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Effect of Acute Calcium Influx after Mechanical Stretch Injury *In Vitro* on the Viability of Hippocampal Neurons

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ABSTRACT

We use a new in vitro model to examine the effect of mechanical deformation on neurons. We examined acute changes in cytosolic calcium concentrations ($[Ca^{2+}]_i$) caused by a rapid stretch of cultured hippocampal neurons, using mechanical loading conditions that mimic brain deformations during trauma. We found that stretch-injury of neurons induces a strain-dependent increase in $[Ca^{2+}]_i$. Remarkably, the extent of this calcium response exceeded the levels initiated by chemical toxicity with NMDA (100 μM) or glutamate (5 mM) exposure. Propidium iodide labeling at 24 h following stretch showed neuronal death occurred only at the most severe level of mechanical injury. Although NMDA-induced toxicity could be inhibited in calcium free media or by treatment with MK-801, stretch-induced neuronal death was not similarly reduced with either treatment. Unexpectedly, reduction of the acute stretch-induced calcium transient with calcium-free media or MK-801 resulted in an increase in neuronal death at lower stretch levels. These data suggest that mechanical stretch can initiate calcium influx in hippocampal neurons, but substantially modulating the early calcium flux from the extracellular space or through the NMDA channel does not provide an effective means for improving neuronal survival.

Key words: calcium; cell death; cell mechanics; hippocampus; neuron; traumatic brain injury

INTRODUCTION

DESPITE THE HIGH PREVALENCE and enormous impact of traumatic brain injury (TBI) on society, little is known of how physical forces translate to injury at the neuronal level. Often, it is assumed that the progression of mechanical injury at the cellular level parallels that of ischemic injury to neurons. For example, an increase in intracellular calcium is considered an important modulator of neuronal loss in both cerebral ischemia and brain trauma (Kristian and Siesjo, 1998; Sapolsky, 2001). Accordingly, a broad range of therapies has been developed to reduce calcium influx in both forms of injury (Leker and Shohami, 2002). Models of mechanical injury to astrocytes and in septo-hippocampal cultures have explored the potential role of calcium in cell death after traumatic injury, showing exacerbation of early measures of injury in reduced calcium conditions (Rzigalinski et al., 1997), and both calpain- and caspase-3-mediated spectrin cleavage (Pike et al., 2000). Although past studies point to how inhibiting calcium influx can reduce cell death using *in vitro* models of hypoxia and neurotoxicity (Choi, 1995), the significance of attenuating the immediate calcium influx in mechanical injury remains unexplored.

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One of the most common and devastating consequences of TBI is memory dysfunction, often linked with selective neuronal damage of the hippocampus following trauma. For even mild TBI, the hippocampus appears exquisitely sensitive to mechanical injury (Smith et al., 1991; Kotapka et al., 1992; Gennarelli, 1994; Kotapka et al., 1994; Colicos et al., 1996; Smith et al., 1997; Dixon et al., 1999). The mechanism for this susceptibility could simply result from an enhanced mechanosensitivity of neurons in the hippocampus, or could occur from the propagation of biochemical signals from mechanically injured neurons to hippocampal neurons that did not receive a mechanical insult at the moment of injury. The effects of mechanical forces on the hippocampus during injury, either synergistically with or separate from any chemically mediated toxicity, may play a large role in the etiology of hippocampal damage in TBI. However, isolating the effect of mechanical forces on the hippocampus *in vivo* is difficult; the anatomical location does not allow one to induce rapid hippocampal deformation without deforming other brain structures.

We utilized a new in vitro system to focus on the possible mechanical vulnerability of hippocampal neurons, using recognized mechanical loading conditions to deform the dissociated cultures of a uniform amount in a manner similar to the rapid tissue deformations that occur in TBI *in vivo* (Margulies et al., 1990; Meaney et al., 1995; Bain and Meaney, 2000). Although the *in vitro* system does not completely mimic the *in vivo* hippocampal architecture, it does allow a precise examination of the potential mechanosensitivity of neurons from the hippocampus. Moreover, by limiting the stretched region to a narrow slit, the stretched portion of the culture received a uniform, uniaxial strain, allowing precise control over the mechanical deformation. The role of calcium as both an initiator and modulator of traumatic neuronal loss has been postulated indirectly as a result of *in vivo* studies (Fineman et al., 1993) and extensive in vitro studies (Choi, 1995; Abdel-Hamid and Tymianski, 1997; Hyrc et al., 1997; Berridge et al., 2000). Others have investigated how mechanical forces can affect the function of cortical neurons and astrocytes (Tavalin et al., 1995; Citron et al., 1997; Rzigalinski et al., 1997; Geddes and Cargill, 2001), but the exact role of calcium in the death of hippocampal neurons is unknown. To evaluate the pathophysiology of dynamic stretch in primary neuronal cultures, we measured acute changes in cytosolic calcium levels caused by the stretch, found the stretch conditions necessary to induce neuronal death, and investigated how calcium flux from the extracellular space, as well as specifically through the NMDA receptor associated channel, would affect the mechanically injured hippocampal cultures.

MATERIALS AND METHODS

Isolation and Culture of Neurons

All procedures involving animal use were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Hippocampal neurons were isolated from E17-E19 Sprague-Dawley rats (Charles River, Wilmington, MA). Isolated hippocampi were incubated in trypsin/DNAse (Sigma, St. Louis, MO, Roche Applied Science, Indianapolis, IN) solution for 20 minutes at 37°C, 5% CO_2, and then triturated in a 10-mL pipette after adding soybean trypsin inhibitor (Gibco, Carlsbad, CA). Cells were centrifuged for 5 min at 1000 RPM and re-suspended in 10 mL of Neurobasal media (NB, Gibco) supplemented with 2% B-27 (Gibco), 0.5 mM L-glutamine (l-gln, Gibco), 25 μM L-glutamate (L-glu, Sigma), and 1% Penicillin-Streptomycin solution (10,000 units penicillin and 10,000 μg streptomycin per mL, Pen/Strep, Gibco), and filtered serially through Nitex mesh (Cross Wire Cloth, Bellmawr, NJ). Hippocampal suspensions were diluted and plated in plating media consisting of NB media supplemented with 2% B-27, 0.5 mM L-glu, 25 μM L-gln, 1% Pen/Strep, and 5% fetal bovine serum (FBS, Hyclone, Logan, UT).

