JOURNAL OF NEUROTRAUMA
Volume 23, Number 2, 2006

Pp. 193–204

Mechanisms and Consequences of Neuronal Stretch Injury *In Vitro* Differ with the Model of Trauma

DONNA M. GEDDES-KLEIN,[1] KIMBERLY B. SCHIFFMAN,[1] and DAVID F. MEANEY[1,2]

ABSTRACT

The deformation to the brain that occurs during traumatic brain injury (TBI) results in a complex strain distribution throughout the brain tissue. Recently, many *in vitro* models of neuronal injury have been developed to simplify the mechanics which occur during TBI. We hypothesized that the type of mechanical insult imparted onto neurons would significantly alter both the mechanism and severity of the neuronal response to injury. In this study, primary cortical neurons were cultured on an elastic substrate and subjected to graded levels (0%, 10%, 30%, 50%) of either uniaxial (cells stretched in one direction only) or biaxial (cells simultaneously stretched in two directions) stretch. We found that neurons stretched in either injury paradigm exhibited immediate increases in intracellular free calcium ($[Ca^{2+}]_i$), but the magnitude of the $[Ca^{2+}]_i$ rise was nearly an order of magnitude higher in biaxially stretched neurons compared to uniaxially stretched neurons. Moreover, while the $[Ca^{2+}]_i$ transient after uniaxial stretch was blocked with specific channel antagonists (APV, CNQX, nimodipine, TTX), a substantial $[Ca^{2+}]_i$ transient persisted in biaxially stretched neurons. We theorized that the additional calcium influx after biaxial stretch entered through nonspecific pores/tears formed in the membrane, since biaxially stretched neurons exhibited significant uptake of carboxyfluorescein, a molecule typically impermeant to cell membranes. Despite the large $[Ca^{2+}]_i$ transients, neither injury profile resulted in death within 24 h of injury. Interestingly, though, uniaxially stretched neurons exhibited enhanced $[Ca^{+2}]_i$ influx following NMDA stimulation 24 h after trauma, compared to both control and biaxially stretched neurons. These data point out that the type of mechanical insult will influence the acute mechanisms of injury *in vitro*, can cause differences in the response to potential secondary excitotoxic injury mechanisms, and emphasizes the need to further study how these mechanical conditions can separately affect cell fate following mechanical injury.

Key words: cytosolic calcium; *in vitro* models; NMDA receptors; permeability

INTRODUCTION

THE UNDERLYING BIOMECHANICAL FACTORS that contribute to the injury patterns observed in traumatic brain injury (TBI) are becoming more defined. Very early biomechanical work suggested that brain injuries occurred principally from pressure gradients within the brain during impact, and that simulating these pressures in animal models would accurately replicate the mechanisms of injury (Denny-Brown and Russel, 1941; Gurdjian and Webster, 1943). Later work expanded this view and showed that the head motions occurring at the mo-

Departments of [1]Bioengineering and [2]Neurosurgery, University of Pennsylvania, Philadelphia, Pennsylvania.

ment of injury result in not only pressures, but also intracranial deformations throughout the brain (Holbourn, 1943; Ommaya et al., 1967). These intracranial deformation patterns are complex, can vary over time, and are now thought to be a primary contributing factor to the lesions that appear in the brain following TBI (Zhang et al., 2001). Many experimental and computational tools are now available to examine these intracranial deformations during the rapid head motions, and to examine how these deformations are simulated in experimental TBI models (Gennarelli, 1994; Meaney et al., 1995; Ueno et al., 1995; Shreiber et al., 1997; Nishimoto and Murakami, 1998; Zhang et al., 2004). Current work is directed towards determining how this new information can be used to define new standards for head protection, as well as improving the biomechanical fidelity of existing experimental models to human TBI (Takhounts et al., 2003; Zhang et al., 2004).

An important adjunct to these biomechanical studies is the recent development of several *in vitro* models of neuronal injury to directly assess the effects of biomechanical input parameters (e.g., strain, strain rate, pressure) on immediate and longer term cell function. These models use dissociated cultures and, less frequently, organotypic cultures to study the biomolecular and electrophysiological effects of mechanical loading on the central nervous system (Tavalin et al., 1995; LaPlaca et al., 1997; Morrison et al., 2000; Geddes and Cargill, 2001; Lusardi et al., 2004b). The models can use a pressure loading, a rapid stretch injury, a rapid fluid shear stress, or a combination of these conditions to mimic the forces experienced within the brain parenchyma during TBI. These cellular models of trauma provide valuable advantages over animal models, as both the mechanical and biochemical environment of the cells can be precisely controlled. The use and utility of these models has steadily expanded in the past decade, and findings from these models have expanded our knowledge on the fundamental mechanisms of injury that can contribute to the sequelae of TBI.

Although valuable information can be learned from *in vitro* models of trauma, it is important to understand how the findings from these models may be sensitive to the type of mechanical loading used in each model. Results from these *in vitro* models are sometimes conflicting, suggesting that differences in the mechanical loading and temporal profile of the mechanical insult may significantly alter the cellular response. For example, increases in intracellular free calcium concentration ($[Ca^{2+}]_i$) appear across nearly all injury models, and the degree of the $[Ca^{2+}]_i$ after stretch consistently changes in proportion to the magnitude of applied strain (Cargill and Thibault, 1996; LaPlaca et al., 1997; Weber et al., 1999; Geddes and Cargill, 2001; Lusardi et al., 2004b). However, the mechanism initiating the increase in $[Ca^{2+}]_i$ seems to vary according to the method used to create the mechanical injury. Geddes et al. (2003) showed that neurons exposed to 30% stretch simultaneously in two perpendicular directions respond with an immediate increase in plasma membrane permeability, causing a corresponding elevation in $[Ca^{2+}]_i$. Alternatively, changes in the NMDA receptor are thought to underlie most of the increases in $[Ca^{2+}]_i$ in other models that use a rapid stretch applied in only direction, a biaxial stretch applied more slowly, or a rapidly applied fluid shear stress across the cell surface (LaPlaca and Thibault, 1998; Weber et al., 1999; Lusardi et al., 2004a). These differing mechanisms for immediate $[Ca^{2+}]_i$ elevations have important implications for designing treatments for TBI, as one finding suggests that cell membrane repair is critically important, while a second group of studies suggests that NMDA receptor activation is the principal mechanism.

In this study, we hypothesized that the type of mechanical insult imparted onto the neurons would significantly alter both the mechanism and severity of the neuronal response to injury. Primary cortical neurons were cultured on an elastic substrate and subjected to graded levels (0%, 10%, 30%, 50%) of either uniaxial (cells stretched in one direction only) or biaxial (cells stretched in two directions) stretch. Cellular responses to various stretch paradigms were assessed by immediate alterations in plasma membrane permeability and $[Ca^{2+}]_i$ as well as alterations in viability and NMDA-induced $[Ca^{2+}]_i$ responses 24 h after injury. Our data show that biaxial stretch is much more likely to produce an immediate and significant change in membrane permeability, while uniaxial stretch will cause immediate elevations in $[Ca^{2+}]_i$ through specific channels that include NMDA receptors. In addition, we find that uniaxial stretch injury leads to a significantly enhanced response to secondary NMDA stimulation 24 h after injury, compared to both biaxially stretched and unstretched neurons. Taken together, these findings show that the local mechanical factors that occur within a brain region during injury have important immediate and delayed consequences on the neuronal response to TBI.

