Published in final edited form as:

Eur J Neurosci. 2008 April ; 27(7): 1659–1672. doi:10.1111/j.1460-9568.2008.06156.x.

Traumatic brain injury causes a long-lasting calcium (Ca^{2+})-plateau of elevated intracellular Ca levels and altered Ca^{2+} homeostatic mechanisms in hippocampal neurons surviving brain injury

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Abstract

Traumatic brain injury (TBI) survivors often suffer chronically from significant morbidity associated with cognitive deficits, behavioral difficulties and a post-traumatic syndrome and thus it is important to understand the pathophysiology of these long-term plasticity changes after TBI. Calcium (Ca^{2+}) has been implicated in the pathophysiology of TBI-induced neuronal death and other forms of brain injury including stroke and status epilepticus. However, the potential role of long-term changes in neuronal Ca^{2+} dynamics after TBI has not been evaluated. In the present study, we measured basal free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in acutely isolated CA3 hippocampal neurons from Sprague–Dawley rats at 1, 7 and 30 days after moderate central fluid percussion injury. Basal $[\text{Ca}^{2+}]_i$ was significantly elevated when measured 1 and 7 days post-TBI without evidence of neuronal death. Basal $[\text{Ca}^{2+}]_i$ returned to normal when measured 30 days post-TBI. In contrast, abnormalities in Ca^{2+} homeostasis were found for as long as 30 days after TBI. Studies evaluating the mechanisms underlying the altered Ca^{2+} homeostasis in TBI neurons indicated that necrotic or apoptotic cell death and abnormalities in Ca^{2+} influx and efflux mechanisms could not account for these changes and suggested that long-term changes in Ca^{2+} buffering or Ca^{2+} sequestration/release mechanisms underlie these changes in Ca^{2+} homeostasis after TBI. Further elucidation of the mechanisms of altered Ca^{2+} homeostasis in traumatized, surviving neurons in TBI may offer novel therapeutic interventions that may contribute to the treatment and relief of some of the morbidity associated with TBI.

Keywords

acute dissociation of neuron; calcium dynamics; Fura-2/Fura-FF; Sprague–Dawley rats

Introduction

Traumatic brain injury (TBI) is a common cause of mortality and morbidity, especially for people younger than 45 years of age in developed nations (Ghajar, 2000; Bruns & Hauser, 2003). Over 40% of TBI survivors experience long-term disabilities including cognitive problems, epilepsy, headaches, behavioral difficulties, employment disabilities and a post-traumatic syndrome following injury (Capruso & Levin, 1992; McAllister & Arciniegas, 2002; Corrigan *et al.*, 2004). TBI can also cause an increased risk for developing neurological conditions such as Alzheimer's disease, Parkinson's disease and other brain disorders that become more prevalent with aging (Centers for Disease Control and Prevention, 2002; National Institute of Neurological Disorders and Stroke, 2002). Thus, it is important to study mechanisms underlying the pathophysiological process following TBI to develop new strategies to prevent the disabling and costly sequelae of TBI.

Alterations in intracellular calcium concentration ($[Ca^{2+}]_i$) and Ca^{2+} homeostatic mechanisms have been implicated in the pathophysiology of TBI and other types of brain injuries. These alterations in Ca^{2+} systems are thought to mediate neuroplasticity changes in neurons surviving brain injury (Delorenzo *et al.*, 2005). TBI, like status epilepticus and stroke, has been shown to damage and kill neurons by excessive stimulation of glutamate receptors followed by a concomitant overwhelming increase in free $[Ca^{2+}]_i$ during the injury phase (McIntosh *et al.*, 1997; Raghupathi, 2004; Delorenzo *et al.*, 2005). In the rat pilocarpine and hippocampal neuronal culture models of status epilepticus, our laboratory has demonstrated that $[Ca^{2+}]_i$ remains elevated and Ca^{2+} homeostatic mechanisms are altered in surviving hippocampal neurons for weeks after the initial injury (Pal *et al.*, 1999, 2000; Raza *et al.*, 2001, 2004). Evidence has indicated that these prolonged changes in neuronal Ca^{2+} dynamics (the Ca^{2+} plateau) play an important role in mediating the persistent neuronal plasticity changes and chronic epilepsy following status epilepticus-induced brain injury (Delorenzo *et al.*, 2005). Numerous studies have also demonstrated alterations in $[Ca^{2+}]_i$ dynamics in the acute time period following TBI (Faden *et al.*, 1989; Fineman *et al.*, 1993; Tymianski & Tator, 1996; McIntosh *et al.*, 1997; Sahuquillo *et al.*, 2001). However, there have as yet been no studies on the chronic effects of TBI on $[Ca^{2+}]_i$ dynamics in traumatized but surviving neurons.