Cells were plated at 750,000 cells/mL (0.5 mL/well) onto thin, clear, flexible elastic membranes (Specialty Manufacturing, Saginaw, MI). The membranes were stretched with a known pre-tension across the surface of custom designed, stainless steel wells that fit into the cell stretch apparatus. Each stainless steel well was polished and passivated prior to use. Each well contained only one culture surface area, and was designed to accommodate support culture for the hippocampal neurons throughout the culturing process. To promote cell attachment, membranes were first coated with 1 μg/cm^2 poly-L-lysine (PLL, Sigma), and then with 1 μg/mL laminin (Collaborative Biomedical, Bedford, MA). To provide glial cell support, support cell cultures (500,000 cells/mL) were prepared on matrigel treated (1:8 dilution in ddH_2O, Collaborative Biomedical) 12 mm millicell wells (Millipore, Bedford, MA). Three hours following plating, hippocampal cultures were fed with the plating media, and 7-day-old support cultures were placed on the neuronal cultures. Both the neuronal and support cultures were fed at 3 days *in vitro* (DIV) with NB media supplemented with B-27, L-gln, 5 μM cytosine β-d-arabinofuranoside (AraC, Sigma), and 5% FBS. At 4 and 7 DIV, cultures were fed with NB media supplemented with B-27, L-gln, and 5% FBS. Support cultures were fed at 3 and 6 DIV with NB media supplemented with B-27, L-glu, and FBS. Once placed onto silastic cultures, the support cultures received the same feeding and media as the hippocampal cultures. All experiments were performed with either

10- or 11-day-old neuronal cultures following removal of the millicell support culture.

Uniaxial Stretch of Cultured Neurons

Each individual culture well of hippocampal neurons was stretched only once and used for either calcium imaging or viability determination. The cell stretching system was designed to apply a linearly increasing stretch across all pulse durations. The pressure onset time, and therefore the rate of applied strain, was selected between 20.5 msec and 1.354 sec prior to each experiment. The fastest onset time, 20.5 msec, was chosen to simulate the rapid deformations that occur with traumatic brain injury (Meaney et al., 1995). The slow onset time, 1354 msec, was selected to approximate physiologic motion of the cervical spinal cord during a normal "head-nodding" movement (Yuan et al., 1998). The middle onset time, 205 msec, provided an intermediate onset rate between traumatic and normal physiologic deformations.

To stretch a hippocampal culture, a cover plate was fastened to the cell culture well, forming a sealed chamber directly on the microscope containing the cultured cells and supporting salt solution. While cells were cultured on the entire 2.54 cm^2 substrate area, the deformable region was limited to an 1.5 × 18 mm rectangle using a stainless steel mask placed beneath the culture (Fig. 1A). The chamber pressure was increased for the selected onset time, causing the elastic substrate and the plated cells to stretch in direct proportion to the pre-set chamber pressure; the cells were rapidly returned to an unstretched state by releasing pressure from the sealed chamber (Fig. 1B). The chamber pressure transient for each experiment was monitored using a dynamic pressure transducer (Entran, Fairfield, NJ). Microscopic examination of hippocampal neurons immediately before stretch (Fig. 1C) and following a >50% strain (Fig. 1D) demonstrated that cell attachment was maintained following stretch.

Quantification of Substrate Strain

Mechanical injury to the cultured cells was quantified as substrate strain, which is described as the maximum percent increase in the width of the culture region during pressurization. For example, a strain of 100% would indicate that the width of the stretch region doubled as a result of the applied chamber pressure loading. The culture substrate used was elastic, returning to within 0.22 ± 0.35% (SD) of its prestretched position following substrate strain >50%. The substrate was thin (0.02 or 0.07 mm) to eliminate inertial effects triggered by the rapid transient pressure pulse used to deform the culture. The rectangular strain region resulted in a uniform strain perpendicular to the length of the slit over 80% of the region, and a negligibly small strain parallel to the slit. The ends of the slit were not used for evaluation, as the strain field was not consistent near the ends.

We utilized four distinct stretch levels, with the highest level (>50%) above the mechanical threshold for *in vivo* vascular and axonal damage (Bain and Meaney, 2000) and within the range of stretch examined in other cell stretch injury models (Ellis et al., 1995; Cargill and Thibault, 1996; Pike et al., 2000; Geddes and Cargill, 2001). The three remaining levels were within the range associated with *in vivo* traumatic injury (Meaney et al., 1995; Shreiber et al., 1999), as well as two groups below this threshold (3–6%, 1–2%). No overlap in the stretch levels among the groups occurred. For each culture, the peak substrate deflection (d) caused by the applied chamber pressure was used to compute the substrate strain (ϵ) applied to the cultured cells:

$$\epsilon = \left(\frac{0.5\theta}{\sin(0.5\theta)} - 1 \right) * 100\% \qquad (1)$$

$$\text{where} \quad \theta = 2\pi - 4 \sin^{-1}\left(\frac{0.5w}{\sqrt{d^2 + (0.5w)^2}} \right),$$

d = centerline deflection as calculated based on the measured peak chamber pressure, and w = width of the exposed culture area (1.5 or 2 mm) (Smith et al., 1999).

