Neuronal Plasma Membrane Integrity is Transiently Disturbed by Traumatic Loading

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ABSTRACT: The acute response of neurons subjected to traumatic loading involves plasma membrane disruption, yet the mechanical tolerance for membrane compromise, time course, and mechanisms for resealing are not well understood. We have used an in vitro traumatic neuronal injury model to investigate plasma membrane integrity immediately following a high-rate shear injury. Cell-impermeant fluorescent molecules were added to cortical neuronal cultures prior to insult to assess membrane integrity. The percentage of cells containing the permeability marker was dependent on the molecular size of the marker, as smaller molecules gained access to a higher percentage of cells than larger ones. Permeability increases were positively correlated with insult loading rate. Membrane disruption was transient, evidenced by a membrane resealing within the first minute after the insult. In addition, chelation of either extracellular Ca^{2+} or intracellular Ca^{2+} limited membrane resealing. However, injury following chelation of both extracellular and intracellular Ca^{2+} caused diminished permeability as well as a greater resealing ability compared to chelation of extracellular or intracellular Ca^{2+} alone. Treatment of neuronal cultures with jasplakinolide, which stabilizes filamentous actin, reduced permeability increases, while latrunculin-B, an actin depolymerizing agent, both reduced the increase in plasma membrane permeability and promoted resealing. This study gives insight into the dynamics of neuronal membrane disruption and subsequent resealing, which was found to be calcium dependent and involve actin in a role that differs from non-neuronal cells. Taken together, these data will lead to a better understanding of the acute neuronal response to traumatic loading.

KEYWORDS: Cell injury, cortical neuron, calcium, neurotrauma, plasma membrane

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Introduction

Traumatic brain injury (TBI) results from stresses and strains that surpass tissue thresholds, leading to macroscopic as well as microscopic structural failure[1,2] and lead to transient or persistent neurological deficits.[3] Although mechanical insults can directly kill cells at suprathreshold levels (eg, catastrophic tearing or transection), sublethal insults can lead to more subtle plasma membrane damage that manifests as increases in membrane permeability, or mechanoporation.[4-6] An increase in axolemmal permeability has been reported as one of the earliest biophysical events following TBI7[9] and has been observed in in-vitro models of neural injury,[10-14] as well as animal models of both TBI[6,9,15] and traumatic spinal cord injury (SCI).[16-18]

Nonspecific mechanoporation may contribute to many of the acute cellular responses following a mechanical insult, such as a loss in ion homeostasis, influx of extracellular molecules, and leakage of cytosolic constituents.[10,19-22] Furthermore, plasma membrane damage has been associated with conduction block,[23] aberrant cell signaling,[24] protease activation,[9] alterations in cellular morphology and cytoskeletal integrity,[7,8,25] and delayed cell death.[6,15]

The mechanical threshold for membrane damage in brain is not fully understood; however, there is evidence for a relationship between loading intensity and degree of membrane damage in many settings. For example, patterns of stress and strain using finite-element analysis have been correlated to axolemmal permeability,[26] supporting the hypothesis that acute membrane damage is linked to mechanical loading in a severity-dependent manner. Stresses generated by ultrasound-induced cavitation have been shown to be responsible for increases in membrane permeability of both prostate cancer cells[27] and corneal endothelial cells[28] in a severity-dependent manner. The size of membrane disruptions in stretched neurons has been shown to increase with increasing strain and strain rate.[10] In addition to the severity of loading parameters, the mode of injury can determine the amount of membrane permeability changes, with biaxial stretch eliciting higher permeability alterations than uniaxial stretch[29] and shear injury causing more membrane permeability alterations compared to compression injury.[14]

Acute membrane permeability may also be associated with transient dysfunction with little or no cell death, which may be due to sublethal injury and/or a dynamic ongoing repair process. Evidence of axolemmal damage has been observed after optic nerve stretch, despite no overt macroscopic destruction.[30] We have previously shown that there is a relationship between degree of mechanoporation and cell death;[14] however, not all permeable cells die,[6] suggesting membrane repair. Delayed introduction of permeability markers in both in-vitro[10] and in-vivo[9,15] models of TBI also support an active repair capacity post-TBI. The study of mechanical disruption of the plasma membrane and resealing mechanisms in

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non-neuronal cell types may provide insight into potential mechanisms of membrane repair post-TBI. For example, high shear stress extrusion of oocytes through a syringe needle causes disturbances in the plasma membrane that reseal in the presence of extracellular Ca^{2+}.[31] This resealing mechanism, first proposed by McNeil et al[32] and later refined,[33] is initiated by extracellular Ca^{2+} influx through tears or pores of the plasma membrane, leading to depolymerization of the actin cortical cytoskeleton and activation of Ca^{2+}-mediated vesicular docking. Subsequently, the docking and fusion of vesicles to the plasma membrane promotes resealing by either patching up the tear[31,33-35] or by decreasing membrane tension.[36]

In this study, we use an in-vitro model of TBI in which primary cortical neurons are subject to short duration, high-rate shear stress in the presence of permeability markers. The goals are to determine mechanical thresholds for neuronal plasma membrane permeability and to investigate the roles of calcium and the actin cytoskeleton in membrane permeability alterations and resealing dynamics. The results contribute to our understanding of neuronal plasma membrane damage and provide potential targets for membrane repair strategies.

Methods

Neuronal cell culture

All procedures involving primary cell harvest were approved by the Georgia Institute of Technology IACUC. Cortices were dissected from embryonic day 18 Sprague–Dawley rats (Charles River, Wilmington, MA), rinsed with Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) without Ca^{2+}/Mg^{2+} followed by incubation with trypsin (2.5 g/L plus 1 mM EDTA; Invitrogen) for 10 minute. Trypsin solution was then removed and tissue rinsed twice with Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum. Cortices were dissociated in a solution of Neurobasal medium (Invitrogen) containing DNAse (0.15 mg/mL; Invitrogen) by brief agitation. Cells were counted and seeded (density = $1.00 - 1.25 \times 10^5$ cells/cm^2) onto customized glass plates for the cell shearing injury device (CSID). Prior to cell seeding, glass plates were briefly flamed and coated with a poly-L-lysine solution (0.0023%w/v; Sigma, St. Louis, MO) for at least 12 h at 37°C, 95% relative humidity. Cells were cultured in Neurobasal medium supplemented with B-27 (2%v/v; Invitrogen), Glutamax (0.5 mM; Invitrogen), and 1000 units/L of penicillin/1 mg/L of streptomycin/2.5 µg/L of amphotericin (Sigma) and used at 7 days in vitro (DIV). The resulting cell phenotype by this seeding and culturing method yields 90% to 95% neurons (confirmed by immunostaining for neuronal-specific cytoskeletal marker tau-5), with the remaining cells exhibiting morphological characteristics of glial cells (verified by glial fibrillary acidic protein immunostaining). In addition, cells at 7 DIV exhibit electrical bursts, as assessed with microelectrode arrays.[11]