We measured basal neuronal $[Ca^{2+}]_i$ and studied Ca^{2+} homeostatic mechanisms in acutely isolated CA3 hippocampal neurons 1, 7 and 30 days after moderate central fluid percussion injury using the ratiometric Ca^{2+} indicators Fura-2 and Fura-FF (Grynkiewicz *et al.*, 1985; Raza *et al.*, 2001, 2004). The results demonstrate that TBI caused chronic alterations in both $[Ca^{2+}]_i$ levels and Ca^{2+} homeostatic mechanisms in hippocampal neurons surviving brain injury. Initial studies evaluating the mechanisms underlying the altered Ca^{2+} homeostasis indicate that necrotic or apoptotic cell death and abnormalities in Ca^{2+} influx and efflux mechanisms could not account for these changes, suggesting that long-term changes in Ca^{2+} buffering or Ca^{2+} sequestration/release may contribute to the alterations in Ca^{2+} dynamics in the surviving TBI neurons and offer new insights into our understanding of the pathophysiology of TBI.

Materials and methods

Animals

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (Hilltop Laboratory Animals, Scottsdale, PA, USA) weighing 100–150 g were used. Animals were housed individually at 20–22 °C with a 12/12-h light–dark cycle (lights on 06:00–18:00 h) and had free access to food and water.

Fluid percussion injury device and conditions

The fluid percussion injury device used to produce experimental concussive brain injury was identical to that used previously on rodents (Lyeth *et al.*, 1990; Delahunty *et al.*, 1995). In brief, the injury device consisted of a Plexiglas cylindrical reservoir 60 cm in length and 4.5 cm in diameter. A rubber-covered Plexiglas piston mounted in O-rings was fitted to one end of the reservoir. The opposite end of the reservoir had a 2-cm-long metal housing coupled to a pressure transducer (Entran Devices, Inc., Fairfield, NJ, USA) and a male Luer-Loc fitting. This fitting was connected to a female Leur-Loc fitting implanted over the exposed dura of the rat just prior to injury. Injury was produced by the impact of a swinging pendulum against the piston of the device. This impact injected a small volume of isotonic saline into the closed cranial vault, producing a very brief displacement and deformation of brain tissue. The pressure pulse was monitored by the pressure transducer and stored on a digital oscilloscope (Tektronix 5111, Beaverton, OR, USA).

Surgical preparation and injury

Animals were surgically prepared 24 h before sham injury or TBI. After placement in a stereotaxic frame under sodium pentobarbital (60 mg/kg) anesthesia, the scalp was incised sagittally and soft tissue displaced laterally. A 4.8-mm-diameter central craniotomy was performed over the sagittal suture midway between the bregma and lamda sutures. Two nickel-plated screws were placed in burr holes 1 mm rostral to the bregma and 1 mm caudal to the lamda sutures. A modified, female Luer-Loc syringe hub was placed over the intact dura and secured with cyanoacrylate and dental adhesives. The scalp was sutured over the injury tube, bacitracin was applied to the incision and the animal was returned to its home cage.

Twenty-four hours after surgical preparation, animals were anesthetized with 4% isoflurane in a carrier gas of 30% O₂/70% N₂O. The scalp was incised and the injury tube was coupled to the injury device. Animals in the TBI group received a moderate (2.0 ± 0.02 atm, 202650 ± 2026.5 Pa) central fluid percussion injury. Animals in the sham group were coupled to the injury device, but no fluid pulse was delivered. After TBI or sham treatment, the scalp was sutured and animals were returned to their cages. Control animals received neither anesthesia nor surgery.

Acute isolation of CA3 hippocampal neurons

Hippocampal CA3 neurons were acutely isolated by methods previously described by our laboratory (Raza *et al.*, 2001, 2004). Briefly, animals were injected with MK-801 (1 mg/kg) intraperitoneally 15 min prior to anesthesia with halothane to inhibit *N*-methyl-D-aspartate (NMDA) receptor activation and increase neuronal cell viability. Following anesthesia, animals were killed by decapitation, and the brains were rapidly removed, dissected and placed in a 4 °C oxygenated (95% O₂/5% CO₂) artificial cerebrospinal fluid composed of (in mM) 201 sucrose, 3 KCl, 26 NaHCO₃, 1.025 NaHPO₄, 6 MgCl₂, 0.2 CaCl₂ and 10 glucose. MK-801 (1 μM) was added to all solutions to increase neuronal viability and was removed 15 min prior to recording. No differences in the yield of the neurons or [Ca²⁺]_i levels were observed when a reversible NMDA receptor blocker, APV (1 μM), was used instead of MK-801 and neurons were only exposed to these agents for less than 20 min during the isolation procedure.