Fluorescent microspheres (nominally 1 μm diameter, Molecular Probes, Eugene OR) were used to measure the membrane strain directly. Silastic membranes coated with PLL as for primary cell plating were incubated in CSS containing a 1:100 dilution of microspheres for 10 min. Following incubation, cultures were rinsed twice and used for strain measurements. Digital images (1280 × 1024) were taken using a DXM2000 (Nikon) with a 40× objective plus an additional 2× magnification from the Multi-Image-Module (Nikon). Calibration using a stage micrometer demonstrated a 1:1 aspect ratio in the acquired images, with 12.5 pixels per 1 μm. All bead experiments were done with 0.05-mm-thick membranes and a 1.5-mm slit plate in place. Images were analyzed with Scion Image (Frederick, MD), which reported the centroid of each microsphere, excluding clusters of microspheres. Images of beads were captured before and during static inflation. Microsphere pairs with centroids closer than 100 pixels were also excluded from the analysis. As predicted, the strain applied to the substrate was aligned along one direction (i.e., uniaxial) with the measured strain across the width of the region significantly higher than the strain in the transverse direction ($n = 46$; $p < 0.001$ by t-test analysis). At a fixed pressure, measurement of bead geometry using different bead pairs showed a uniform measured strain across the

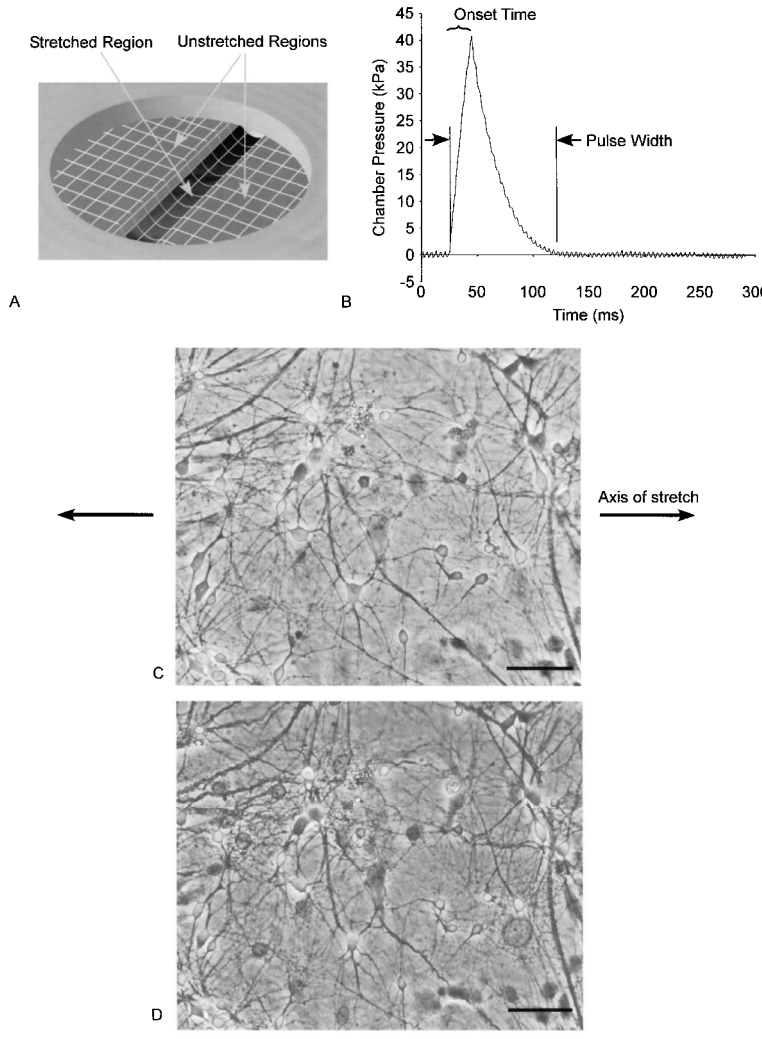


FIG. 1. Cells plated onto a flexible silastic membrane are subjected to mechanical stretch. Displacement of the culture area (represented by a grid in **A**) is restricted to a slit, centered under the culture area. The culture well is placed into a sealed chamber and a transient positive pressure pulse is applied (83 KPa, maximum), resulting in a uniform elastic deformation of the unrestricted culture surface. Changes in cytosolic calcium, indicated by fura-2, are monitored in the stretched region. (**B**) Chamber pressure trace for 20.5-msec onset, duration of 100 msec. The shape of the chamber pressure trace was uniform across all onset times (20.5 msec, 205 msec, and 1.35 sec). A typical culture before (**C**) and immediately following (**D**) a stretch >50%. Bar = 50 μm.

deformed substrate ($\epsilon_x = 11.94 \pm 0.49\%$, $\epsilon_y = 0.98 \pm 0.71\%$ n = 46). Moreover, strain measurements showed the substrate strain was properly predicted by using measurements of membrane deflection and equation 1.

Fura-2 Calcium Imaging

The acute cellular response was measured using the ratioable fluorescent calcium dye fura-2AM (Molecular Probes, Eugene, OR) (Grynkiewicz et al., 1985). Preliminary experiments with the optically clear substrate showed the membrane beneath the hippocampal cultures contributed no background fluorescence to the imaging used in these studies. Cultures were loaded at 37°C for 45 min with 5 μM fura-2, rinsed, and then allowed to sit for 10 min prior to imaging in control saline solution (CSS: 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 25 mM HEPES, 15 mM glucose, pH = 7.4, osmolarity adjusted to 330 mOsm with sorbitol) supplemented with any experiment-specific pharmacologic agent. MetaFluor (Universal Imaging, Downingtown, PA) software was used for all image capture and analy-

sis. Images were captured using an intensified 8-bit Hamamatsu CCD (Optical Apparatus, Ardmore, PA). The excitation shutter and filter wheel were controlled using a Lambda 10-2 (Sutter Instruments, Novato, CA). Images at 340 nm and 380 nm were captured at 2-sec intervals for 1 min prior to stimulus; capture continued for 5 min at 2-sec intervals following the stimulus; ratio analysis was performed offline. Three measures were taken from the fura-2 ratio for each cell: pre, peak, average, and final (see Fig. 3A below). The pre value is the average response for the cell prior to stretch. The peak value is the maximum ratio measured in the 40 sec following the stimulus for stretched and agonist controls. The average value is the average response over 5 min following stimulus, and the final value is the measure for the cell at 5 min following stimulus. All neurons in the field were included in the initial analysis. In some cases, the F_{380} signal dropped to zero following stimulus; in these cases, the neuron's continued presence on the membrane was confirmed using the F_{340} signal, and it was excluded from further analysis. No cells permanently detached from the membrane following stretch injury.