MATERIALS AND METHODS

Neuron Isolation and Cell Culture

Animal procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of Pennsylvania. Cortical neurons were isolated from embryonic day 18 Sprague Dawley rats (Charles River Laboratories, Wilmington, MA). Pregnant

rats were anesthetized with CO_2 and sacrificed via cervical dislocation. Embryos were surgically removed from the adult rat and cortices were isolated. Tissue was incubated in trypsin (0.3 mg/mL; Sigma, St. Louis, MO) and DNAse (0.2 mg/mL; Roche Applied Science, Indianapolis, IN) solution for 20 min at 37°C and then triturated in media containing soybean trypsin inhibitor (1 mg/mL, Invitrogen Corp., Carlsbad, CA). Neurons were centrifuged and re-suspended in Neurobasal media (Invitrogen Corp.) supplemented with 2% B-27 (Invitrogen Corp.), 2.0 mM L-glutamine (Invitrogen Corp.), 1% Penicillin-Streptomycin (Invitrogen Corp.), and 25 μM L-glutamic acid (Sigma). Cells were then filtered through Nitex mesh (Cross Wire Cloth, Bellmawr, NJ) and diluted to 0.75 million cells/mL for plating. Cells were plated onto flexible silicone substrates (seven parts Sylgard 186 and four parts Sylgard 184; 0.001" thick; Ellsworth Adhesives, Norristown, PA) that were previously coated overnight with poly-L-lysine (5 μg/mL; Sigma) and incubated at 37°C, 5% CO_2. Four days after plating, media was changed to Neurobasal media supplemented with B-27 and 2 mM L-glutamine. All experiments were performed on 10- or 11-day-old cultures, to be consistent with previous studies of *in vitro* models of neuronal stretch (Zhang et al., 1996; Goforth et al., 1999; Weber et al., 1999; Arundine et al., 2003; Lusardi et al., 2004b).

In Vitro *Model of TBI*

TBI was simulated using a custom-designed *in vitro* model (Lusardi et al., 2004b). Neurons cultured on silicone substrates were placed atop a stainless steel plate inside a sealed chamber. Substrates were stretched by applying a rapid pressure pulse (onset time = 15 msec) of varying pressures, in order to adjust the applied strain magnitude between 10%, 30%, and 50% (Table 1). Analysis of the strain field across the membrane surface was based on past numerical simulations of both membrane geometries (Morrison et al., 1998; Lusardi et al., 2004b). These numerical simulations, validated with measurements of the membrane strain, were used as a guide in selecting the input pressures and, in turn, the peak membrane strain for both testing configurations. In all experiments, imaged cells were located within 25% of the center of the substrate in order to ensure fully uniaxial or biaxial strain fields. The onset time of the pressure pulse was held constant at 15ms across all strain magnitudes so that strain rates were consistent with those typically used to simulate TBI (Geddes and Cargill, 2001; Lusardi et al., 2004b). Only slightly higher pressures were required to achieve biaxial strain magnitudes that were equivalent to uniaxial stretch. The geometry of the stainless steel plate was varied in order to adjust the strain state, as illustrated in Figure 1. A stainless steel plate with a 6 mm × 18 mm rectangular slit was used when cells were stretched in one direction only, or uniaxially. In both configurations, we use the applied membrane strain to describe the severity of injury and not the strain within individual cells, which is likely smaller based on past studies (Barbee et al., 1994; LaPlaca and Thibault, 1997). A plate with a circle of 18 mm in diameter was used when cells were stretched equally in all directions, or biaxially. Only the neurons cultured within the open region of the stainless steel plate were stretched. Since the strain in the two perpendicular directions using the biaxial geometry changes with position on the substrate (Morrison et al., 1998), we examined regions only from the center of the membrane, where equal strains are applied in both directions. In addition, control experiments were performed in which cells were loaded into cell stretching system but not stretched. Each individual well of cortical neurons was stretched only once and used for only one study.

Table 1. Pressures Required to Achieve Substrate Strain

	10%	*30%*	*50%*
Uniaxial stretch	1.4 ± 0.1 psi	2.9 ± 0.1 psi	5.1 ± 0.1 psi
Biaxial stretch	1.3 ± 0.1 psi	2.4 ± 0.1 psi	4.3 ± 0.1 psi

The magnitude of the substrate strain was increased by raising the chamber pressure applied to the neurons. Slightly more pressure was necessary to induce biaxial strains that were equal in magnitude to uniaxial strains.

$[Ca^{2+}]_i$ Measurement

The calcium sensitive fluorescent dye, Fura-2AM (Molecular Probes, Eugene, OR), was used to determine immediate stretch-induced alterations in $[Ca^{2+}]_i$. Neurons were incubated in 5 μM Fura-2 AM for 40 min and then rinsed three times with saline (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM HEPES, 15 mM glucose). Cells were excited alternately at 340 and 380 nm, and an image of the emission from each excitation wavelength was collected using a 14-bit Hamamatsu digital camera (C4742-98; Universal Imaging, Inc., West Chester, PA). In all experiments, 1 min of pre-stretch baseline data were recorded. Neurons were stretched, and $[Ca^{2+}]_i$ was monitored continuously for 2 min following stretch. The emission intensity images were post-processed to calculate changes in $[Ca^{2+}]_i$ in regions of interest in the images using MetaFluor software (Universal Imaging, Downingtown, PA). All cells within the field of view were analyzed for each experi-

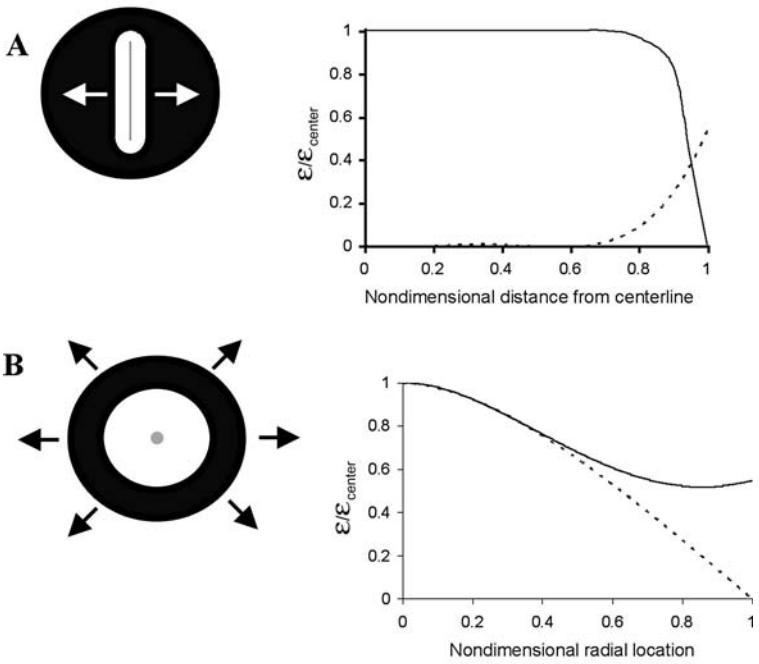

FIG. 1. How the strain state was altered by varying the geometry of the rigid plate (shown in black) beneath the silicone substrates. The two principal nominal strains, normalized to the maximum value at the center of the membrane (shown in gray), are shown for the two membrane geometries (50% maximum stretch condition). The first and second principal strains are denoted by solid and dashed lines, respectively. (**A**) Pressurizing only a 6 × 18mm approximately rectangular region of the silicone substrate produces a membrane deformation field with a much larger principal stretch across the width of the slit than its length. Because of this difference in principal stretch ratios, we refer to this configuration as the uniaxial method. (**B**) Alternatively, pressurizing an 18-mm-diameter circular region of the silicone substrate produces a membrane deformation field with an equal principal stretch value at the center of the membrane (i.e., biaxial), with this ratio in the principal stretch changing with distance from the center of the membrane.