Injury

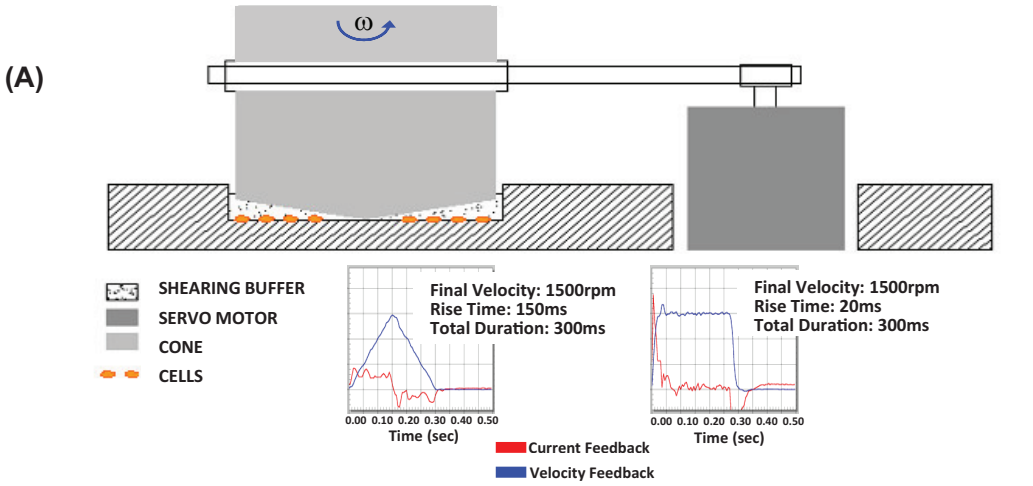
Neuronal cell cultures were subjected to a high magnitude, short duration, pulse of fluid shear stress using the CSID[11] (Figure 1A). The CSID consists of a servo motor-driven cone (0.5°) that rotates the shearing buffer between the cone and cell plate, thereby rapidly transferring momentum from the rotating cone to the cells, producing a uniform shear stress across the culture plate. Experiments were conducted by removing culture plates from the incubator, rinsing them with HBSS, and mounting them in the CSID. The cone was lowered until its apex contacted the center of cell plate. Shearing buffer, HBSS (with or without Ca^{2+}/Mg^{2+}) and permeability markers, was added through a perfusion port, slowly filling the gap between the cone and the plate. The insult parameters of shear stress magnitude and duration were 140 dynes/cm^2 and 300 ms, respectively. The rise time, which is defined to be the length of time for the cone to reach maximum velocity, was either 20 ms (high rate) or 150 ms (low rate). Cells were immediately removed from the CSID and subjected to permeability assessment. Groups of 3 to 8 cultures were used for each condition. A separate set of cultures was used to assess cell death by measuring lactate dehydrogenase (LDH) release (LDH quantification kit; Sigma, St. Louis, MO) for injury and sham injury conditions at 6 and 24 h after injury (n = 4).

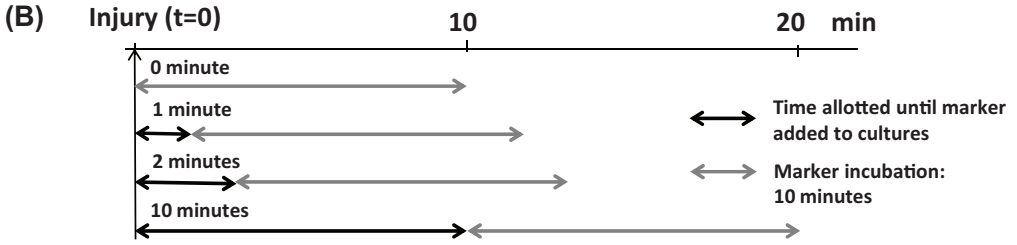
Plasma membrane permeability assessment

The shearing buffer consisted of HBSS with Ca^{2+}/Mg^{2+} and either calcein (629 Da; 3.2×10^{-4} M; Sigma) or fluorescein-5-isothiocyanate (FITC)-conjugated dextran (10^{-4} M) molecules of different sizes (42 and 464 kDa; Sigma). Cells were incubated at 37°C in buffer containing permeability marker for 10 minutes prior to injury. Sham cultures were placed in the CSID and loaded with the shearing buffer after the cone was lowered as in the injured cultures, but the cone did not rotate. Sham cultures were also incubated for 10 minutes with the shearing buffer after they were taken from the CSID. Naïve controls were cultures that were not placed in the CSID, but were exposed to shearing buffer containing the permeability marker for 10 minutes. These plates were used to establish baseline uptake of permeability marker. In order to reveal the resealing time course; cultures were injured without the presence of permeability marker, which was then added to cells at 1, 2, or 10 minutes after injury (calcein only). Calcein- or FITC-conjugated dextrans were in contact with cells for a total of 10 minutes for all groups (Figure 1B). Following incubation with the permeability marker, all plates were rinsed with HBSS.

Chelation of calcium ions (Ca^{2+})

A Ca^{2+}-free extracellular environment was attained by rinsing cells with HBSS without Ca^{2+}/Mg^{2+} and using shearing buffer supplemented with 1 mM EGTA (Sigma) and calcein (Figure 1B). Dimethyl BAPTA-AM (Molecular Probes Inc., Eugene, OR), a potent cell-permeant Ca^{2+} chelator, was used to chelate





(C)

Permeability Marker	Time allotted after injury for addition (minutes)			
	0	1	2	10
Calcein	N,B,E,EB,J,L	N,B,E,EB,J,L	N	N
FITC-Dextrans (42kDa and 464kDa)	N	-	-	-

Figure 1. (A) Schematic of the cell shearing injury device (CSID), exaggerated illustration of the cone angle and cells. The servo-motor current and velocity traces for 150 and 20 ms rise times are shown below the device schematic. The *x*-axis is time and the *y*-axis is current and angular velocity (arbitrary units removed). The shear stress magnitude (140 dyn/cm^2) and total insult duration (300 ms) were constant for both loading rates. (B) Timeline of neuronal plasma membrane assessment. To temporally assess permeability following mechanical insult calcein was present during injury (0 minutes) or added at 1, 2, or 10 minutes following injury. Total incubation of cells with calcein (3.2×10^{-4} M) was 10 minutes following addition for all groups. (C) Summary of the experimental condition and the type of permeability marker added at different times following insult. B indicates dimethyl BAPTA-AM; E, EGTA; EB, EGTA + dimethyl BAPTA-AM; FITC, fluorescein-5-isothiocyanate; J, jasplakinolide; L, latrunculin-B; N, no treatment. See Methods for treatment durations.

intracellular Ca^{2+}. Cells were pretreated for 75 minutes with 60 μM of dimethyl BAPTA-AM in HBSS containing Ca^{2+}/Mg^{2+}. Cultures were then rinsed 3 times with HBBS with or without Ca^{2+}/Mg^{2+}. Cells were then injured with the CSID shearing buffer containing calcein (Figure 1C). For the evaluation of the resealing capability cells were injured without the permeability marker, which was then added 1 minute following injury. Ca^{2+}-free shearing buffer (containing 1 mM EGTA) plus dimethyl BAPTA-AM was also used for separate groups to determine the effect of depleting both intracellular and extracellular Ca^{2+}.