Culture Viability Assay

Sister cultures (10 DIV) were used to measure culture viability at one timepoint following stretch. Propidium Iodide (PI, Molecular Probes) was used as a marker of membrane permeability, considered an indicator of cell death, and fura-2AM as an indicator of live cells. Previous experiments have shown PI to be a marker of "cell injury" acutely following stretch injury (McKinney et al., 1996) and a likely measure of membrane permeability (either transient or permanent) rather than cell death. However, mechanically damaged membranes seal within hours of stretch (Tavalin et al., 1995; LaPlaca et al., 1997; Pike et al., 2000; Shi and Pryor, 2000). Unlabeled cultures were injured following the same protocol and appropriate CSS as those used for fura-2 experiments. Cultures were rinsed in CSS, and allowed to sit for 10 min prior to insult. At 30 min following insult, the CSS was replaced with NB media supplemented with B-27 and l-gln, and returned to the incubator. Recognizing that PI measurements may be confounded by cells detatching from the substrate over the 24-h post-stretch period, we conducted experiments examining several time points (30 min, 6, and 18 h) following stretch. None of the cultures showed evidence of interim neuronal loss. Moreover, examination of cultures exposed to 5 mM glu demonstrated a density of PI positive neurons similar to live neurons in untreated cultures. Finally, there was no evidence of cellular debris in the supernatant at any time point following stretch. For these reasons, we felt that the PI labeling at 24 h was a reasonable reflection of the total cell death. After 23 h, cells were loaded with 5 μM fura-2 for 1 h, the cells were rinsed with CSS, and 2 μg/mL PI was added to the culture. Images of PI and fura-2–labeled cells were collected from the stretched region for each culture well. Cell counting was conducted in a blinded fashion, and the cell counting information was analyzed to assess viability changes with applied stretch, chemical stimulation, or treatment. The percent of dead cells was calculated as (no. cells PI positive)/(no. cells PI positive + no. cells fura-2 positive) $\times$100. There was no co-localization of fura-2 and PI.

Miscellaneous Compounds

NMDA (Sigma) was prepared from a frozen 10 mM stock solution in ddH_2O. L-glu (Sigma) was prepared from a frozen 10 mM stock solution in CSS. MK-801 (Sigma) was prepared from a frozen 10 mM stock in ddH_2O.

Statistics

For calcium imaging of mechanically injured neurons, a minimum of three separate culture wells were used for each experimental group studied, with an average of 50 $\pm$ 20 cells analyzed per culture well. For viability determination in the stretched cultures, one stretched field was collected from at least three wells per stretch condition. For viability determination in the agonist-treated cultures, three fields were selected from each of at least three cultures for imaging. To avoid cell isolation bias, cultures used for each experimental group were spread across a minimum of two separate cell isolations. An initial statistical analysis using a nested design showed that neither cell isolation nor well ID were significant factors in the analysis, and therefore data within each experimental group was grouped accordingly.

Statistical analyses were performed using SAS V.8 (Cary, NC). Since the peak calcium levels varied over three orders of magnitude, the fura-2 ratio data were analyzed using a generalized linear model (McCulloch and Searle, 2001) with a log link function; the natural log of the mean of the data was modeled as a linear function of the factors. The variance at any combination of main effects (strain, onset, treatment, and region) was modeled as the square of the mean of the data. Wald tests were used to determine significant differences between specific combinations of factors. Logistic regression (Venables and Ripley, 1999) was used to model the PI data. For both fura-2 and viability data, a Bonferroni correction was used to adjust for multiple comparisons (Kutner et al., 1996).

RESULTS

Experimental Controls

No detectable changes in acute free cytosolic calcium were recorded in cells exposed to a transient pressure pulse with no substrate deformation. Moreover, these single transient pressure pulses did not adversely affect neuronal viability 24 h after insult. We continued to perform these pressurization experiments for each experimental condition, and report them as "0% strain." Sham experiments were also performed for each condition to demonstrate that the stretch or agonist induced changes were not an experimental artifact.

Acute Neuronal Response to Magnitude and Rate of Applied Strain

The minimum strain level required to trigger an acute increase in the free cytosolic calcium ($[Ca^{2+}]_i$) in hippocampal cultures decreased when the strain was applied more rapidly to the culture (Fig. 2). For cultures exposed to strains applied "slowly" (onset time of 1.35 s), strains 12–17% or greater were required to trigger an acute $[Ca^{2+}]_i$ response (Fig. 2A, $p < 0.001$). The strain level for a significant calcium influx to occur dropped to 3–6% for the moderate onset time (205 msec, Fig. 2B, $p < 0.001$), and dropped again to 1–2% for the fastest onset of 20.5 msec (Fig. 2C, $p < 0.001$). Since it most closely resembles conditions resulting in traumatic injury to nervous tissue and shows the greatest sensitivity to stretch, we used the 20.5 msec-onset time for the remainder of the studies described.