Hippocampal slices 450 μm in thickness were sectioned on a 12° agar ramp using a Vibratome sectioning apparatus (Series 200, Technical Products International, St. Louis, MO, USA). Slices were then incubated for 10 min in an oxygenated dissociation medium [(in mM) 120 NaCl, 5 KCl, 6 MgCl₂, 0.2 CaCl₂, 25 glucose, and 20 piperazine-N,N'-bis[2-ethanesulfonic acid], pH adjusted to 7.2 with NaOH] at 34 °C. Slices were treated with 8 mg/mL Protease XXIII in dissociation medium for 6–8 min and then thoroughly rinsed. The CA3 region was visualized with a dissecting microscope and 1-mm² punches were excised and triturated with

a series of Pasteur pipettes of decreasing diameter at 4 °C, resulting in an even suspension of acutely isolated CA3 hippocampal cells that were then plated on poly-L-lysine-coated glass coverslip chambers (Nunc, Naperville, IL, USA) and incubated at 37 °C in a 5% CO₂/95% air atmosphere. This procedure yielded numerous viable neurons for experimentation, as shown in Fig. 3 (Raza *et al.*, 2001,2004).

Measurement of [Ca²⁺]_i

As described previously by our laboratory (Raza *et al.*, 2001, 2004), changes in neuronal [Ca²⁺]_i were measured using the ratiometric, high-affinity ($K_d \approx 224$ nM) fluorescent Ca²⁺ indicator, Fura-2 (Grynkiewicz *et al.*, 1985). Fura-2-AM (1 μM, Molecular Probes, Eugene, OR, USA) was dissolved into the cell suspension (0.1% DMSO) just prior to plating. After 45 min, dye loading was terminated with three washes with a recording solution [(in mM) 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 CaCl₂, 1 MgCl₂, 2 μM glycine, pH 7.3, osmolarity adjusted to 325 mOsm with sucrose) and incubated for an additional 15 min to allow for complete cleavage of the AM moiety from Fura-2.

Acutely isolated CA3 hippocampal neurons were visualized on an inverted microscope (Olympus IX 70, Olympus America, Melville, NY, USA) using a 20×, 0.7 numerical aperture, fluorite water-immersion objective (Olympus America) maintained at 37 °C with a heated stage (Harvard Apparatus Inc., Holliston, MA, USA). Fura-2 was excited with a 75-W xenon arc lamp (Olympus America) with alternating wavelengths of 340 and 380 nm filtered through a Sutter Filter Wheel (Sutter Instruments Co., Novato, CA, USA). Fluorescent emission at 510 nm was captured through a Fura filter cube (Olympus America) with a dichroic at 400 nm emission using a cooled digital CCD camera (LSR AstroCam Ltd, Cambridge, UK).

The Temporal Module of the Perkin Elmer Life Sciences Imaging Suite (Version 4.0, Perkin Elmer, Boston, MA, USA) was used to control image acquisition and processing. Image pairs were captured and corrected for non-specific background fluorescence by subtracting images acquired from non-indicator-loaded plates. Specific regions of interest were designated for each neuron in the microscope field. Ratio measurements for individual neurons were taken at 10-s intervals for 60 s, averaged and calibrated according to the Grynkiewicz equation (Grynkiewicz *et al.*, 1985). Individual neurons from multiple experiments were pooled to calculate mean ± SEM.

An *in situ* Ca²⁺ calibration curve was employed to convert fluorescence ratios to Ca²⁺ concentrations. Baseline ratios of Fura-2-loaded hippocampal neurons were measured for ~5 min in recording solution. The solution was changed to a calibration buffer [100 mM KCl, 10 mM Ca-EGTA, 1 mM free Mg²⁺, 10 mM MOPS, pH = 7.2 supplemented with the ionophore, 5 μM bromo-A23187 (Molecular Probes)]. This high-Ca²⁺ calibration buffer (~39 μM Ca²⁺) was used to measure the maximum ratio value, R_{max} . The bath solution was then changed to a low-Ca²⁺ calibration buffer (~0 μM Ca²⁺) (100 mM KCl, 10 mM free EGTA, 1 mM free Mg²⁺, 10 mM MOPS, pH = 7.2) supplemented with 5 μM bromo-A23187 to determine the minimum ratio value, R_{min} . These ratio values, after background correction, were used to calculate [Ca²⁺]_i using the equation: $[Ca^{2+}]_i = (K_d S_{f2}/S_{b2})(R - R_{min})/(R_{max} - R)$ where R is the 340/380-nm signal ratio at any time, S_{f2} is the absolute value of the corrected 380-nm signal at R_{min} , S_{b2} is the absolute value of the corrected 380-nm signal at R_{max} and the K_d is 224 nM.

Glutamate stimulation

Glutamate challenge was accomplished using a modified double barrel 'sewer pipe' perfusion technique (Gibbs *et al.*, 1997) in which control and glutamate solutions flowed out of parallel Teflon tubes (0.2 mm internal diameter) in a laminar pattern. Rapid (< 200 ms) and complete solution changes at a constant flow rate were effected by moving the tube assembly laterally

in relation to the neuron under study with no cross-contamination evident between tubes as glutamate application was terminated completely through lateral movement of sewer pipes.