ment. The average number of cells imaged per field of view was 34 ± 2, and ranged from 12 to 49. Three to seven experimental wells were used for each experimental condition, and all experiments were performed across at least two isolations. It should be noted that the percent of neurons responding to stretch varied across different cortical isolations, though trends remained the same.

In a subsequent study, the mechanism of the $[Ca^{2+}]_i$ peak after 50% stretch was investigated by blocking sodium channels with 1 μM tetrodotoxin (TTX; Sigma), AMPA receptors with 2 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma), L-type voltage gated calcium channels with 10 μM nimodipine (Sigma), and NMDA receptors with 25 μm D(−)-2-amino-5-phosphonopentanoic acid (APV; Sigma). Drugs were added 5 min prior to injury and left on for the remainder of the $[Ca^{2+}]_i$ imaging period.

$[Ca^{2+}]_i$ Calibration and Analysis

$[Ca^{2+}]_i$ was calibrated according to Equation 1, where R is the recorded Fura-2 ratio, K_D is the dissociation constant of Fura-2 (140 nM), R_{min} is the minimum Fura-2 ratio, R_{max} is the maximum Fura-2 ratio, and Q is the ratio of the 380-nm intensity upon attaining R_{min} to that upon attaining R_{max} (Grynkiewicz et al., 1985). To determine R_{max} , neurons were treated with the calcium ionophore, A-23187 (100 μM; Molecular Probes). $[Ca^{2+}]_i$ was then reduced with 1 mM BAPTA AM (Molecular Probes) diluted in calcium-free saline, and R_{min} was determined. R_{min}, R_{max}, and Q were determined experimentally to be 0.18, 14.6, and 9.04, respectively.

$$[Ca^{2+}]_i = K_D Q \frac{R - R_{min}}{} \quad (1)$$

Normalized peak $[Ca^{2+}]_i$ after stretch was assessed for all cells individually and defined as the peak $[Ca^{2+}]_i$ over the first minute after stretch divided by the baseline $[Ca^{2+}]_i$ prior to stretch. Neurons exhibiting normalized $[Ca^{2+}]_i$ after stretch greater than 2, representing increases greater than 100% of the $[Ca^{2+}]_i$ prior to stretch, were considered to show a $[Ca^{2+}]_i$ "response" to stretch.

Detection of Alterations in Plasma Membrane Permeability

Plasma membrane permeability after mechanical stretch was assessed by evaluating uptake of the normally impermeant fluorescent molecule, carboxyfluorescein (CBF, MW = 380 Da, radius = 0.5 nm; Sigma). This technique was previously used to detect permeability changes in both electroporated cells (Bartoletti et al., 1989; Gift and Weaver, 2000) and stretched cells (Geddes et al., 2003). Previous studies have shown that stretch-induced membrane permeability changes were repaired by ten minutes after stretch (Geddes et al., 2003). Therefore, CBF was left on the cells for the entire duration of the proposed permeability change in order to both maximize dye diffusion into cells and to limit diffusion out of cells. Nonetheless, it should be noted that any permeability changes that persisted after the dye was removed were likely left undetected because dye was free to diffuse out of cells. Immediately prior to injury, cells were treated with 100 μM CBF, and nuclei were stained with Hoechst 33342 (20 μg/mL; Molecular Probes, Eugene, OR). Neurons were stretched in the presence of CBF and incubated at 37°C, 5% CO_2 for 10 min. Neurons were then rinsed thoroughly with saline to ensure all extracellular CBF molecules were removed. Similar experiments were repeated 24 h after injury to evaluate membrane repair. Representative fluorescent images were taken from three different areas near the center of the stretched population. Cells positively stained with CBF were later counted and normalized to the total number of Hoechst-positive nuclei.

Functional Assessment 24 h after Stretch

Using parallel sister cultures, viability was assessed in unstretched neurons as well as those stretched either biaxially or uniaxially at 10%, 30%, or 50%. Four wells were used for each experimental condition. To avoid the potential complicating response of adjacent, unstretched neurons on viability, neuronal cultures were patterned on the substrate to only cover the area of deformation used for each type of mechanical injury. Neurons were stretched in saline and incubated at 37°C, 5% CO_2 for 24 h. Viability at 24 h was assessed by staining live cells with 2 μM calcein, AM (Molecular Probes), and dead cells with 2 μM ethidium homodimer-1 (Sigma). Nuclei were also stained with Hoechst to obtain a total cell count. Representative fluorescent images were taken from three different regions near the center of the stretched population of cells. Percent of dead cells were determined by total number of ethidium positive cells normalized to total number of Hoechst positive cells. $[Ca^{2+}]_i$ responses to secondary NMDA insults were also assessed 24 h after no stretch, 50% uniaxial or 50% biaxial stretch. Seven to eight experimental wells were used for each experimental group. $[Ca^{2+}]_i$ was measured as described above for one minute and then neurons were treated with 100 μM NMDA.

Statistical Analysis

Data are presented as means ± SEM. General linear model analysis of variance (ANOVA) was used to assess the independent roles of strain magnitude and strain state. Unpaired two-tailed Student's *t*-tests were performed in order to assess statistical significance versus unstretched controls, and *p*-values of <0.05 were considered significant.

RESULTS

$[Ca^{2+}]_i$ Alterations after Stretch

Figure 2A shows the average $[Ca^{2+}]_i$ transient after either 50% uniaxial or biaxial stretch. In both cases, mechanical stretch injury caused an immediate rise in $[Ca^{2+}]_i$ followed by a gradual recovery period, as observed in other neuronal injury models (LaPlaca et al., 1997; Weber et al., 1999; Geddes and Cargill, 2001; Lusardi et al., 2004b). However, the $[Ca^{2+}]_i$ response after biaxial stretch was nearly an order of magnitude higher than the peak change in $[Ca^{2+}]_i$ following uniaxial stretch. We then compared the peak $[Ca^{2+}]_i$ after stretch (normalized to baseline) for all the applied stretch levels (Fig. 2B). The peak $[Ca^{2+}]_i$ after stretch was proportional to the applied strain magnitude ($p < 0.001$), but also significantly depended on the direction of the applied stretch ($p < 0.001$). $[Ca^{2+}]_i$ peaks were rare after either uniaxial or biaxial 10% stretch. Following 30% stretch, $[Ca^{2+}]_i$ responses were only evident after biaxial stretch. At the most severe level of stretch (50%), $[Ca^{2+}]_i$ peaks were evident after both uniaxial and biaxial stretch, but were much greater in magnitude after biaxial stretch. The $[Ca^{2+}]_i$ peak after 50% uniaxial stretch was only 1.6 ± 0.1 times greater than baseline $[Ca^{2+}]_i$, compared to 5.6 ± 0.7 following 50% biaxial stretch.