To draw a comparison between our mechanically triggered response and a more common chemical neurotoxic response, we compared our stretch-activated calcium transients across different stretch groups to established agonist-evoked increases in $[Ca^{2+}]_i$ (Fig. 3B). We found glutamate (5 mM) and NMDA (100 μM) stimulated acute increases in $[Ca^{2+}]_i$ ($p < 0.001$). However, we found that the acute stretch-induced peak increase in fura-2 ratio was greater than the effect of chemical insults that have been used in toxicity experiments in cultured hippocampal neurons (Choi, 1987; Vergun et al., 2001). The response to glutamate was not significantly different from the 12–17% or >50% strains ($p > 0.15$). We found that a 100 μM NMDA stimulus evoked a peak change in fura-2 ratio similar to the peak for a 3–6% strain. Beyond a 3–6% stretch level, the peak changes in fura-2 ratio exceeded the peak change caused by NMDA application ($p < 0.001$). With a k_d of 140 nM in salt, fura-2 is saturated at approximately 1 μM calcium (a likely condition in these experiments). To verify that the increasing ratios measured using fura-2 were representative of actual in-

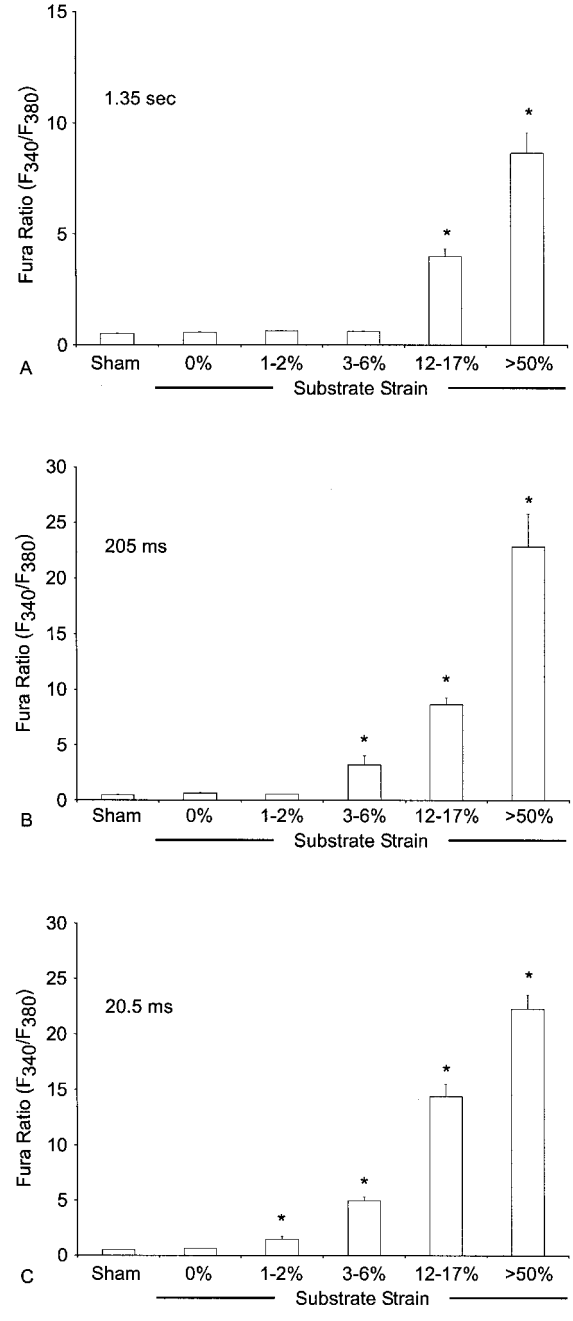
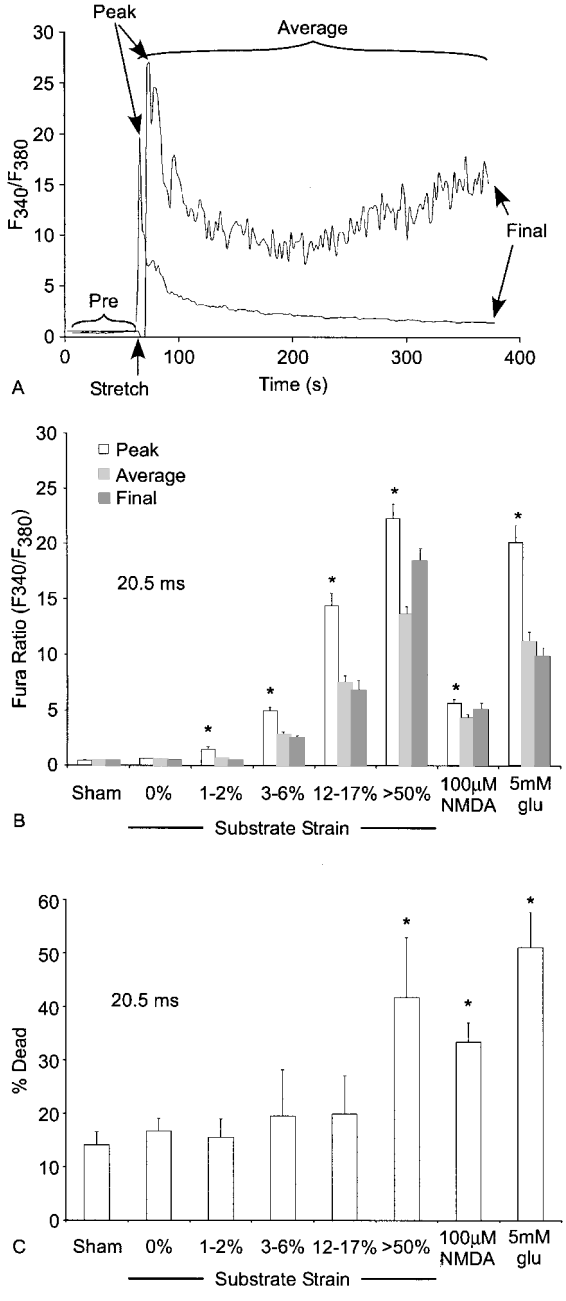


FIG. 2. Hippocampal neurons are sensitive to both the magnitude and rate of applied dynamic mechanical strain. Fura-2 ratios (F340/F380) were acquired every 2 sec, with a peak value determined in the 40 sec immediately following stretch. The peak fura-2 measured in neurons following strains applied over 1.35 sec (**A**) were significantly different from the pressure sham (0% substrate strain) for the 12–17% and >50% strain groups ($^*p < 0.001$). When strain was applied more quickly, over 205 msec, the neurons responded at and above substrate strains of 3–6% (B, $^*p < 0.001$). At a "traumatic" onset, 20.5 msec, cells exposed to substrate strain as low as 1–2% showed a significant peak increase in the fura-2 ratio (**C,** $^*p < 0.001$).

creases in $[Ca^{2+}]_i$, a subset of experiments was run using the fura-2 analog fura-5F (k_d 400 nM, Molecular Probes, Eugene, OR). At stretch levels (12–17%) showing the closest changes to NMDA stimulation, the results using fura-5 confirmed the trend of increased ratios reflecting a significantly higher $[Ca^{2+}]_i$. The fura-5F experiments showed that the increased ratios measured with fura-2 did reflect actual increases in $[Ca^{2+}]_i$. To facilitate analysis across a range of calcium changes that include moderate calcium increase, all further experiments were run using fura-2.