To assess neuronal Ca^{2+} homeostatic mechanisms, $[\text{Ca}^{2+}]_i$ measurements were made before, during and after brief glutamate exposure ($50 \mu\text{M}$, 4 min) using the low-affinity indicator, Fura-FF ($K_d \approx 20 \mu\text{M}$). Measurements made with Fura-FF were reported as 340/380-nm signal ratios. Fura-2, because of its high affinity for Ca^{2+} , functions reliably when Ca^{2+} concentrations are in the nanomolar to low micromolar range (Haugland, 1996; Raza *et al.*, 2001; Sun *et al.*, 2004). With its low affinity for Ca^{2+} , Fura-FF is a better indicator for measuring higher Ca^{2+} concentrations without being saturated. Micromolar $[\text{Ca}^{2+}]_i$ values were generated using the original K_d value ($20 \mu\text{M}$) to estimate the peak $[\text{Ca}^{2+}]_i$ values upon glutamate stimulation (see Fig. 4) (Hyrc *et al.*, 2000; Raza *et al.*, 2001; Sun *et al.*, 2004).

Histology and image analysis

Animals were perfused transcardially under sodium pentobarbital anesthesia (150 mg/kg) with 250 mL of saline, followed by 500 mL of 4% paraformaldehyde on post-injury day 7. Brains were removed, placed in 4% paraformaldehyde and then in phosphate buffer for 24 h each before sectioning. Coronal brain slices were cut ($40 \mu\text{m}$) through the dorsal hippocampus using a Vibratome sectioning apparatus. Sections were taken at least $80 \mu\text{m}$ apart to avoid recounting the same neurons in different slices. Serial sections were mounted on glass slides, air-dried, stained with cresyl violet, dehydrated and covers-lipped with Permount. Sections from animals in both sham and TBI groups ($n = 4$) were mounted on each slide and processed in parallel to minimize technical variance. Cresyl violet-stained neurons in the CA3 region of the hippocampus were counted per unit area ($150 \times 230 \mu\text{m}$) by an independent examiner blinded to the study conditions. Individual counts from at least four serial sections at least $80 \mu\text{m}$ apart were made and averaged as neurons per region for each animal.

Cell death assay

Neuronal death in acutely isolated neurons was assessed using fluorescein diacetate–propidium iodide and annexin–propidium iodide microfluorometry. Neurons labeled with fluorescein diacetate–propidium iodide or annexin–propidium iodide were quantified by means of the Ultraview image analysis software package (Version 4.0, Perkin Elmer Life Sciences), and percent neuronal death was calculated as the number of neurons labeled by propidium iodide divided by the sum of the number of neurons labeled by propidium iodide and those labeled by fluorescein diacetate or annexin. This procedure has been established to evaluate the effects of isolation procedures on neuronal death (Raza *et al.*, 2004).

Data analysis

Student's *t*-test or one-way analysis of variance (ANOVA) were applied when appropriate to compare $[\text{Ca}^{2+}]_i$ or cell-death among groups. The Tukey test was used for all *post-hoc* comparisons. For comparing the distributions of $[\text{Ca}^{2+}]_i$ levels and also the differences in basal $[\text{Ca}^{2+}]_i$ levels we used a two-way ANOVA with type (sham vs. TBI) and day after TBI as the two factors. In addition, the population distributions were also compared using the Kolmogorov–Smirnov test, which provides a more rigorous evaluation of distributions and evaluates if the data in the populations compared followed a normal distribution. The decay curves in Fig. 9 were generated using standard procedures (Raza *et al.*, 2001) by calculating the mean $[\text{Ca}^{2+}]_i$ levels at 15-s intervals following the glutamate load and then normalizing these mean $[\text{Ca}^{2+}]_i$ levels to a maximum value of 1.0 using SigmaPlot. Statistical tests were run using SigmaStat 2.0 and graphs generated with SigmaPlot 8.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant for all data analysis. We used 4–6 animals for each experimental condition or time point studied. Dissociating the hippocampal slices routinely yielded 10–15 healthy, phase-bright neurons that were used for the recordings, yielding a

minimum of five neurons for each animal. The means of each group of neurons from each animal were used to evaluate results and conduct statistical analysis. Descriptions of individual experimental numbers are given in the figure legends. Viable neurons included in the study had a smooth surface and a pyramidal-like morphology with processes. Non-viable neurons were swollen or circular and the surface was uneven and irregular. Data from each animal were first subjected to an outlier test, pooled together in respective groups and ultimately represented as total number of cells studied.