The percent of neurons responding to stretch (defined by a peak increase in $[Ca^{2+}]_i$ greater than 100%; Fig. 2C) also synergistically depended on the magnitude and direction of applied strain ($p < 0.001$). While less than 25% of neurons stretched uniaxially responded to a 50% peak stretch injury, the majority of neurons stretched biaxially responded to either 30% or 50% stretch. Specifically, 50% uniaxial stretch caused 18 ± 3% of neurons to respond to stretch with more than a doubling in $[Ca^{2+}]_i$,

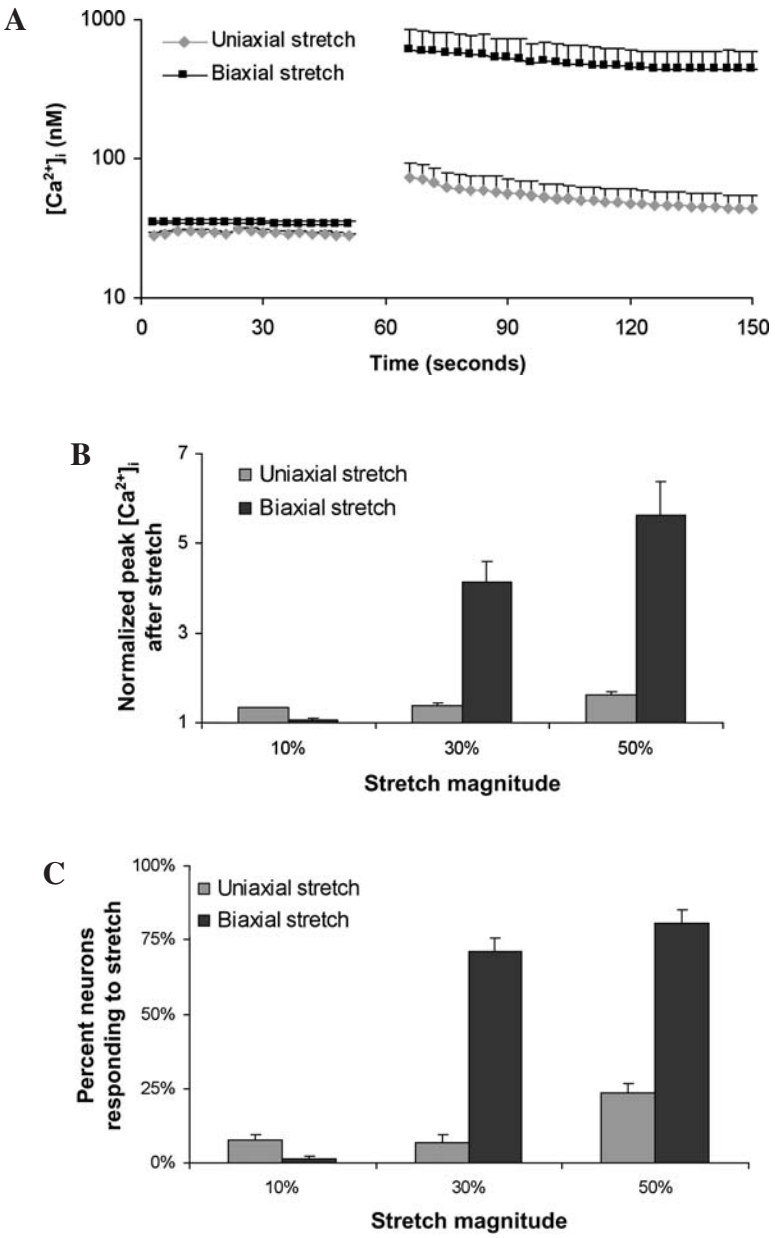

FIG. 2. (**A**) The average $[Ca^{2+}]_i$ transients are shown for a neurons subjected to either 50% uniaxial or biaxial stretch. (**B**) The normalized peak $[Ca^{2+}]_i$ after stretch is summarized for all stretch levels that were tested. (**C**) The percent of neurons responding to stretch (defined by a peak increase in $[Ca^{2+}]_i$ greater than 100%) after graded levels of stretch are shown after either uniaxial or biaxial stretch. As strain magnitude is increased in either the uniaxial or biaxial direction, both the magnitude and the percent of neurons responding to stretch increases ($p < 0.001$).

compared to $81 \pm 4\%$ of neurons subjected to biaxial stretch.

Next, we investigated the mechanism of $[Ca^{2+}]_i$ transients in response to either 50% uniaxial or biaxial stretch. We assessed the influence of specific membrane receptors/channels on $[Ca^{2+}]_i$ peaks after stretch by pretreating neurons with saline solution containing antagonists directed towards NMDA receptors (25 μm APV), L-type voltage gated calcium channels (10 μM nimodipine), sodium channels (1 μM TTX), and AMPA receptors (2 μM CNQX). We used this cocktail, rather than a series of single inhibitors, to account for the $[Ca^{2+}]_i$ changes that could occur from receptor/channel mechanisms reported in previous models of *in vitro*

stretch injury (Weber et al., 1999; Wolf et al., 2001; Lusardi et al., 2004a). Following 50% uniaxial stretch, $[Ca^{2+}]_i$ peaks were significantly reduced when neurons were pretreated with the aforementioned cocktail ($p < 0.001$; Fig. 3A). Moreover, the percent of neurons responding to uniaxial stretch with increases in $[Ca^{2+}]_i$ was reduced from $44.4 \pm 2.4\%$ to only $8.9 \pm 1.5\%$ ($p < 0.001$; Fig. 3B). Pre-treatment of neurons with the cocktail also significantly reduced both the normalized peak $[Ca^{2+}]_i$ and percent neurons responding to 50% biaxial stretch ($p < 0.001$; Figure 3A, B). However, large $[Ca^{2+}]_i$ transients were still evident after 50% biaxial stretch, with $62.6 \pm 3\%$ of cocktail-treated neurons still exhibiting $[Ca^{2+}]_i$ peaks after stretch. These results suggest that the activation of NMDA receptors, AMPA receptors, and/or voltage gated calcium and sodium channels together contribute to stretch-induced $[Ca^{2+}]_i$ transients following both uniaxial and biaxial stretch.