Actin depolymerization and polymerization

Actin depolymerization was initiated by pretreatment of cell cultures with latrunculin-B (25 μM; Sigma) for 60 minutes. Actin polymerization was promoted by pretreating cell cultures with jasplakinolide (5 μM; Molecular Probes) for 5 minutes, while either adding permeability marker prior to injury or 1 minute postinjury to probe for the effect of actin stability on both permeability and subsequent resealing. In both cases, chemical treatments were followed by rinses with HBSS containing Ca^{2+}/Mg^{2+} prior to injury (Figure 1C). A separate set of cultures was used to further probe the role of actin stabilization in resealing by applying jasplakinolide to cultures 1 minute after the insult for 5 minute under Ca^{2+}/Mg^{2+}-free (containing 1 mM EGTA) conditions, followed by 10 minute of calcein incubation in the presence of Ca^{2+}/Mg^{2+}.

Quantification of plasma membrane permeability and statistical analysis

To analyze the degree of plasma membrane permeability changes, cell cultures that were subjected to the above protocols

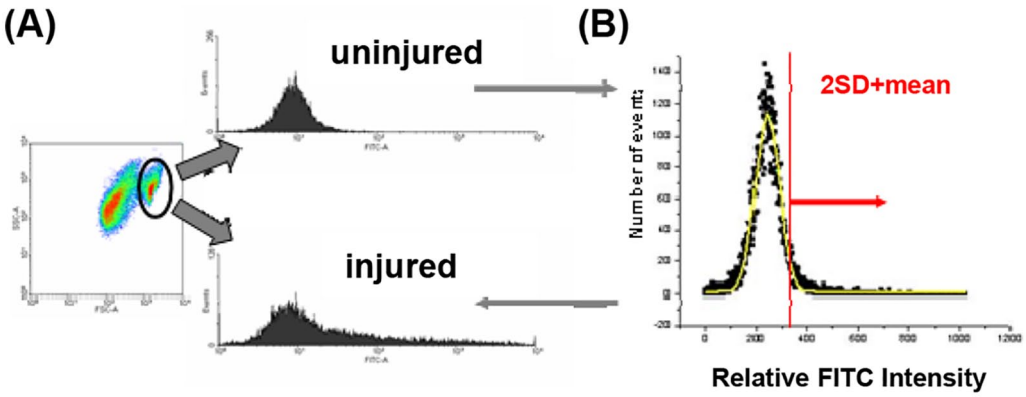


Figure 2. Quantification of positive cells using flow cytometry analysis: (A) histograms on the left were representative fluorescent data from uninjured populations (upper) and injured ones (lower). (B) Threshold of uptake was assigned from examination of the Gaussian distribution for uninjured controls. Cells that present higher fluorescence than the threshold (vertical line) were considered positive cells. FITC indicates fluorescein-5-isothiocyanate.

were rinsed with HBSS containing Ca^{2+}/Mg^{2+} and then treated with trypsin (2.5 mg/mL) followed by removal from the glass plates via gentle scraping. Trypsin was removed from cells following centrifugation at 1000*g* for 10 minutes at 4°C. Pellets containing cells were then re-suspended in phosphate-buffered saline (PBS, pH = 7.4, 4°C). Ten minutes before suspended cells were counted, propidium iodine (PI; 1.0 mg/mL; Molecular Probes) and Hoechst 33342 (0.5 mg/mL; Molecular Probes) were added. A Beckton Dickinson LSR Flow Cytometer was then used to determine fluorescent intensity from at least 10 000 cells per sample. Using WinMDI (Scripps, San Diego, CA) flow cytometry quantification software, live cells were gated from the rest of the particles in solution using a side-scatter versus forward scatter dot plot. In order to ensure that only live cells were being read, Texas Red and UV histograms were used to exclude cells that were permeable to PI. Cells counted for the presence of permeability markers excluded PI, indicating complete resealing and viability at the time of flow cytometry. Subsequently, the intensity of individual cells gated in the latter step was displayed in a histogram of FITC intensity (~533 nm emission) (Figure 2A). Using a similar permeability quantification method previously published,[31] data analysis was accomplished by first fitting the FITC histograms obtained from uninjured control samples with a Gaussian curve, using Origin Software (OriginLab Corp., Northampton, MA). The center (peak value) and standard deviation of the normal distribution curve were then used to find the threshold for the given experiment. The threshold was defined as the peak of the naïve control Gaussian curve plus 2 standard deviations to the right, which excludes approximately 95% of that population (assuming a normally distributed curve). The average threshold for naïve control samples was used to define the percentage of positive cells in treatment groups. Positive cells were defined to be cells located to the right of the threshold (Figure 2B). The uptake ratio was quantified for experiments with chemical pretreatments. The uptake ratio is defined as the percentage of positive cells at 1 minute following injury normalized by the percentage of positive cells at 0 min. These ratios are only shown when the percentage positive cells at 1 minute is significantly different than the values at 0 minute, and they were used to compare resealing independent of initial insult. Even when using this semiquantitative method, there were some variations in the baseline of experiments. Each experiment was repeated at least 3 times and contained untreated, uninjured, and injured, controls that were compared to treated groups in order to account for day-to-day variations. Statistical analysis was accomplished by analysis of variance (ANOVA), followed by Tukey's pairwise comparison.

Results

Neuronal membrane permeability increases due to mechanical insult

Fluid shear stress-induced injury caused an immediate increase in neuronal plasma membrane permeability as evidenced by dye uptake directly following the insult. This increase was dependent on the size of the molecule used as the permeability marker ($P < .05$; Figure 3), suggesting that a range of membrane defect sizes are produced by this form of neuronal injury. We chose to use calcein for the majority of this study due to its smaller size and higher percentage of cells affected (40%-50%) compared to dextrans, permitting us to see a wide range of decreases and increases in permeability. Injured neurons presented good neurite attachment, preserving most of the fine network arbor after the insult (Figure 4A). The calcein uptake within a culture was heterogeneous, and we commonly found neighboring cells containing varying amounts of calcein (Figure 4B). These conditions did not cause significant cell death at 6 and 24 hours postinjury in neuronal cultures subjected to the same insult parameters (data not shown).