Results

Moderate central fluid percussion injury caused elevations in dissociated hippocampal neuronal $[Ca^{2+}]_i$ 1 day after TBI

To investigate the role of Ca^{2+} in the subacute period of TBI, we measured neuronal $[Ca^{2+}]_i$ 1 day after moderate central fluid percussion injury using the high-affinity, ratiometric Ca^{2+} indicator Fura-2 in acutely isolated hippocampal neurons from control, sham and TBI animals. Acutely isolated CA3 hippocampal neurons harvested 1 day after TBI manifested a mean basal $[Ca^{2+}]_i$ of 300.03 ± 19.99 nM ($n = 7$ animals), significantly ($P < 0.001$) higher than mean basal $[Ca^{2+}]_i$ in CA3 hippocampal neurons harvested from age-matched control (137.53 ± 13.42 nM, $n = 5$ animals) and sham animals (152.26 ± 21.52 nM, $n = 5$ animals, Fig. 1A). No significant differences were observed between the control and sham groups.

Analysis of the population distributions of basal $[Ca^{2+}]_i$ revealed values ≤ 200 nM in approximately 88% of control and sham neurons. In contrast, only 35% neurons 1 day post-TBI had basal values ≤ 200 nM indicating a shift in the population of neurons from the TBI animals to higher basal $[Ca^{2+}]_i$ levels (Fig. 2A). This rightward shift in the distribution of Ca^{2+} values in TBI neurons was significantly different from sham neurons (Fig. 2, two-way ANOVA, $P < 0.001$ and Kolmogorov–Smirnov test, $P < 0.0001$).

Moderate central fluid percussion injury caused alterations in hippocampal neuronal Ca^{2+} homeostatic mechanisms 1 day after TBI

In order to investigate the effects of TBI on alterations in Ca^{2+} homeostatic mechanisms, the ability of dissociated neurons to handle a given load of Ca^{2+} was assessed by challenging them with glutamate ($50 \mu\text{M}$) for 4 min. The Fura-FF ratio values for the glutamate recovery curves for sham and 1-day post-TBI neurons are shown in Fig. 3A. In response to glutamate stimulation, both the sham and the 1-day TBI neurons produced corresponding increases in $[Ca^{2+}]_i$, as evidenced by the increased Fura-FF ratio. Upon removal of glutamate, sham neurons ($n = 5$ animals) promptly restored $[Ca^{2+}]_i$ to preglutamate levels. By contrast, $[Ca^{2+}]_i$ in TBI neurons ($n = 5$ animals) remained elevated throughout the period of recording (and even out to 30 min) following glutamate removal (Fig 3 and Fig 5). The peak free $[Ca^{2+}]_i$ values in both sham and TBI neurons in response to the glutamate stimulation are shown in Fig. 4. Glutamate treatment ($50 \mu\text{M}$ glutamate for 4 min) elicited peak $[Ca^{2+}]_i$ values of 13.57 ± 0.82 and $13.92 \pm 0.86 \mu\text{M}$ in the sham and 1-day TBI neurons, respectively, that were not statistically different ($n = 5$ animals, t -test, $P = 0.1$).

In addition, when challenged with sublethal glutamate, the sham neurons were able to buffer $90.2 \pm 4.5\%$ of glutamate-induced Ca^{2+} load. In contrast, TBI neurons were able to buffer only $30.7 \pm 9.7\%$ of Ca^{2+} to the resting levels (Fig. 6). The injured neurons could not handle the elevated free $[Ca^{2+}]_i$ load as efficiently as the control neurons and thus were exposed to prolonged elevated $[Ca^{2+}]_i$ levels. Thus, dissociated hippocampal neurons 1 day post-TBI demonstrated higher baseline $[Ca^{2+}]_i$ levels and prolonged elevations of $[Ca^{2+}]_i$ following excitatory stimulation.

Moderate central fluid percussion injury caused persistent elevations in basal neuronal $[Ca^{2+}]_i$ 1 week after TBI

To investigate the potential role of Ca^{2+} in the subacute period of TBI, we also measured neuronal $[Ca^{2+}]_i$ 7 days after moderate central fluid percussion injury. Acutely isolated CA3 hippocampal neurons harvested 7 days after TBI manifested a mean basal $[Ca^{2+}]_i$ of 268.92 ± 14.81 nM ($n = 7$ animals), significantly ($P < 0.001$) higher than mean basal $[Ca^{2+}]_i$ in CA3 hippocampal neurons harvested from age-matched control (158.87 ± 13.35 nM, $n = 5$ animals) and sham animals (142.61 ± 7.74 nM, $n = 5$ animals, Fig. 1B). No statistical differences were observed between the control and sham groups.