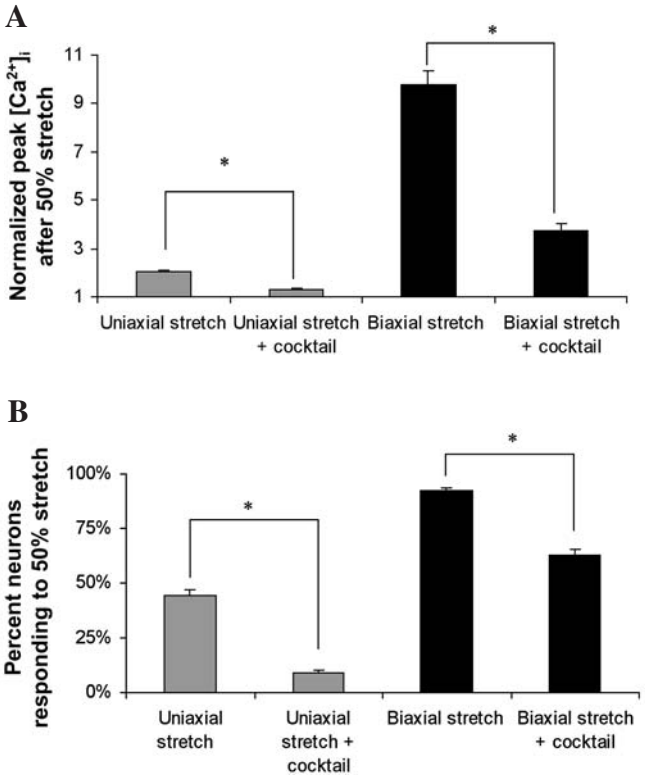

FIG. 3. Normalized peak $[Ca^{2+}]_i$ after 50% stretch (**A**) and percent of neurons responding to 50% stretch with increases in $[Ca^{2+}]_i$ (**B**) are shown for neurons stretched either biaxially or uniaxially in the presence or absence of a cocktail of channel antagonists to block NMDA receptors, AMPA receptors, and L-type voltage gated calcium and sodium channels. The $[Ca^{2+}]_i$ response in uniaxially stretched neurons was completely blocked in the presence of the cocktail whereas that in biaxially stretched neurons were only partially blocked. $*p < 0.001$.

However, additional mechanisms of calcium entry likely occur in response to 50% biaxial stretch, and this alternative mechanism accounts for a large fraction of calcium influx following biaxial stretch injury.

Stretch-Induced Changes in Plasma Membrane Permeability

$[Ca^{2+}]_i$ transients after stretch may also arise as a result of increases in plasma membrane permeability, thereby allowing influx of calcium through non-specific pores/tears in the membrane. We determined if stretched neurons exhibited increases in plasma membrane permeability by evaluating uptake of the normally impermeant molecule, CBF. Representative micrographs are shown in Figure 4A for neurons subject to control, 50% uniaxial or 50% biaxial stretch. CBF positive cells (denoted by bright staining) are only evident in the stretched neurons, with the majority of the staining evident in the neurons stretched biaxially. The percent of neurons permeable to CBF is summarized in Figure 4B. CBF uptake significantly depended on both the applied strain magnitude ($p < 0.001$) and direction of strain ($p < 0.001$). Moreover, only neurons subjected to 50% biaxial strain showed a significant elevation in CBF uptake, compared to all other groups tested ($p < 0.01$). By 24 h after 50% stretch injury, no significant uptake of CBF was evident in either uniaxially ($p = 0.48$) or biaxially stretched neurons ($p = 0.38$), indicating that permeability changes were indeed transient.

Viability Changes after Stretch

With these immediate changes in $[Ca^{2+}]_i$ occurring following the two forms of stretch injury, we proceeded to examine if either injury paradigm caused changes in neuronal viability 24 h after stretch. Despite the increases in plasma membrane permeability after biaxial injury, we did not observe any co-localization of ethidium homodimer 1 and calcein in the neurons at any of the stretch levels tested. Interestingly, no significant differences in viability were observed after either 50% uniaxial or biaxial stretch, compared to control conditions (Fig. 5). These results suggest that any permeability changes that were evident immediately after stretch had been repaired, leading to no significant uptake of the ethidium homodimer at 1 day after stretch injury.

Calcium Influx through NMDAR after Stretch

Although neuronal stretch was not lethal in either uniaxial or biaxial stretch, the overall functional capacity of stretched neuronal cultures remained unclear. Therefore, we assessed alterations in calcium flux through NMDA receptors 24 h after stretch by treating cells with 100 μM

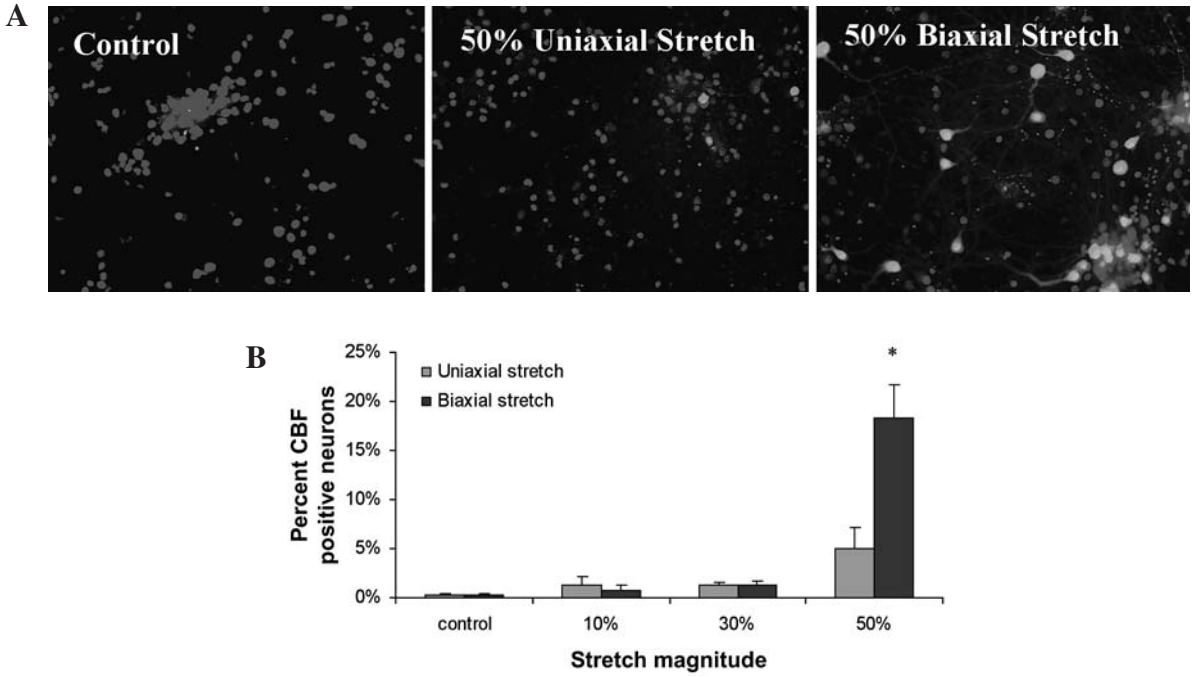

FIG. 4. (**A**) Representative micrographs illustrate carboxyfluorescein (CBF) uptake in neurons after control, 50% uniaxial, or 50% biaxial stretch. CBF-positive cells are indicated by bright staining, and Hoechst-positive nuclei are shown in gray. (**B**) The percent of CBF-positive cells after graded levels of either uniaxial or biaxial stretch is shown. Significant CBF uptake was only evident in neurons after 50% biaxial stretch, compared to control. $*p < 0.01$ compared to control.