The temporal profile of neuronal membrane permeability to calcein indicated that resealing occurs rapidly following the insult (Figure 5). The percentage of cells positive for calcein was reduced fourfold within the first minute following injury. A smaller portion of the cell population, however, remained permeable to calcein for at least 2 minutes. By 10 minutes, the percent of positive cells was not statistically different than

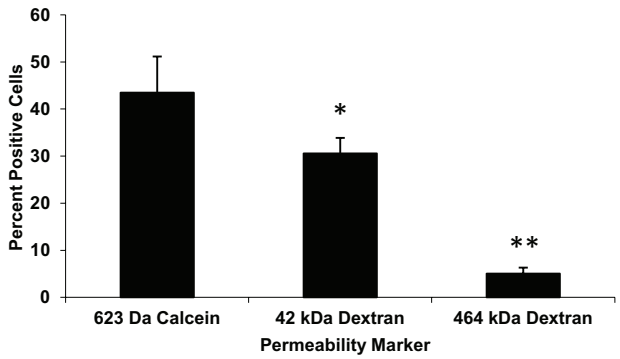


Figure 3. Percent-positive cells decreased as the size of the marker molecule increased following equal mechanical insult (140 dynes/cm^2 shear stress magnitude, 20 ms rise time). Permeability markers were present during injury. Dextran concentrations were 10^{-5} M.
*Significantly different from calcein group ($P < .05$), and **42 kDa group ($P < .0005$) group. Error bars represent SD. n = 3 to 5 cultures/group.

uninjured controls. Furthermore, the increase in permeability to calcein was rate dependent, with higher rate insults (20 ms rise time) causing a larger increase in membrane permeability than lower rates (150 ms rise time).

Role of Ca^{2+} in neuronal plasma membrane resealing

Permeability marker (calcein) exclusion over time was dependent on the presence of extracellular Ca^{2+} in the media, with chelation of extracellular Ca^{2+} resulting in the loss of resealing ability by a large percentage of neurons (Figure 6). When permeability dye was present at the time of the insult, the percentage of calcein-positive cells was comparable between the Ca^{2+}-free and normal (Ca^{2+}-containing) groups. As in the previous experiment, when permeability marker was

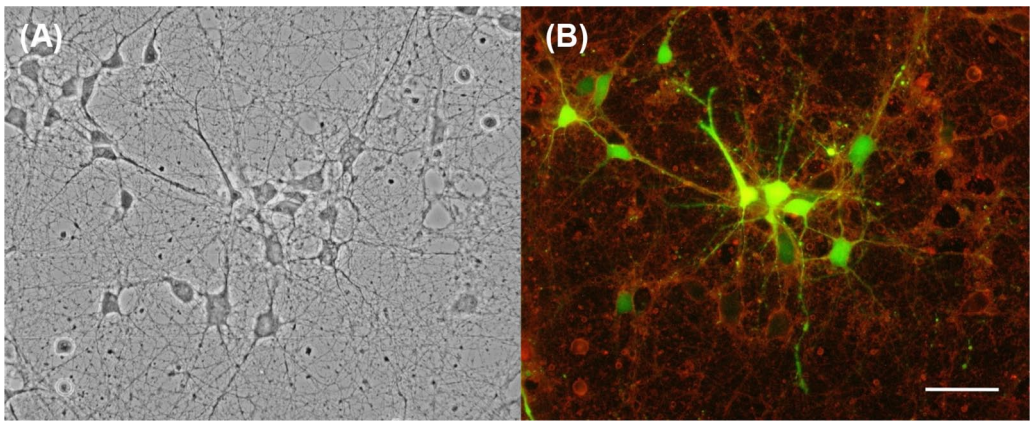


Figure 4. Representative neuronal culture injured in the presence of the fluorescent permeability marker calcein: (A) phase micrograph shows preservation of cellular integrity following insult. (B) Fluorescence micrograph of the same field shows cells which contained calcein (~520 nm). Notice the differential uptake of calcein within cells (white arrows highlight examples of cells with no uptake). Cultures were treated with a 50 µg/mL solution of Di-8 ANEPPS (red) for 10 minutes as a counterstain. Injury parameters: shear stress = 140 dynes/cm^2; rise time = 20 ms. Scale bar = 50 µm. Di-8 ANEPPS indicates Di-8 aminonaphthylethenylpyridinium; EM, emission wavelength.

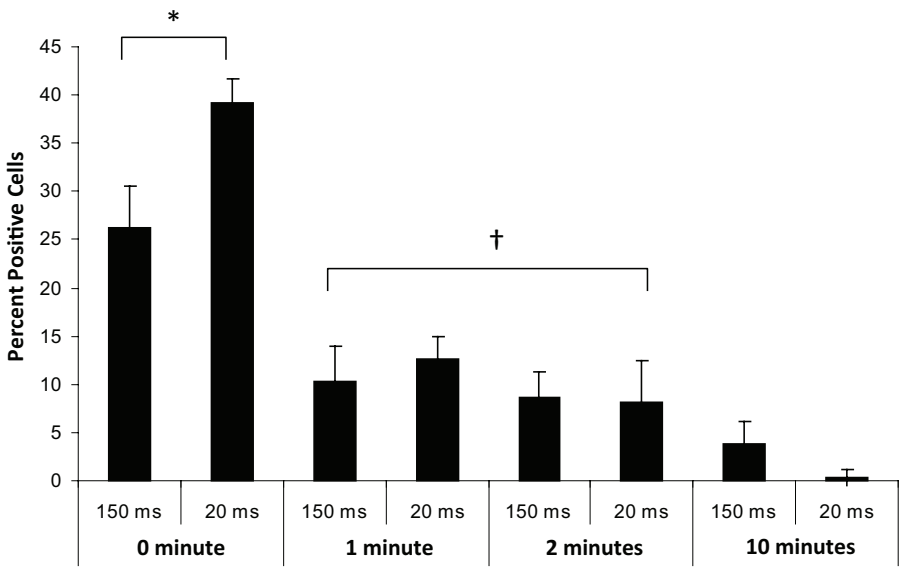


Figure 5. Percent of positive cells was highest when calcein was present during injury (0 minute). This increase was rate dependent. Cells rapidly resealed following injury, reducing the percentage of positive cells fourfold within the first minute. Total calcein incubation time was 10 minutes for all samples. Shear stress for all injuries: 140 dynes/cm^2; rise times: 20 and 150 ms.
*Significantly different $P < .001$. †Significantly different from uninjured controls (zero percent uptake; not shown) $P < .05$. Error bars represent SD. n = 4 to 5 cultures/group.