Analysis of the population distributions of basal $[Ca^{2+}]_i$ revealed values ≤ 200 nM in 80% of control and sham neurons. In contrast, only 36% of neurons 7 days post-TBI had basal values ≤ 200 nM. Thus, a shift in the population of neurons from the TBI animals to higher basal $[Ca^{2+}]_i$ levels observed at 1 day was also observed 7 days following TBI (Fig. 2B). This rightward shift towards higher Ca^{2+} values in TBI neurons at day 7 was significantly different from sham neurons (Fig. 2, two-way ANOVA, $P < 0.001$ and Kolmogorov–Smirnov test, $P < 0.0001$). To our knowledge, these results provide the first direct evidence for a chronic alteration in $[Ca^{2+}]_i$ levels at 1 week following TBI.

Moderate central fluid percussion injury caused persistent alterations in hippocampal neuronal Ca^{2+} homeostatic mechanisms 1 week after TBI

We also investigated Ca^{2+} homeostatic mechanisms 7 days following TBI. Similar to the 1-day TBI neurons, the 7-day TBI and sham neurons ($n = 5$ animals each) responded to glutamate stimulation ($50 \mu\text{M}$ glutamate for 4 min) with increased Fura-FF ratio. However, upon removal of glutamate, whereas sham neurons restored the $[Ca^{2+}]_i$ ratio to preglutamate levels, the 7-day TBI neurons demonstrated persistent elevations in $[Ca^{2+}]_i$ ratio throughout the duration of recording (Fig. 3B). As shown in Fig. 4, peak free $[Ca^{2+}]_i$ values elicited upon glutamate stimulation in sham and 7-day TBI neurons were 13.47 ± 0.63 and $13.79 \pm 0.78 \mu\text{M}$, respectively, and were not statistically different ($n = 5$ animals, t -test, $P = 0.12$).

In addition, upon stimulation with glutamate, age-matched sham neurons buffered $89.85 \pm 6.42\%$ of the glutamate-induced Ca^{2+} load. In contrast, 7-day TBI neurons buffered $36.73 \pm 9.08\%$ of the given Ca^{2+} load, thereby demonstrating a significant ($P < 0.005$) inability to restore resting $[Ca^{2+}]_i$ (Fig. 6). Thus, CA3 neurons 7 days post-TBI still display altered ability to restore elevated $[Ca^{2+}]_i$ to basal levels following excitatory stimulation.

Basal neuronal $[Ca^{2+}]_i$ 1 month after moderate central fluid percussion injury

Thirty days after moderate central fluid percussion injury, we measured neuronal $[Ca^{2+}]_i$ to determine if the alterations in neuronal Ca^{2+} homeostasis observed 1 and 7 days post-injury persisted into a chronic setting after TBI. In contrast to the 1-day and 1-week time periods after TBI, the basal $[Ca^{2+}]_i$ of acutely isolated CA3 hippocampal neurons 30 days post-TBI (132.03 ± 11.22 nM, $n = 7$ animals) and 30 days post-sham operation (142.11 ± 10.56 nM, $n = 5$ animals) were not statistically different ($P = 0.514$, Student's t -test, Fig. 1C).

The distribution of $[Ca^{2+}]_i$ in neurons from sham and 30-day post-TBI animals were similar, with 75% and 85% of basal neuronal $[Ca^{2+}]_i \leq 200$ nM, respectively (Fig. 2C). There were no significant differences in the distributions of Ca^{2+} values between sham and TBI neurons 3 days following the TBI injury (Fig. 2, two-way ANOVA, $P = 0.26$ and Kolmogorov–Smirnov test, $P = 0.35$). These results indicate that moderate TBI caused persistent elevations of neuronal $[Ca^{2+}]_i$ for the first week after injury, but after several weeks the neuronal $[Ca^{2+}]_i$ levels were not significantly different from controls.

Moderate central fluid percussion injury caused persistent alterations in hippocampal neuronal Ca^{2+} homeostatic mechanisms 1 month after TBI

Having observed that $[\text{Ca}^{2+}]_i$ levels returns to baseline or near-baseline 30 days post-TBI, we investigated if the alterations in Ca^{2+} homeostatic mechanisms and an inability to restore resting Ca^{2+} observed 1 day and 7 days following TBI still persisted 30 days post-TBI. Upon stimulation with sublethal glutamate ($50 \mu\text{M}$, 4 min), $[\text{Ca}^{2+}]_i$ levels were elevated, as indicated by increased Fura-FF ratio; however, these age-matched sham neurons promptly restored Fura-FF ratio following glutamate removal. In contrast, TBI neurons continued to display elevated $[\text{Ca}^{2+}]_i$ ratio despite glutamate removal (Fig. 3C, $n = 5$ animals each). Peak free $[\text{Ca}^{2+}]_i$ values (Fig. 4) elicited upon glutamate stimulation in sham and 3-day TBI neurons were 11.34 ± 0.84 and $11.85 \pm 0.65 \mu\text{M}$, respectively ($n = 5$ animals), and were not significant.