NMDA and measuring the corresponding $[Ca^{2+}]_i$ change. 100 μM NMDA was chosen in order to activate the majority of NMDA receptors, without saturating the Fura-2 response outside of the calcium sensitive range, and to match our previous studies (Lusardi et al., 2004a). By 24 h after all stretch conditions, $[Ca^{2+}]_i$ returned to normal levels as no significant differences in baseline $[Ca^{2+}]_i$ were evident. Upon application of NMDA, more than 75% of cells responded to NMDA with increases in $[Ca^{2+}]_i$, independent of stretch condition (Fig. 6A). Despite showing an initial acute peak in $[Ca^{2+}]_i$ nearly five times less than a biaxial stretch of equal magnitude, cells subjected to uniaxial stretch exhibited enhanced $[Ca^{2+}]_i$ after NMDAR stimulation compared to both control cells and biaxially stretched neurons ($p < 0.001$; Fig. 6B). Cells stretched biaxially also exhibited enhanced calcium flux through NMDAR when compared to control cells ($p < 0.001$; Fig. 6B).

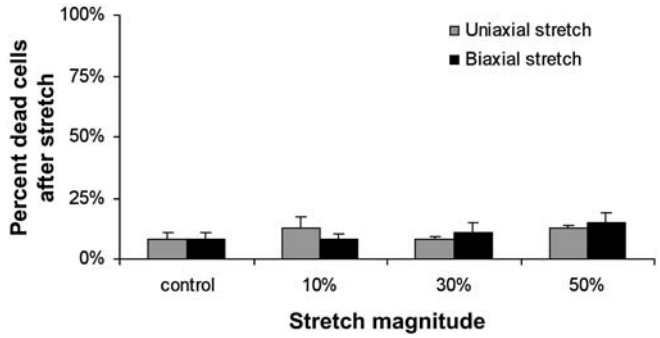

FIG. 5. Neuronal death after graded levels of either uniaxial or biaxial stretch or control is shown. No changes in cell death were observed after any level or type of neuronal stretch.

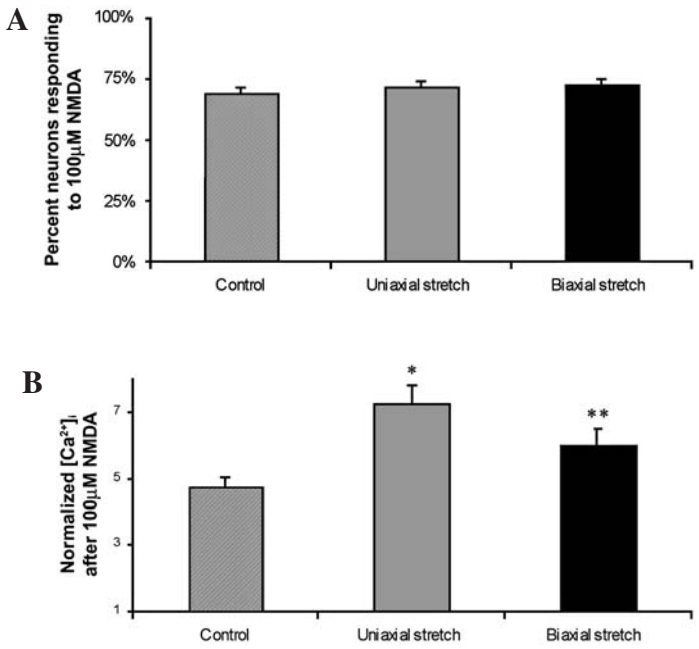

FIG. 6. Percent of neurons responding (**A**) and normalized peak $[Ca^{2+}]_i$ to secondary insults (**B**) of 100 μM of NMDA at 24 h after either 50% uniaxial or biaxial stretch are shown. No change in the percent of neurons responding to NMDA between stretch paradigms was evident. Uniaxially stretched neurons exhibited an enhanced $[Ca^{2+}]_i$ response to NMDA compared to both control neurons and biaxially stretched neurons. Biaxially stretched neurons exhibited an increased response to NMDA stimulation compared to control neurons only. *$p < 0.001$ compared to control, and biaxially stretched neurons; **$p < 0.001$ compared to control neurons.

DISCUSSION

The use of *in vitro* models of TBI can provide valuable information in understanding the initial cellular mechanisms of trauma, eventually leading to new insights for developing pharmaceutical therapies. In this study, we compared the immediate responses of primary cortical neurons to either rapid uniaxial or biaxial stretch—two commonly used paradigms of trauma *in vitro*. Both types of stretch occur in TBI, yet very little is known on the distinguishing features between these two types of mechanical injury. In both forms of stretch, cells responded with immediate rises in $[Ca^{2+}]_i$. However, the mechanism of the initial increase in $[Ca^{2+}]_i$ varied substantially between the two stretch conditions. The immediate $[Ca^{2+}]_i$ increase following uniaxial stretch was caused by the activation of specific calcium channels, since it was completely blocked when NMDA receptors, AMPA receptors, and voltage gated calcium and sodium channels were simultaneously antagonized. In contrast, a large $[Ca^{2+}]_i$ response to biaxial stretch persisted when these same channels were pharmacologically blocked. The remaining calcium influx after biaxial stretch was explained by an increase in non-specific permeability of the cell membrane, as evidenced by the increased uptake in the cell impermeable molecule, CBF, in these mechanically injured neurons. Although neither uniaxial nor biaxial stretch resulted in cell death at 24 h, uniaxially stretched neurons exhibited enhanced response to NMDA stimulation 24 h following injury when compared to both unstretched and biaxially stretched neurons.

Increases in $[Ca^{2+}]_i$ have been reported within seconds of trauma induced by fluid shear stress (LaPlaca et al., 1997), biaxial stretch (Weber et al., 1999; Geddes and Cargill, 2001), and uniaxial stretch (Lusardi et al., 2004b). Similarly, long-lasting $[Ca^{2+}]_i$ increases have been observed following various animal models of trauma (Fineman et al., 1993; Nilsson et al., 1996; Xiong et al., 1997). As the magnitude of $[Ca^{2+}]_i$ transients has been shown to be directly related to the applied strain magnitude and rate (LaPlaca et al., 1997; Weber et al., 1999; Geddes and Cargill, 2001; Lusardi et al., 2004b), the $[Ca^{2+}]_i$ is generally considered one of the initiating factors in transducing a mechanical insult into a cellular response. Perhaps the most frequently studied mechanism of calcium influx in mechanically injured neurons is the NMDA receptor, activated after fluid shear stress (LaPlaca and Thibault, 1998), mild to moderate biaxial stretch (Ahmed et al., 2002) and uniaxial stretch (Lusardi et al., 2004a). Additionally, the AMPA receptor can show

a dramatic loss in its desensitization properties (Goforth et al., 1999), leading to conditions that can increase $[Ca^{2+}]_i$ through voltage gated channels. Indeed, in our model of uniaxial injury, inhibiting these receptors and channels nearly abolished any increase in $[Ca^{2+}]_i$. Importantly, no significant increases in membrane permeability were observed at any level of uniaxial stretch at the strain rates used in this study. These data emphasize that uniaxial stretch injury is centrally mediated by the activation of membrane-based receptors and channels