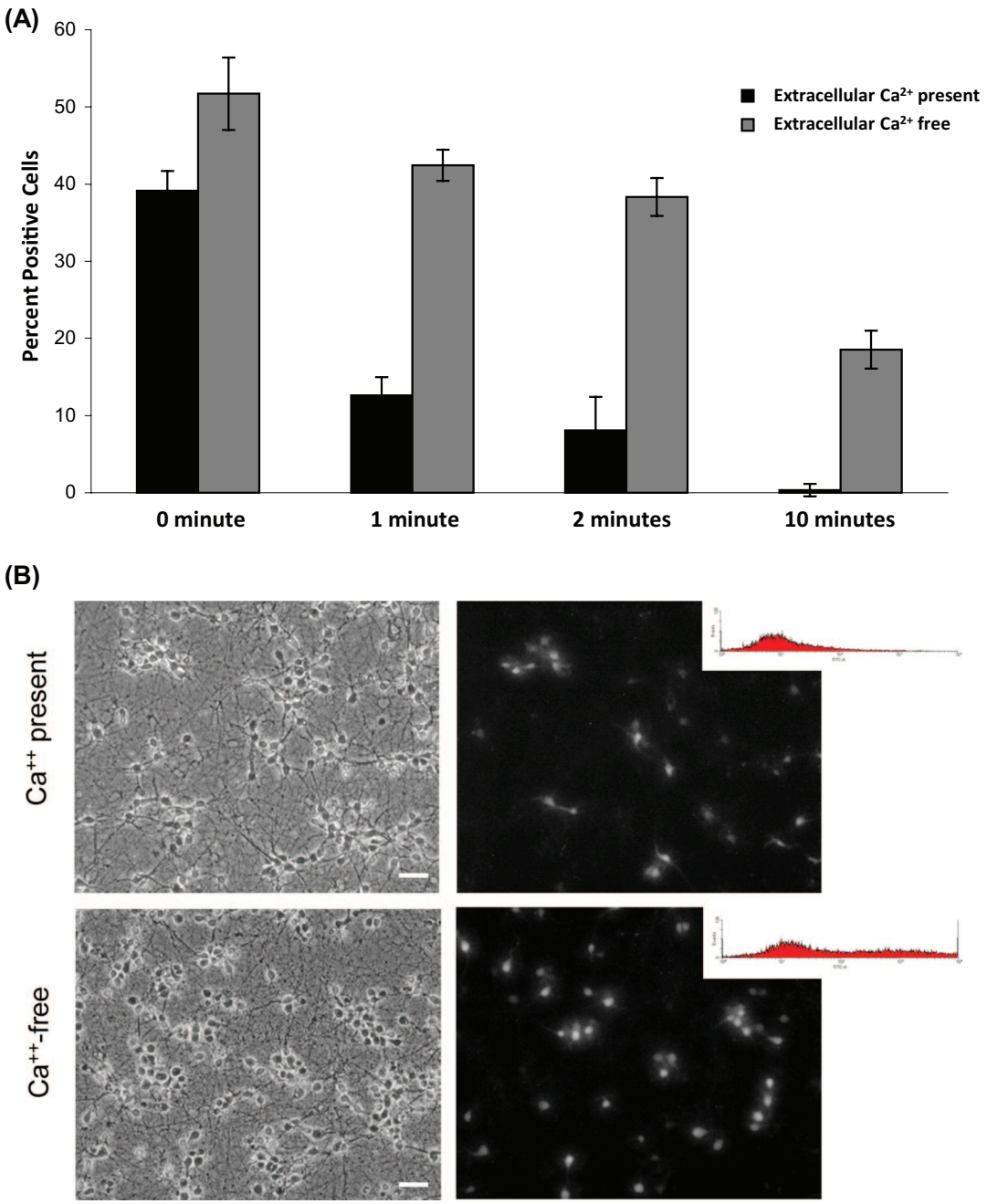


Figure 6. Dependence of neuronal plasma membrane resealing on extracellular Ca^{2+}: (A) the ability of a large percentage of neuronal cells to rapidly reseal within the first minute was lost in the absence of extracellular Ca^{2+}. Even though the average percentage of positive cells at 10 minutes was reduced by about one-third of the permeability level seen when the marker was present during injury, this value was still significantly larger than uninjured controls. Total calcein incubation was 10 minutes for all samples. Ca^{2+}-free samples were injured and incubated in HBSS buffer containing 1 mM EGTA with or without calcein. For all injuries: Shear stress = 140 dynes/cm^2, rise time = 20 ms. General linear model shows that both extracellular Ca^{2+} and time of marker introduction are significant variables ($P < .0001$ for both). Error bars represent SD. n = 4 to 5 cultures/group. (B) Representative photomicrographs of neuronal cultures following shear injury in the presence of Ca^{2+} (top row) and in Ca^{2+} buffer (bottom row). Both cultures were injured in the presence of calcein (0-minute condition). Both cultures were injured in the presence of calcein (0-minute condition). Phase contrast images show that cells in both conditions were well adhered after the injury. Both cultures had calcein in the media during the injury (0-minute condition). Right images show permeable cells following injury under fluorescent microscopy (emission 530 nm), with more permeable cells in calcium-free conditions. Representative histograms obtained from flow cytometry in each condition are shown of the right. Scale bar = 50 µm.

added to the media at 1, 2, or 10 minutes postinsult in the presence of Ca^{2+}, dye was excluded from the cells, indicated that membrane resealing took place. When extracellular Ca^{2+} was chelated, however, permeability marker entered the cells even with delayed marker addition. The longer the delay, the less permeable the cells were; when added 10 minutes postinjury, 18% of the cells were still permeable, versus 1% of the cells in Ca^{2+}-containing buffer.