Moreover, 30-day sham neurons buffered $88.7 \pm 12.42\%$ of the glutamate-induced Ca^{2+} load. In contrast 30-day post-TBI neurons buffered $54.68 \pm 14.24\%$ of the given Ca^{2+} load (Fig. 6). These results indicate that while dissociated hippocampal neurons 30 days post-TBI demonstrates near baseline Ca^{2+} levels, the injury has caused permanent alterations in Ca^{2+} homeostatic mechanisms (Fig 1C, Fig 3C and Fig 6). These findings indicate that TBI has a long-lasting effect on Ca^{2+} dynamics and suggests that these effects may be permanent.

Elevated basal $[\text{Ca}^{2+}]_i$ was not caused by neuronal death in the subacute time period after moderate central fluid percussion injury

Moderate central fluid percussion injury has been well characterized and shown not to induce significant neuronal death in the rat hippocampus (Lyeth *et al.*, 1990; Delahunty *et al.*, 1995). Nevertheless, to confirm this finding under the conditions of the current study and to exclude the possibility that increased neuronal $[\text{Ca}^{2+}]_i$ 7 days after injury was caused by the process of delayed neuronal death, we assayed CA3 hippocampal neuronal survival 7 days after injury in sham and TBI animals. No statistical differences in the count of cresyl violet-stained neurons per unit area in sham-operated (31.44 ± 2.27 neurons per region) or TBI animals (34.13 ± 1.06 neurons per region) were observed when analysed by an examiner blinded to group ($P = 0.325$, $n = 4$ animals, Student's *t*-test, Fig. 7A). In addition, the distribution of neuronal $[\text{Ca}^{2+}]_i$ 7 days post-TBI did not reveal a large population of neurons with exceedingly high Ca^{2+} levels. These results demonstrated that neuronal cell death was not a significant factor in the hippocampal area sampled in causing the altered Ca^{2+} dynamics observed 7 days post moderate TBI.

In addition to using cresyl violet-stained neurons per unit area to evaluate neuronal injury, we also evaluated cell death in dissociated neurons using fluorescein diacetate–propidium iodide stains. No significant differences in cell death were observed between neurons dissociated from age-matched sham and TBI animals ($P = 0.6$, $n = 5$ animals, Student's *t*-test, Fig. 7B and C). Fewer than 5% of the isolated neurons manifested cell death in both the groups. These results demonstrate that cell death was not the cause of elevated $[\text{Ca}^{2+}]_i$ in the acutely isolated neurons observed in this study. Furthermore, the results also demonstrate that the isolation procedure did not significantly affect the viability of the neurons.

To provide a further rigorous assessment of the issue of neuronal death or delayed cell death contributing to elevated basal $[\text{Ca}^{2+}]_i$ in the subacute time period after moderate central fluid percussion injury, we also measured percentage cell death in the acutely isolated neurons using the annexin–propidium iodide technique (Raza *et al.*, 2001). This method allowed for the evaluation of both necrosis (acute cell death) and apoptosis (delayed cell death) in acutely isolated neurons. Percentage dead or apoptotic neurons were determined at 7 days post TBI. As shown in Fig. 7C, no significant increase in neuronal death or apoptosis was observed at 7 days post TBI ($P = 0.8$, $n = 5$ animals, Student's *t*-test, Fig. 7C), a time point when $[\text{Ca}^{2+}]_i$ levels were elevated and Ca^{2+} homeostatic mechanisms were still altered (Fig 1B and Fig 3C).

The percentage of dead or apoptotic neurons in control and TBI injured preparations was less than 4%. These results demonstrate that very few neurons were undergoing cell death in either the control or the TBI group and that the differences in numbers of apoptotic neurons were not a prominent feature of the acutely isolated neuronal populations at 7 days. Thus, these results demonstrate that neuronal injury from apoptotic processes could not account for the alterations observed in Ca^{2+} dynamics in surviving TBI neurons. Previously published data from the VCU brain injury group and others have demonstrated that ATP levels, although depleted immediately after trauma in this fluid percussion model, recover to control levels within 1–3 h after injury, indicating the ATP depletion was also not a major factor mediating the altered Ca^{2+} dynamics in surviving TBI neurons observed in the present study (Vink *et al.*, 1987, 1994; Marklund *et al.*, 2006; Holloway *et al.*, 2007; Zhou *et al.*, 2007).

Mechanisms underlying elevated $[\text{Ca}^{2+}]_i$ levels and altered Ca^{2+} homeostasis in the moderate central fluid percussion injury model

One possible explanation for the inability of the TBI neurons to restore resting $[\text{Ca}^{2+}]_i$ as rapidly as control neurons could be that the TBI manifested increased Ca^{2+} entry or load during the glutamate stimulation. To evaluate this possibility, we determined the peak free $[\text{Ca}^{2+}]_i$ levels following glutamate stimulation in TBI and sham neurons in the presence or absence of Ca^{2+} entry inhibitors (DeLorenzo *et al.*, 2005; Blaustein & Lederer, 1999). All the experiments were performed on 7-day post-TBI and sham neurons, a time point at which both the elevated Ca^{2+} levels and alterations in Ca^{2+} homeostatic mechanisms are still present and cells have recovered from any deficits in ATP levels and metabolic status that might be present at the immediate post-trauma period (Vink *et al.*, 1987, 1994; Marklund *et al.*, 2006; Holloway *et al.*, 2007; Zhou *et al.*, 2007).