In contrast, a large $[Ca^{2+}]_i$ transient persisted after biaxial stretch in the presence of glutamate receptor antagonists, suggesting an additional mechanism for the $[Ca^{2+}]_i$ transient in biaxially stretched neurons. We hypothesized that the stretch-induced $[Ca^{2+}]_i$ transient in biaxially stretched neurons using a narrow range of strain rates was through non-specific pores/tears in the membrane, as observed in various other *in vitro* models of biaxial stretch and fluid shear stress over a broader rate of strain rates (Tavalin et al., 1995; LaPlaca et al., 1997; Geddes et al., 2003; Serbest et al., 2005) and animal models of trauma (Pettus et al., 1994; Stone et al., 2004). Indeed, we observed uptake of the normally impermeant molecule, CBF, in 20% of neurons immediately after 50% biaxial stretch but not 24 h after stretch, suggesting that biaxially stretched neurons did exhibit transient increases in plasma membrane permeability. As nearly 50% of neurons stretched under identical conditions exhibited calcium increases even in the presence of a cocktail of calcium channel antagonists, it is likely that significantly more than 20% of neurons actually exhibited altered membrane permeability. One possible explanation for the underestimation is due to the large size difference between calcium and CBF (the molecular size of CBF is nearly ten times that of calcium) so that some membrane tears may be large enough to allow calcium influx but not CBF entry. Additionally, neurons with alterations in permeability that had not been fully repaired by ten minutes after stretch may not have been detected as CBF may have diffused out of neurons upon rinsing.

Varying the strain state also resulted in differences in cell function, despite no change in cell death. We evaluated cell function by measuring NMDA-induced calcium influx 24 h after trauma, as a means of investigating how stretched neurons respond to secondary insults. Previous researchers have shown that secondary NMDA insults after *in vitro* trauma result in both immediate hypersensitivity to NMDA induced calcium influx (Weber et al., 1999) and secondary susceptibility to NMDA stimulation (Arundine et al., 2003). Intriguingly, we found that uniaxially stretched neurons were hypersensitive to NMDA insults 24 h after trauma compared to both unstretched and biaxially stretched neurons. Enhanced calcium flux through NMDA receptors after trauma may arise from alterations in receptor expression or a reduction in the magnesium block of NMDA receptors, both of which occur after trauma (Zhang et al., 1996; Furukawa et al., 2000; Kumar et al., 2002; Osteen et al., 2004). We were initially surprised that biaxially stretched neurons were not more sensitive than uniaxially stretched neurons to post-injury NMDA treatment because the $[Ca^{2+}]_i$ response to trauma was substantially elevated in biaxially stretched neurons. However, it is possible that the increase in permeability may result in rapid membrane turnover, and therefore may cause a relative reduction in NMDA receptor expression when compared to the condition causing no change in membrane permeability (i.e., uniaxial stretch). The differences in NMDA stimulation between the two mechanical conditions warrants more study, since these differences may highlight how tissue deformation fields may influence the vulnerability of the brain to secondary excitotoxic insults.

With the ability to precisely define the mechanical and biochemical environment, *in vitro* models of trauma can provide valuable insight into the mechanisms that transduce a mechanical insult into a cellular response. However, it is important to note that *in vitro* models of trauma drastically reduce the complex environment of the brain (neurons, glia, extracellular matrix, vasculature and cerebrospinal fluid) into a simple monolayer of neurons. While neurons were initially thought to be the major component of the brain, it is now clear that the surrounding glia dramatically modulate both physiologic and pathologic neuronal processes. Additionally, neurons are cultured from embryonic rodent brains, making it nearly impossible to match the maturational state of adult neurons, despite the fact that they exhibit the same ion channels and neurotransmitter receptors. Therefore, it is imperative study both *in vitro* and *in vivo* models of trauma in parallel.

In summary, we found that calcium enters neurons through two distinct routes: (a) mechano-activated ion channels/receptors (NMDA, AMPA, voltage gated sodium, calcium) and (b) stretch-induced pores/tears formed in the plasma membrane. Both uniaxial and biaxial stretch cause calcium influx through mechano-activated ion channels/receptors. Alternatively, we found that calcium flux through non-specific pores in the membrane occurred predominately in biaxially stretched neurons. These differing pathways lead to surprisingly different consequences on neuronal function after trauma. These data show that broad reduction of abnormally high $[Ca^{2+}]_i$ may not be effective at modulating cell fate, since specific calcium entry points appear to have a preferred or enhanced role in the processes that lead to abnormal cell function. Moreover, these results demonstrate the im-

portance of understanding the mechanical loading conditions that occur in humans in order to develop the most appropriate animal and cellular models of TBI.

ACKNOWLEDGMENTS

Funds for this work were provided by NIH NS35712, NIH NICHD 41699, and an NRSA fellowship to D.M.G.

REFERENCES

AHMED, S.M., WEBER, J.T., LIANG, S., et al. (2002). NMDA receptor activation contributes to a portion of the decreased mitochondrial membrane potential and elevated intracellular free calcium in strain-injured neurons. J. Neurotrauma **19,** 1619–1629.

ARUNDINE, M., CHOPRA, G.K., WRONG, A., et al. (2003). Enhanced vulnerability to NMDA toxicity in sublethal traumatic neuronal injury *in vitro*. J. Neurotrauma **20,** 1377–1395.

BARBEE, K.A., MACARAK, E.J., and THIBAULT, L.E. (1994). Strain measurements in cultured vascular smooth muscle cells subjected to mechanical deformation. Ann. Biomed. Eng. **22,** 14–22.

BARTOLETTI, D.C., HARRISON, G.I., and WEAVER, J.C. (1989). The number of molecules taken up by electroporated cells: quantitative determination. FEBS Lett. **256,** 4–10.

CARGILL, R.S., 2ND, and THIBAULT, L.E. (1996). Acute alterations in $[Ca^{2+}]_i$ in NG108-15 cells subjected to high strain rate deformation and chemical hypoxia: an *in vitro* model for neural trauma. J. Neurotrauma **13,** 395–407.

DENNY-BROWN, D., and RUSSEL, W.R. (1941). Experimental cerebral concussion. Brain **64,** 92–164.

FINEMAN, I., HOVDA, D.A., SMITH, M., YOSHINO, A., and BECKER, D.P. (1993). Concussive brain injury is associated with a prolonged accumulation of calcium: a ^{45}Ca autoradiographic study. Brain Res. **624,** 94–102.

FURUKAWA, Y., OKADA, M., AKAIKE, N., HAYASHI, T., and NABEKURA, J. (2000). Reduction of voltage-dependent magnesium block of *N*-methyl-D-aspartate receptor-mediated current by *in vivo* axonal injury. Neuroscience **96,** 385–392.

GEDDES, D.M., and CARGILL, R.S. (2001). An *in vitro* model of neural trauma: device characterization and calcium response to mechanical stretch. J. Biomech. Eng. **123,** 247–255.

GEDDES, D.M., CARGILL, R.S., 2ND, and LAPLACA, M.C. (2003). Mechanical stretch to neurons results in a strain rate and magnitude-dependent increase in plasma membrane permeability. J. Neurotrauma **20,** 1039–1049.

GENNARELLI, T.A. (1994). Animate models of human head injury. J. Neurotrauma **11,** 357–368.

GIFT, E.A., and WEAVER, J.C. (2000). Simultaneous quantitative determination of electroporative molecular uptake and subsequent cell survival using gel microdrops and flow cytometry. Cytometry **39,** 243–249.