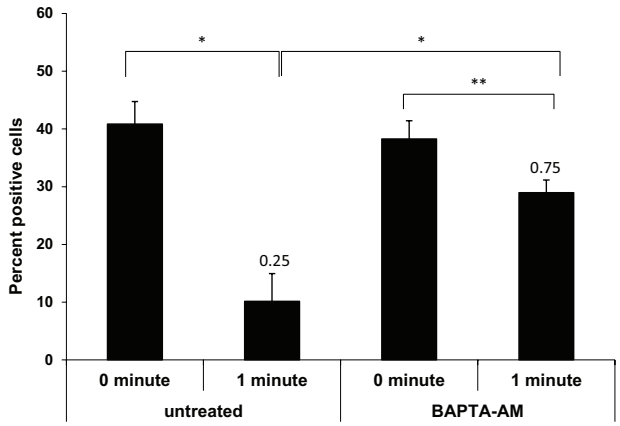


Figure 7. BAPTA-AM pretreatment did not have an effect in the increase of permeability due to the mechanical insult under normal extracellular Ca^{2+}. Resealing, however, was affected by intracellular chelation of Ca^{2+}, resulting in a larger percentage of cells that did not reseal to calcein entry by 1 minute after insult when compared to untreated group (*$P < .0001$), **$P < .05$. Uptake ratios are seen on top of 1-minute groups. Error bars represent SD. n = 4 to 5 cultures/group.

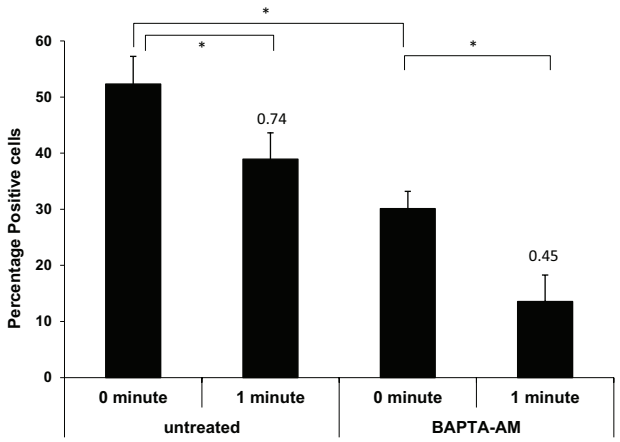


Figure 8. When extracellular Ca^{2+} was absent during shearing, BAPTA-AM pretreatment reduced the permeability increase due to mechanical insult when compared to untreated group (*$P < .0001$), also sheared in absence of extracellular Ca^{2+}. Pretreatment also increased resealing, which is manifested by the lower percentage of positive cells to calcein 1 minute after insult compared to untreated group. Uptake ratios are seen on top of 1-minute groups. Error bars represent SD. n = 8 cultures/group.

The role of intracellular Ca^{2+} in neuronal plasma membrane disruption and resealing following injury was elucidated by chelating intracellular Ca^{2+} stores using dimethyl BAPTA-AM prior to injury. In the presence of extracellular Ca^{2+}, chelation of intracellular Ca^{2+} followed by injury with permeability marker present (0 minute, BAPTA-AM) resulted in the same amount of calcein uptake as compared to the untreated (ie, both intracellular and extracellular Ca^{2+} present) group (0 minute, untreated) (Figure 7). However, resealing of neuronal plasma membranes in the presence of extracellular Ca^{2+} was affected by chelation of intracellular Ca^{2+}. Adding the permeability marker 1-minute following injury resulted in a higher uptake ratio (0.75) in the treated group (1 minute, BAPTA-AM) compared to the untreated (ie, both intracellular and extracellular Ca^{2+} present) group (0.25) (1 minute, untreated), indicating impaired membrane resealing (Figure 7). Pretreatment with dimethyl BAPTA-AM followed by chelation of extracellular Ca^{2+} using EGTA and subsequent injury resulted in a lower percentage of permeable cells at the time of injury (Figure 8). In the total absence of the divalent cation inside and outside the cells, they regained, at least in part, the ability to reseal following injury, as cell uptake ratio was 0.45 (absence of intracellular and extracellular Ca^{2+}) with delayed permeability marker compared to 0.75 (absence of extracellular Ca^{2+}) (Figure 8). Dimethysulfoxide (DMSO; Sigma), the vehicle for dimethyl BAPTA-AM (0.06%v/v), has been reported to increase resealing of plasma membrane at low extracellular Ca^{2+} concentrations.[37] However, control experiments using cells that were pretreated with DMSO only (0.06%v/v) were not significantly different than the nontreatment group (data not shown). Also, similar results were found when using FITC-conjugated dextran molecules as permeability markers (data not shown), indicating that this phenomenon is not a result of calcein interaction with the agents used.

Role of actin cytoskeleton in neuronal plasma membrane resealing

In order to elucidate the role of actin in neuronal plasma membrane disruption and subsequent resealing from shear stress injury, we pretreated the cells with latrunculin-B, in order to depolymerize actin. Latrunculin-B pretreatment decreased permeability marker uptake of calcein at 0 minute (25%) compared to untreated, injured cells (56.5%) (Figure 9). In addition, when latrunculin-B was added 1-minute following injury to examine resealing, only 10.5% of the cells were permeable versus 24% for untreated, injured cultures (Figure 9). While the total number of permeable cells was lower for both pretreatment and delayed treatment with latrunculin-B, the relative degree of resealing was the same in treated versus untreated (injured) cultures (0.42 for both) (Figure 9). Latrunculin-B alone (no injury) resulted in significant marker uptake compared to untreated, uninjured cells, suggesting that cytoskeleton disruption alone alters membrane permeability (Figure 9).

To further elucidate the role of the cortical actin cytoskeleton in resealing, actin was stabilized using jasplakinolide, which is cell permeant and has been shown to stabilize filamentous actin (f-actin),[38] thereby promoting actin polymerization.[31,38] Jasplakinolide treatment was hypothesized to decrease resealing since cortical actin may obstruct the cytosolic vesicles docking with the plasma membrane—a crucial step in resealing.[31] Both pretreatment and treatment following injury did not deter at least some degree of resealing of neuronal plasma membrane (Figure 10). Pretreatment with jasplakinolide

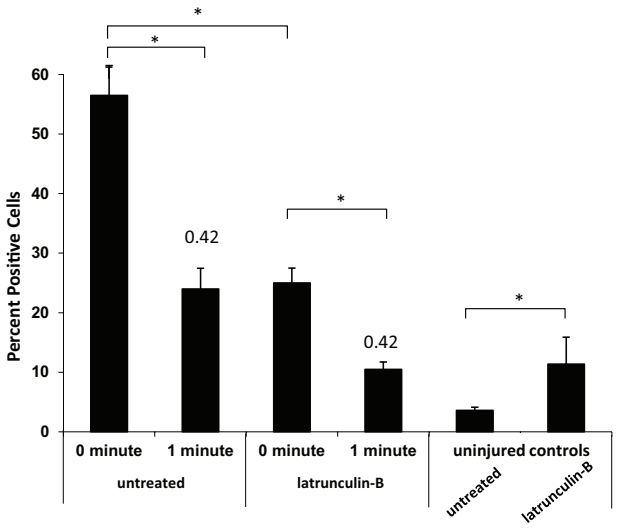


Figure 9. Pretreatment with latrunculin-B reduced the increase in percentage of positive cells due to mechanical insult when compared to untreated group (*$P < .0001$). Latrunculin-B-treated group contained less-positive cells at 1 minute, although the uptake ratios were the same for treated and untreated groups at that time. Uninjured control cultures that were untreated show less than 5% of cell uptake calcein, but uninjured cultures treated with latrunculin-B took up significantly more calcein. Error bars represent SD. n = 3 to 4 cultures/group.

