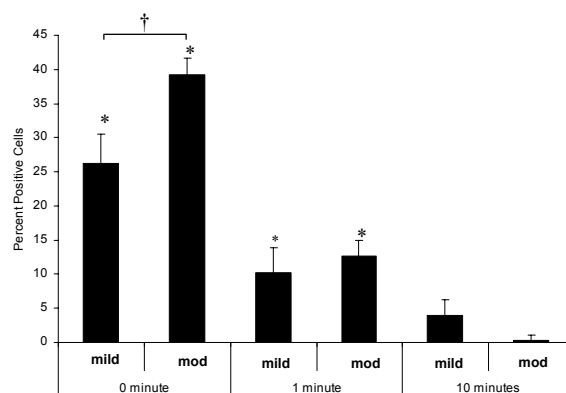


Figure 1. Neuronal plasma membrane permeability increases following a fluid shear stress insult were rate-dependent. Moderate insult induced a higher percentage of neurons to become permeable to calcein than the mild parameter. * Significantly different than uninjured controls. † Significantly different than other injury level.

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

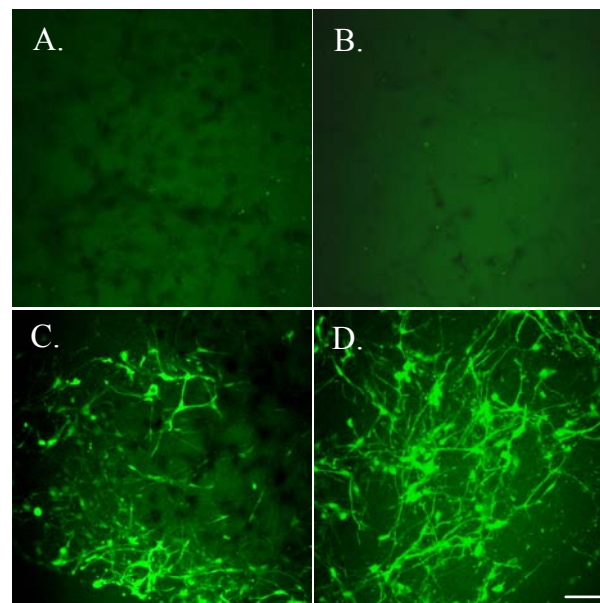


Figure 2. Acute permeability alterations in 3-D neural cultures. Cells were subjected to static control conditions (A) or 0.50 shear strain at strain rates of 1 s⁻¹ (B), 10 s⁻¹ (C), or 30 s⁻¹ (D). There was modest calcein uptake (which is normally cell impermeant) following quasi-static deformation; 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).

2). The percentages of calcein-positive cells were significantly increased versus static controls for all loading rates evaluated (Figure 3). 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.

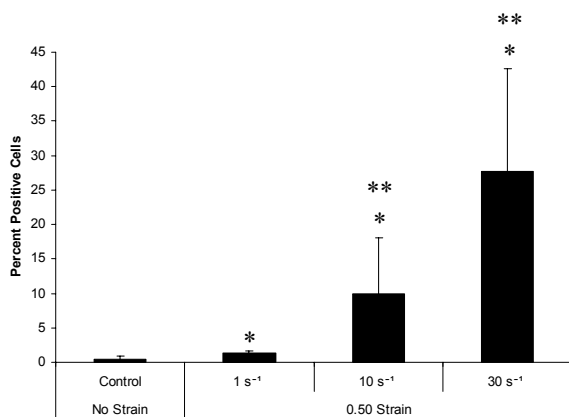


Figure 3. The percentage of calcein-positive cells following static control or variable rate deformation. All strain rates had a significant increase in the percentage of calcein-positive cells compared to controls (*, $p < 0.05$). Furthermore, the moderate and high rate deformation regimes were significantly higher than the quasi-static deformation, signifying a rate dependence in the acute permeability response (**, $p < 0.05$).

Membrane Disruption is Linked to Electrophysiological Dysfunction Following Shear Stress Injury

Following shear stress insult, permeable cells and non-permeable cells were observed within close proximity of each other (Figure 4), illustrating the heterogeneous response to trauma. Although permeable cells had resting membrane potentials similar to non-permeable cells and non-injured controls (-50 to -70 mV), no excitable properties (either spontaneous or invoked) were observed (Figure 4B). Non-permeable cells from the same culture exhibited spontaneous action potentials (Figure 4C).

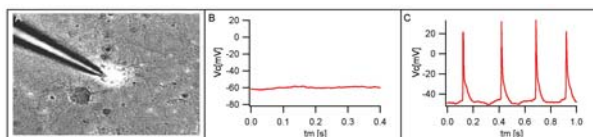


Fig. 4. Electrophysiological alterations in permeable cells following mechanical insult by fluid shear stress. (A) Patch clamp pipette in a cell permeable to calcein with non-permeable neighboring cell under incandescent and fluorescent light (em 530 nm). (B) Recording from a permeable cell that has a resting membrane potential of -60 mV but does not exhibit excitable properties. (C) Spontaneous action potentials from a non-permeable cell in the same culture.

In addition, the average input conductance was greater in permeable cells than non-permeable cells and both were elevated over non-injured controls. This supports our

hypothesis that permeable cells may have altered electrophysiological properties following injury; however, further investigation is required to determine the extent and mechanism.

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 events or the link to electrophysiological function is not well known. 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 effective 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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