GOFORTH, P.B., ELLIS, E.F., and SATIN, L.S. (1999). Enhancement of AMPA-mediated current after traumatic injury in cortical neurons. J. Neurosci. **19,** 7367–7374.

GRYNKIEWICZ, G., POENIE, M., and TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260,** 3440–3450.

GURDJIAN, E.S., and WEBSTER, J.E. (1943). Experimental head injury with special reference to the mechanical factors in acute trauma. Surg. Gynecol. Obstet. **76,** 623–634.

HOLBOURN, A.H.S. (1943). Mechanics of head injuries. Lancet **2,** 438–441.

KUMAR, A., ZOU, L., YUAN, X., LONG, Y., and YANG, K. (2002). *N*-Methyl-D-aspartate receptors: transient loss of NR1/NR2A/NR2B subunits after traumatic brain injury in a rodent model. J. Neurosci. Res. **67,** 781–786.

LAPLACA, M.C., and THIBAULT, L.E. (1997). An *in vitro* traumatic injury model to examine the response of neurons to a hydrodynamically-induced deformation. Ann. Biomed. Eng. **25,** 665–677.

LAPLACA, M.C., and THIBAULT, L.E. (1998). Dynamic mechanical deformation of neurons triggers an acute calcium response and cell injury involving the *N*-methyl-D-aspartate glutamate receptor. J. Neurosci. Res. **52,** 220–229.

LAPLACA, M.C., LEE, V.M., and THIBAULT, L.E. (1997). An *in vitro* model of traumatic neuronal injury: loading rate-dependent changes in acute cytosolic calcium and lactate dehydrogenase release. J.. Neurotrauma **14,** 355–368.

LUSARDI, T.A., WOLF, J.A., PUTT, M.E., SMITH, D.H., and MEANEY, D.F. (2004a). Effect of acute calcium influx after mechanical stretch injury *in vitro* on the viability of hippocampal neurons. J.. Neurotrauma **21,** 61–72.

LUSARDI, T.A., RANGAN, J., SUN, D., SMITH, D.H., and MEANEY, D.F. (2004b). A device to study the initiation and propagation of calcium transients in cultured neurons after mechanical stretch. Ann. Biomed. Eng. **32,** 1546–1558.

MEANEY, D.F., SMITH, D.H., SHREIBER, D.I., et al. (1995). Biomechanical analysis of experimental diffuse axonal injury. J. Neurotrauma **12,** 689–694.

MORRISON, B., 3RD, MEANEY, D.F., and McINTOSH, T.K. (1998). Mechanical characterization of an *in vitro* device designed to quantitatively injure living brain tissue. Ann. Biomed. Eng. **26,** 381–390.

MORRISON, B., 3RD, MEANEY, D.F., MARGULIES, S.S., and McINTOSH, T.K. (2000). Dynamic mechanical stretch of organotypic brain slice cultures induces differential genomic expression: relationship to mechanical parameters. J. Biomech. Eng. **122,** 224–230.

NILSSON, P., LAURSEN, H., HILLERED, L., and HANSEN, A.J. (1996). Calcium movements in traumatic brain injury: the role of glutamate receptor-operated ion channels. J. Cereb. Blood Flow Metab. **16,** 262–270.

NISHIMOTO, T., and MURAKAMI, S. (1998). Relation between diffuse axonal injury and internal head structures on blunt impact. J. Biomech. Eng. **120,** 140–147.

OMMAYA, A.K., YARNELL, P., HIRSCH, A.E., and HARRIS, E.H. (1967). Scaling of experimental data on cerebral concussion in sub-human primates to concussion threshold for man. Presented at the 13th Stapp Car Crash Conference, Society of Automotive Engineers, Anaheim, CA.

OSTEEN, C.L., GIZA, C.C., and HOVDA, D.A. (2004). Injury-induced alterations in *N*-methyl-D-aspartate receptor subunit composition contribute to prolonged ^{45}calcium accumulation following lateral fluid percussion. Neuroscience **128,** 305–322.

PETTUS, E.H., CHRISTMAN, C.W., GIEBEL, M.L., and POVLISHOCK, J.T. (1994). Traumatically induced altered membrane permeability: its relationship to traumatically induced reactive axonal change. J. Neurotrauma **11,** 507–522.

SERBEST, G., HORWITZ, J., and BARBEE, K. (2005). The effect of poloxamer-188 on neuronal cell recovery from mechanical injury. J. Neurotrauma **22,** 119–132.

SHREIBER, D., BAIN, A.C., and MEANEY, D.F. (1997). *In vivo* thresholds for mechanical injury to the blood–brain barrier. Presented at the 41st Stapp Car Crash Conference, Society of Automotive Engineers, Lake Buena Vista, FL.

STONE, J.R., OKONKWO, D.O., DIALO, A.O., et al. (2004). Impaired axonal transport and altered axolemmal permeability occur in distinct populations of damaged axons following traumatic brain injury. Exp. Neurol. **190,** 59–69.

TAKHOUNTS, E., EPPINGER, R., CAMPBELL, J.Q., TANNOUS, R., POWER, E., and SHOOK, L. (2003). On the development of the SIMon finite element head model. Stapp Car Crash J. **47,** 107–133.

TAVALIN, S.J., ELLIS, E.F., and SATIN, L.S. (1995). Mechanical perturbation of cultured cortical neurons reveals a stretch-induced delayed depolarization. J. Neurophysiol. **74,** 2767–2773.

UENO, K., MELVIN, J.W., LI, L., and LIGHTHALL, J.W. (1995). Development of tissue level brain injury criteria by finite element analysis. J. Neurotrauma **12,** 695–706.

WEBER, J.T., RZIGALINSKI, B.A., WILLOUGHBY, K.A., MOORE, S.F., and ELLIS, E.F. (1999). Alterations in calcium-mediated signal transduction after traumatic injury of cortical neurons. Cell Calcium **26,** 289–299.

WOLF, J.A., STYS, P.K., LUSARDI, T., MEANEY, D., and SMITH, D.H. (2001). Traumatic axonal injury induces calcium influx modulated by tetrodotoxin-sensitive sodium channels. J. Neurosci. **21,** 1923–1930.

XIONG, Y., GU, Q., PETERSON, P.L., MUIZELAAR, J.P., and LEE, C.P. (1997). Mitochondrial dysfunction and calcium perturbation induced by traumatic brain injury. J. Neurotrauma **14,** 23–34.

ZHANG, L., RZIGALINSKI, B.A., ELLIS, E.F., and SATIN, L.S. (1996). Reduction of voltage-dependent Mg^{2+} blockade of NMDA current in mechanically injured neurons. Science **274,** 1921–1923.

ZHANG, L., YANG, K.H., and KING, A.I. (2001). Biomechanics of neurotrauma. Neurol. Res. **23,** 144–156.

ZHANG, L., YANG, K.H., and KING, A.I. (2004). A proposed injury threshold for mild traumatic brain injury. J. Biomech. Eng. **126,** 226–236.

Address reprint requests to:
Donna M. Geddes-Klein, Ph.D.
University of Pennsylvania
3320 Smith Walk
120 Hayden Hall
Philadelphia, PA 19104-6392

E-mail: dmgeddes@seas.upenn.edu