The constant application of the NMDA over the five-minute monitoring period resulted in a sustained elevation in cytosolic calcium. Although the mechanical stretch was applied very rapidly, only strain of 1–2% showed a return of the cytosolic calcium to resting, pre-stretch values 5 min after stretch. While the $[Ca^{2+}]_i$ peak was transient for cultures exposed to stretch beyond 3%, the calcium levels measured 5 min following stretch had subsided to levels that matched or exceeded the levels measured after NMDA stimulation. Individual cultures exposed to strain >50% resulted in fura-2 levels that only transiently subsided; by 5 min following stretch, the average fura-2 ratio had begun to rise again (Fig. 3B).

Delayed Neuronal Response to Strain

We measured the viability of stretched cells 24 h following the stretch and compared the results to known toxic doses of glutamate and NMDA. Although strains as low as 1–2% elicited a significant $[Ca^{2+}]_i$ increase, and strains >3–6% exceeded the ratio triggered by the toxic 100 μM NMDA dose, only strain >50% caused significant neuronal death at 24 h following the stretch (Fig. 3C, $p < 0.001$).

Acute Calcium Source Is Primarily Extracellular

By removing calcium from the extracellular media, the threshold for a significant stretch-induced $[Ca^{2+}]_i$ rise increased to 12–17% ($p < 0.001$, Figure 4A). There was

FIG. 3. Temporal changes in cytosolic calcium and neuronal viability after mechanical stretch. Typical cytosolic calcium response (**A**) indicates the three measurements made for each cell: the peak measured within 40 sec following stretch, the average measured over 5 min following stretch, and the final value at 5 min following stretch. (**B**) The elevated $[Ca^{2+}]_i$ measured in neurons exposed to transient stretch at 20.5 msec persists over the 5-minute recording period, with stretch groups above 3–6% resembling the response to chronic application of agonist (100 μM NMDA). Exposure to strain <50% or 5 mM glutamate treatment resulted in a transient peak fura-2 followed by a partial reduction in free cytosolic calcium. The fura-2 measured in neurons exposed to NMDA was uniform over the 5-min measurement period. Strain >50% resulted in the highest peak $[Ca^{2+}]_i$ measurement, followed by a transient decrease in $[Ca^{2+}]_i$ and a final rise in the fura-2 ratio. For the peak value, strain significant at $^*p < 0.001$ compared to the pressure sham (0% substrate strain). (**C**) Cell viability at 24 h following insult is reduced significantly only for stretch >50% and agonist treated cultures. Despite the acute calcium transient measured for stretched cultures (**B**), we found no correlation between the acute stretch-induced calcium response and PI uptake at 24 h. Strain significant at $^*p < 0.001$ compared to the pressure sham (0% substrate strain).

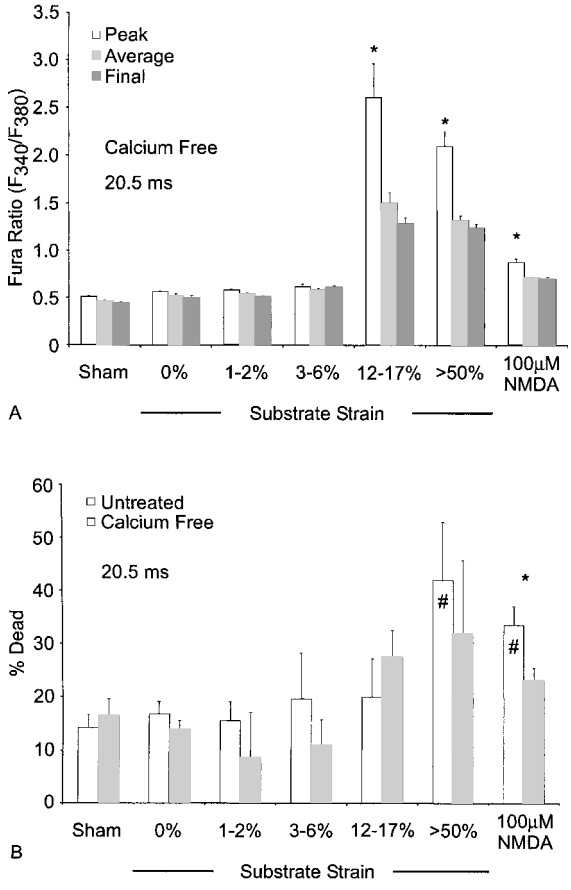


FIG. 4. The influence of extracellular calcium on cytosolic calcium changes and viability after mechanical stretch. (**A**) Cultures stretched in the absence of extracellular calcium showed a complete block of the calcium transient for stretch groups below 12–17%, and a significant reduction ($^{*}p < 0.001$ compared to the stretch-matched untreated group) for the remaining stretch groups. The $[Ca^{2+}]_i$ response of neurons stretched at 12–17% was not significantly different from those stretched >50% for any of the three measures (peak, average, or final). (**B**) When comparing untreated and calcium-free conditions, the reduction in acute $[Ca^{2+}]_i$ was protective only in NMDA-treated cultures ($^{*}p < 0.001$). Compared to treatment-matched sham cultures, the threshold for significant cell death dropped from >50% to the 12–17% strain group for the cells stretched in calcium-free CSS ($^{\#}p < 0.001$).

no stretch-induced $[Ca^{2+}]_i$ increase following stretch levels of 1–2% and 3–6%. While the 100 μM NMDA-induced increase was attenuated significantly ($p < 0.001$), a small but significant increase was observed ($p < 0.001$).