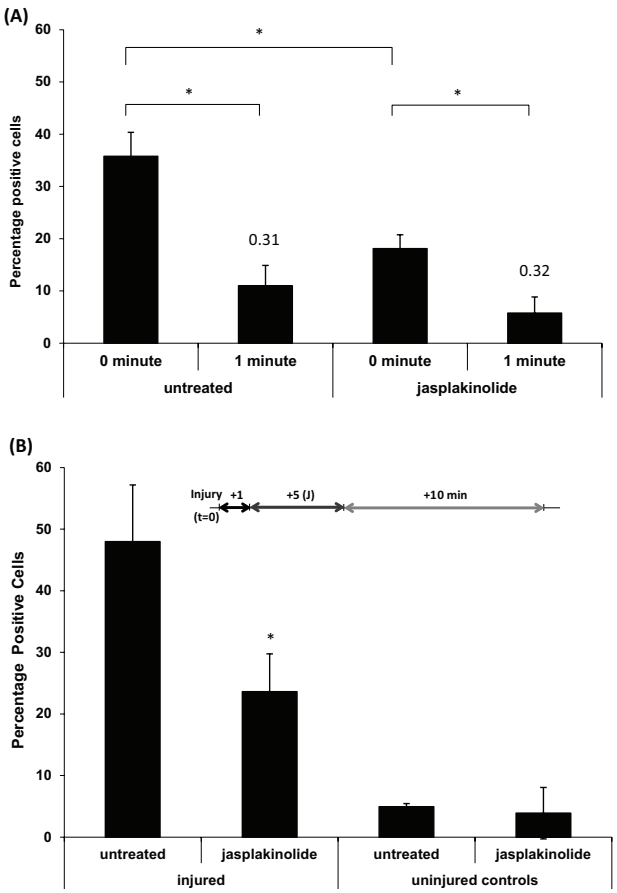


Figure 10. Effect of actin stabilization on membrane permeability and resealing: (A) pretreatment with jasplakinolide reduced increases in the percentage of positive cells to calcein due to mechanical insult (*$P < .0005$). Pretreatment with jasplakinolide did not have an effect in the cellular resealing at 1 minute postinsult when compared to untreated cultures at 1 minute. Uptake ratios are seen on top of 1-minute groups. Error bars represent SD. n = 3 to 5 cultures/group. (B) Treatment of jasplakinolide after insult induced cell resealing, which is evidenced by the lower percentage of positive cells to calcein when the marker is added after treatment when compared to untreated group ($P < .005$). Chart shows time course of this experiment. Black arrow indicates time between insult and addition of jasplakinolide (J), dark gray indicates jasplakinolide treatment, and mid gray indicates calcein incubation in normal Ca^{2+} conditions. Uninjured controls are displayed on the right and show that treatment alone did not significantly change uptake levels. Error bars represent SD. n = 4 to 5 cultures/group.

caused a reduction of positive cells at the time of injury and when calcein was added at 1 minute (Figure 10A). However, uptake ratios of both untreated and treated groups remained the same. Post-treatment of jasplakinolide in extracellular Ca^{2+}-depleted conditions caused a decrease in positive cells compared to untreated cultures when Ca^{2+} was reintroduced (Figure 10B).

Discussion

We found that sublethal shear stress-induced mechanical trauma in neurons caused a flux of extracellular molecules into the cell through nonspecific membrane disruptions. In addition to neurons, fluid shear stress-induced mechanoporation, with both a cone-disk viscometer and fluid microchannels, has been demonstrated in DU145 prostate cancer cells,[39] supporting that these observations are due to a nonspecific phenomenon. Changes in permeability likely result from excessive plasma membrane strains that arise from high-rate shear stress.[40] By varying the size and the molecular mass of the permeability marker, we were able to show differential uptake of the marker based on its molecular size. This suggests that a range of membrane defect size is produced from these insult parameters, consistent with other studies examining traumatic injury-associated mechanoporation with different sized permeability markers.[10,18] If we approximate molecular size for the smallest marker (calcein) and assume that markers are getting into the cell through nonspecific defects, we can estimate the defect size in this model to be on the order of several square nanometers. Membrane pores of this size are sufficient for ions to pass through[41,42] and to elicit electrophysiology dysfunction[43] while still being well below the critical size for survival[44] and resealing capacity.[45]

Permeability marker was predominantly visible in the soma, with relatively few neurites uptaking calcein. While there are obvious volume differences between neuronal cell bodies and neurites, the variable uptake may also be due to a differential strain distribution. We previously found that plasma membrane strains are significantly larger at the cell bodies than at adjacent neurites during a traumatic mechanical insult.[40] Neurons cultured on flat surfaces have their somata resting relatively higher from the plating surface than the neurites, making the cell bodies more vulnerable to shear stress insult at the apical surface (versus, for instance, a substrate stretch

insult). Calcein-filled neurites were observed only in cells that had calcein-positive somata, suggesting that entry was through the cell bodies, possibly due to the larger strains, albeit further studies are needed to confirm this assertion.

The rate dependency of the increase in plasma membrane permeability can be attributed to the viscoelastic properties of cells. The shorter rise time insult results in a more severe injury since the stress is applied more rapidly, allowing less time for relaxation and increasing membrane damage. The observation that higher rates result in more cell damage corroborates results from other in-vitro models that show that the rate is an important factor in injury severity.[10,19,21,46-48] More specifically, the authors have previously shown that shear stress-induced injury results in immediate influx of extracellular calcium.[40]

The addition of permeability marker at various times postinjury allowed us to examine resealing phenomena. The dramatic reduction in the percentage of cells containing calcein when the marker was added to cells 1 minute postinjury indicates a fast initiation of resealing, which is comparable to resealing times reported following cell wounding.[36] Resealing did not differ between the 2 rates, suggesting that both levels of injury were below catastrophic injury levels and did not impair resealing mechanisms. The resealing process occurred rapidly, as no difference in permeability to calcein between injured and uninjured cultures was observed when the marker was added 10 minutes postinjury, a time course consistent with poration caused by acoustic cavitation.[49] The resealing process was impaired when Ca^{2+} and Mg^{2+} were removed from the extracellular buffer, consistent with other studies.[50] The importance of extracellular Ca^{2+} for resealing of the plasma membrane has been demonstrated in numerous studies using many different cell types. For example, a dependency on extracellular Ca^{2+} for the resealing process is evident following nerve transection[51,52] and cell wounding.[34,35] Here, we report that a similar Ca^{2+}-dependent membrane repair mechanism occurs in neurons with smaller membrane defects resulting from a rapid onset, uniform mechanical insult.