The contribution of the NMDA channel in the stretch-induced $[Ca^{2+}]_i$ transient was investigated using MK-801 (Fig. 5A). After pretreating cultures with the noncompetitive, activation-dependent NMDA channel blocker MK-801 (100 μM) for 10 min prior to agonist or stretch, we found a complete block of the NMDA-induced transient and the 1–2% and 3–6% stretch-induced transients, suggesting that the NMDA receptor is a very sensitive

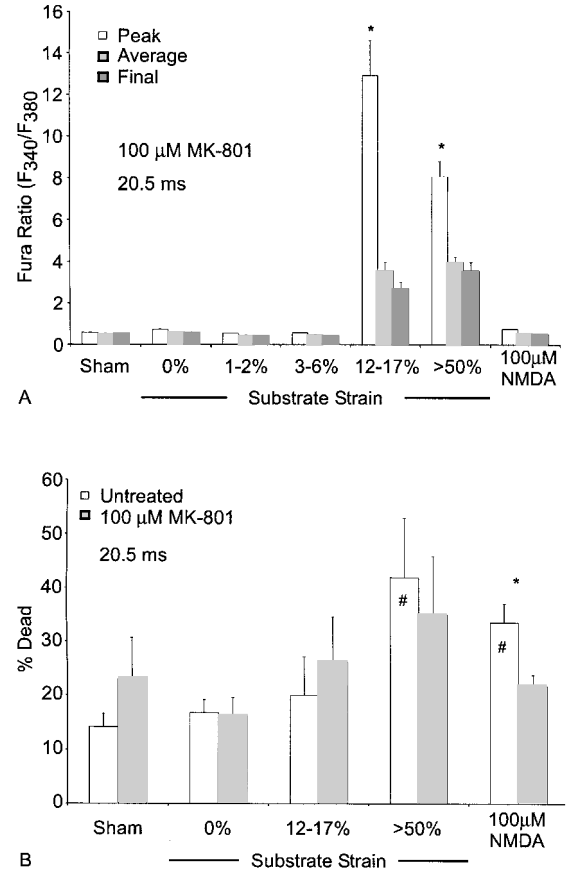


FIG. 5. The role of the NMDA receptor in the modulating the calcium response and viability following mechanical stretch. Cells were stretched in the presence of 100 μM MK-801 and compared to the untreated wells. (**A**) Fura-2 measurements of both stretched and agonist treated cultured show a significant reduction in the cytosolic calcium transient for the MK-801 treated cultures ($p < 0.001$ compared to the stretch matched sham), with a total block of the acute $[Ca^{2+}]_i$ transient both for cultures stretched 1–2% and 3–6%, and in the NMDA cultures ($^{*}p < 0.001$ compared to the treatment-matched 0% strain group). For pretreated cultures exposed to strain >50%, the fura-2 ratio peaked immediately after stretch and gradually decreased over the five minute monitoring period, unlike the gradual rise in calcium observed for untreated cultures exposed to the same stretch level. (**B**) Blocking the acute calcium influx through the NMDA receptor associated channel was protective for agonist insult ($^{*}p < 0.001$), but not for stretch-injured neurons. Cells exposed to 12–17% strain showed a reduction in viability when stretched in MK-801 treated conditions. Cells exposed to >50% strain were not protected by MK-801 treatment ($^{+}p < 0.05$ and $^{\#}p < 0.001$ compared to treatment-matched 0% strain).

strain sensor. At the 12–17% and >50% strains, MK-801 treatment resulted in a partial block of stretch-induced $[Ca^{2+}]_i$ transients (Fig. 5A), suggesting activation of additional strain-sensitive $[Ca^{2+}]_i$ pathways. For the >50% strain, we measured a more marked reduction in peak $[Ca^{2+}]_i$ using MK-801 treatment than for the 12–17% stretch, suggesting a larger relative contribution of the NMDA receptor to the stretch-induced $[Ca^{2+}]_i$ transient. Similar to removing calcium from the extracellular media, pretreatment with MK-801 resulted in a general cytosolic calcium response that peaked immediately, and was followed by a slowly decreasing calcium response over the five minute period following stretch.

Reducing Calcium Influx Does Not Improve Viability at 24 h

Significant improvement in viability was observed by removing calcium from the extracellular media during a 30-min NMDA application (Fig. 4B). However, in the stretch-injured cells, we found that calcium-free conditions for the same duration (30 min) following stretch actually worsened the effect of stretch, shifting the toxic strain from >50% in physiologic salt solution, to 12–17% in calcium-free salt solution. Similarly, no loss in viability was observed in cells treated with MK-801 and then exposed to NMDA for 30 min (Fig. 5B). As we found in the calcium-free conditions, pre-treatment with MK-801 reduced the threshold for mechanically induced neuronal death from >50% to the 12–17% strain group ($p < 0.05$).

DISCUSSION

Here, we present a new *in vitro* model of dynamic stretch-injury of hippocampal neurons that mimics the mechanical loading conditions found in human brain trauma. Employing uniaxial stretch, we found that the pathophysiologic response in neurons was substantially different from that induced by chemical toxicity. In particular, we observed large increases in cytosolic calcium proportional to applied stretch that even exceeded those induced by toxic doses of glutamate and NMDA. Despite the high calcium load following stretch-injury, we only found neuron death at 24 h post-injury at the most extreme stretch condition (>50% strain). Furthermore, this stretch-induced cell death following high strain could not be substantially reversed by either removing extracellular calcium or by blocking influx through the NMDA receptor associated channel in the first thirty minutes following stretch. Paradoxically, at moderate strains of 12–17%, blockade of the acute calcium transient actually increased neuronal death. These observations suggest that increased intracellular calcium in the acute period is not strongly associated with neuron death following mechanical injury.

Biofidelic finite element computer models of the human brain response during impact have shown that a complex strain field occurs within the brain at the moment of injury (King, 2000). Neurons within the brain can exhibit a highly organized architecture. Therefore, it is likely that the direction of applied mechanical deformation may influence the response of the neuron *in vivo*. The simplest form of deformation is uniaxial strain, and we developed our cell stretch model to induce a uniform, uniaxial deformation across a population of hippocampal neurons. In addition to simplifying the mechanical environment using uniaxial strain, the uniformity of the strain field is likely to result in reduced variation in the measured outcomes. Furthermore, we used a range of strains and strain rates that paralleled "real-world" mechanical loading conditions found in brain trauma. In the past, investigators focused on correlating the sites of highest tissue deformation (>30%) with pathologic markers of tissue injury (Zhou et al., 1994). However, our data show that significant calcium signals, rivaling those induced by 100 μM NMDA, can be triggered even for mild stretch levels (3–6%) provided these stretch insults are applied dynamically (20 msec) to cultured hippocampal neurons. These data suggest that neurons within the hippocampus may experience significant calcium influx even under the mildest injury conditions predicted from finite element models. Despite the inability to trigger neuronal death at these lower stretch levels, the resulting increases in calcium are nevertheless sufficient to initiate a number of calcium-mediated processes that could alter cell function, both transiently and long-term (Kampfl et al., 1997; Kristian and Siesjo, 1998; White et al., 2000).

Assessing the response of cultured CNS cells to mechanical stretch has received attention in the past, including the use of biaxial stretch on cortical astrocytes (Ellis et al., 1995; Lamb et al., 1997; Rzigalinski et al., 1997), mixed cortical neuronal and glia cultures (Tavalin et al., 1995; McKinney et al., 1996; Zhang et al., 1996; Tavalin et al., 1997; Goforth et al., 1999), neural like cells (Cargill and Thibault, 1996; Geddes and Cargill, 2001; Wolf et al., 2001), and septo-hippocampal cultures of neurons (Pike et al., 2000). Additionally, the influence of rapid fluid shear stress has also been examined (LaPlaca and Thibault, 1997). As in the current study, a significant amount of this past work examined the impact of stretch on calcium dynamics. Although most previous efforts investigating cell stretch and injury have examined the role of calcium release from intracellular stores in neurons and astrocytes (Rzigalinski et al., 1998; Weber et al., 1999; Floyd et al., 2001), there are recent data that show NMDA receptor activation is partially responsible

for immediate changes in calcium dynamics and mitochondrial membrane potential that occurs in stretch injured cortical neurons (Ahmed et al., 2000, 2002). In our data, the inability to completely block the calcium increase after stretch by removing extracellular calcium suggests that there is also a component of intracellular release in hippocampal cultures subjected to stretch injury, similar to that shown in previous studies.

A major objective of this study was to explore potential "mechanosensitivity" of hippocampal neurons to stretch injury. It has been well established that neurons in the hippocampus are selectively vulnerable to injury following brain trauma in humans and animal models (Kotapka et al., 1992, 1994; Colicos et al., 1996; Smith et al., 1997). However, the relative influence of primary mechanical damage to hippocampal neurons compared to secondary neurotoxicity induced by damage elsewhere is not known. Our reduced model of cellular injury shows that mechanical stretch alone can cause significant calcium influx into hippocampal neurons, exceeding the levels caused by a high level of NMDA stimulation. Recently, it has been shown that calcium influx triggered by biaxial stretch is much larger in hippocampal neurons than cortical neurons (Geddes et al., 2003), pointing to the hippocampus as a vulnerable area that will show calcium-mediated damage following TBI. Even though the mechanical threshold for *in vitro* hippocampal cell death is high, the marked changes in intracellular calcium across the range of stretch conditions highlights the potential mechanosensitivity of the hippocampal neuron, perhaps compromising the ability of the neuron to respond to subsequent insults.

Blocking calcium influx, whether global (calcium free) or targeted to the NMDA receptor is well established in reducing neuronal death from chemical toxicity (Bonfoco et al., 1995; Rothman and Olney, 1995; Kiedrowski, 1999; Hasbani et al., 2001). Recently, though, the central role of calcium in mediating neuronal death has been re-examined. Some investigators have clarified that while calcium influx into cultured cortical neurons via the NMDA receptor is toxic, identical loads via voltage-sensitive Ca^{2+} channels is not (Sattler et al., 1998, 2000). In contrast, our results demonstrated that efforts to remove the immediate calcium influx or blunt the immediate influx through the NMDA receptor in the first thirty minutes following stretch were not effective treatments at mechanically lethal stretch levels. We also found that reduction of the acute calcium transient in the first thirty minutes following stretch at a non-lethal stretch level actually reduced neuronal viability at 24 h. Others have shown a similar exacerbation of death by the blocking acute influx through the voltage gated calcium channels during kainate-induced neurotoxocity (Leski et al., 1999). Although our data suggest that calcium influx occurring within the first 30 min may represent a reasonable marker of the level of mechanical perturbation, the role of this immediate calcium load in mediating neuronal death following trauma is less clear. At the current time, we cannot discount that high stretch may trigger additional mechanical damage that subsequently plays a major role in outcome. We also must consider that both the partial block of $[Ca^{2+}]_i$ with MK-801 and the small elevation in calcium occurring in calcium free conditions imply that additional calcium influx pathways are acutely activated by stretch, as well as the influence of the cytosolic calcium beyond the initial 30 min after stretch. Furthermore, it is possible that the large population of unstretched neurons in the culture have a trophic effect on the stretched neurons, making them less vulnerable to potentially toxic insults. The apparent disparity between modulating the early calcium influx and improving neuronal viability points to another feature distinguishing mechanical and chemical injury to hippocampal neurons.

In summary, our data demonstrate the unique consequences of mechanical deformation of hippocampal neurons using injury levels consistent with deformations that occur in the brain during head trauma. A large calcium influx is induced following deformation that is proportional to the level of stretch. However, the pharmacological manipulation of this calcium influx in the acute phase following stretch does not appear to improve neuronal viability. Unfortunately, the primary mediators or even general mechanisms of neuron death following mechanical deformation remain unknown.

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