The chelation of intracellular Ca^{2+} with BAPTA-AM prior to the insult (in the presence of normal extracellular Ca^{2+} concentrations) also limited resealing, demonstrating that cytosolic Ca^{2+} may also play a role in the repair mechanism following mechanical disruption of the neuronal plasma membrane. Depletion of intracellular Ca^{2+} and the lack of robust resealing may be due, in part, by the potential for cytosolic BAPTA to rapidly chelate Ca^{2+} as it enters the injured cells,[53] hereby reducing the Ca^{2+} available for resealing. The lack of calcium in either the extracellular or intracellular compartment did not affect the initial permeability, suggesting that the cells still received the insult. Even though cells appeared adhered to the slide following the insult (see Figure 6B), because of the key role of divalent ions and actin in cell adhesion,[54] we cannot confirm that the actual stress and strain on the cells is the same for all the treatments. We therefore used a ratio of permeable cells from experiments when marker was added 1 minute after injury to permeable cells when marker was present during the injury (0-minute condition) to examine resealing under conditions with variable initial permeability levels.

When both extracellular and intracellular Ca^{2+} was depleted, cells were less permeability to the marker at the time of the insult (Figure 8), but a higher percentage of the cells were able to reseal compared to cells that had either extracellular or intracellular Ca^{2+} reduced, indicated by the lower uptake ratio. It is possible that the cells were not as injured as other cultures, although examination of actual membrane stress was outside the scope of this study. It is also possible that permeability marker did not remain in the cell because of the diminished resealing. Chelation of intracellular Ca^{2+} by dimethyl BAPTA-AM has been shown to promote decoupling of the actin cytoskeleton and transmembrane adhesion proteins.[55] It may be possible that changes in the Ca^{2+} gradient across the plasma membrane, which is perturbed upon calcium chelation, may play a role in the resealing process, albeit we cannot rule out other mechanisms.[56] For example, decoupling actin from the membrane may reduce plasma membrane tension, in much the same way that actin-depolymerization agents such as cytochalasin-D have been shown to reduce tension and facilitate resealing.[57] Furthermore, reduced membrane tension may be a requirement for resealing.[57]

Latrunculin-B is known to be a powerful actin-depolymerization agent.[58,59] Disturbing the actin cytoskeleton with latrunculin-B had a significant effect on mechanically induced membrane disruptions. The relative decrease in permeability upon actin depolymerization compared to the untreated group may be due to a decrease of cytoskeleton/membrane interactions and reduction in membrane stiffness and tension,[53] making the neurons less vulnerable to the insult. It is possible that a less-stiff cytoskeleton is not damaged as much under these insult conditions, and/or it allows for the plasma membrane to reseal faster by passive means, which can occur even in the absence of extracellular Ca^{2+}.[36,57] This slower calcium-independent mechanism is supported by our results of the temporal profile of resealing under low extracellular Ca^{2+} conditions (see Figure 6). This evidence may help explain why latrunculin-treated cells exhibited reduced permeability but maintained some degree of resealing capacity. This is in contrast, however, to the increased poration observed in latrunculin-treated cells subject to electroporation,[60] albeit the nature of the insults may lead to differing membrane damage and resealing dynamics.[61] Interestingly, latrunculin alone (no injury) resulted in more calcein uptake than no treatment (see Figure 9), supporting the possibility that a less-stiff membrane would allow more marker to pass through as well as be less injured.

Pretreatment of neuronal cultures with the actin polymerization promoter jasplakinolide resulted in a decrease in the percentage of positive cells in injured cultures similar to the observation under actin depolymerizing conditions. This

is in contrast to the expectation that an increase in membrane stiffness would render the cells more vulnerable to the mechanical insult and membrane failure.[62] It is possible that an increase in actin polymerization restricts the cell from experiencing the large strains required for membrane compromise. Our findings suggest that the force required for mechanoporation is likely dependent on both the fluidity of the membrane and stress bearing properties of the actin cytoskeleton. Nonetheless, the complex relationship between membrane tension, cytoskeletal rigidity, and failure criteria for traumatically injured neurons is not fully understood and will require further study.

To further probe the role of actin polymerization in the membrane resealing process, we took advantage of the diminished ability of cells to reseal when extracellular Ca^{2+} is removed. By injuring cultures in low/no extracellular Ca^{2+} before jasplakinolide treatment, we ensured that cells were injured as much as untreated groups and that a relatively large percentage of cells remained permeable throughout the treatment time (ie, reduced resealing capacity). Postinjury treatment with jasplakinolide while membranes were still permeable showed that enhanced actin polymerization was mechanoprotective, perhaps preventing the persistence of membrane permeability and eventually supporting resealing over untreated cultures after addition of extracellular Ca^{2+}. Jasplakinolide treatment of endothelial cells has been reported to decrease resealing of those cells when subjected to scratch wounding.[31] The nature of this type of cell wounding would produce larger membrane defects compared to those observed in this study, which may impede resealing capacity. Further studies are needed to determine the role of the cytoskeleton and membrane-cytoskeleton interactions in neuronal plasma membrane disruption and resealing under traumatic conditions such as those used in this study.

These findings may have an important impact in the study of TBI, as normal electrical function in neurons depends on a tightly regulated ionic concentration gradient across the plasma membrane, which is likely to be altered as a result of increased permeability.[43] Plasma membrane disruption may also cause a dramatic perturbation in other cellular functions, given that molecules much larger than ions are able to cross the plasma membrane. It is not known how these cells are affected by a potential loss in ion homeostasis or how the temporal recovery profile contributes to ultimate function. Taken together, these results indicate that a traumatic CNS insult causes disruption of the plasma membrane and that the degree of mechanoporation and resealing depend, in part, on membrane-actin structure and calcium-dependent mechanisms. Further work on pathomechanotransduction mechanisms should focus on correlating cellular-level stresses and strains to membrane permeability in other neural cell and tissue preparations subjected to mechanical insult in order to determine the threshold for membrane failure and capacity for repair.

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Author Contributions

G.R.P. performed all experiments, developed assays, performed data analysis, helped with study direction and interpretation, and wrote the first manuscript draft. M.C.L. conceived of the idea and participated in study direction and interpretation. Both authors contributed to manuscript revisions and editing.

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