As shown in Fig. 8, the peak free $[\text{Ca}^{2+}]_i$ levels attained after glutamate stimulation were not significantly different between sham and TBI neurons ($n = 5$ animals, $P = 0.7$). To evaluate further the total contribution of Ca^{2+} ($[\text{Ca}^{2+}]_e$) entry to the peak free $[\text{Ca}^{2+}]_i$ levels after a glutamate stimulation, we exposed neurons to glutamate in a Ca^{2+} -free medium. In the presence of low $[\text{Ca}^{2+}]_e$ (0.2 mM CaCl_2), the glutamate-induced peak was almost completely abolished in both control and TBI neurons when compared with normal $[\text{Ca}^{2+}]_e$ (2 mM CaCl_2) ($n = 5$ animals, $P < 0.001$, Fig. 8), suggesting that the majority of the increased free $[\text{Ca}^{2+}]_i$ following glutamate stimulation was coming from the external medium. Studies were then initiated to evaluate the major routes of Ca^{2+} entry.

We found that there were two main components to this glutamate-induced increased free $[\text{Ca}^{2+}]_i$ in both control and TBI neurons. The smaller component was mediated by Ca^{2+} entry through voltage-gated Ca^{2+} channels, as evidenced by a small decrease in Ca^{2+} peak in the presence of the Ca^{2+} channel inhibitor, nifedipine ($n = 5$ animals, $P < 0.001$, Fig. 8). The larger component was mediated by activation of the NMDA-gated Ca^{2+} channel that was significantly reduced in the presence of MK-801 ($n = 5$ animals, $P < 0.001$, Fig. 8). The sum of voltage-gated and NMDA-activated Ca^{2+} entry essentially accounted for all of the glutamate-induced increased free $[\text{Ca}^{2+}]_i$ peak in both control and TBI neurons (Fig. 8). Importantly, there were no significant differences in peak $[\text{Ca}^{2+}]_i$ between sham and TBI neurons in the presence of any of the pharmacological manipulations. These studies demonstrate that TBI in the fluid percussion model did not alter the glutamate-activated NMDA Ca^{2+} channel or voltage-gated Ca^{2+} channel activity in the plasma membrane.

In order further to elucidate the mechanisms underlying the altered neuronal Ca^{2+} homeostasis following TBI, we investigated for possible alterations in mechanisms of Ca^{2+} entry and Ca^{2+} efflux (DeLorenzo *et al.*, 2005). We evaluated Ca^{2+} entry mechanisms by analysing the recovery of $[\text{Ca}^{2+}]_i$ from a glutamate-induced peak free $[\text{Ca}^{2+}]_i$ load in sham and TBI neurons. Glutamate was washed off the cells in Ca^{2+} -free solution or solution containing

pharmacological inhibitors of Ca^{2+} entry. The resultant $[\text{Ca}^{2+}]_i$ decay curves from the highest point after the glutamate exposure under these different conditions were generated using standard procedures (data analysis and Raza *et al.*, 2001). As shown in Fig. 9A, removal of extracellular Ca^{2+} ($n = 5$ animals) following glutamate stimulation could not significantly prevent the TBI-induced delayed $[\text{Ca}^{2+}]_i$ recovery in TBI neurons compared with the sham neurons ($P < 0.01$, t -test), indicating the increase entry of Ca^{2+} from $[\text{Ca}^{2+}]_e$ could not be producing the delayed recovery in TBI neurons compared with sham neurons.

We then investigated the role of altered voltage-gated or NMDA receptor-gated Ca^{2+} channel as possible causes for the delayed recovery of $[\text{Ca}^{2+}]_i$ in the TBI neurons. As shown in Fig. 9B and C, treatments with nifedipine ($5 \mu\text{M}$, $n = 5$ animals) or MK-801 ($10 \mu\text{M}$, $n = 5$ animals) did not prevent delayed recovery of $[\text{Ca}^{2+}]_i$ following glutamate stimulation in TBI neurons in comparison with sham neurons. Taken together, the analyses of free $[\text{Ca}^{2+}]_i$ peak levels (Fig. 8) and Ca^{2+} decay curves (Fig. 9) indicate that the Ca^{2+} influx mechanisms were largely not responsible for the observed alterations in Ca^{2+} homeostatic mechanisms in TBI neurons.

After studying Ca^{2+} influx, we next evaluated the effects of inhibiting Ca^{2+} efflux on the recovery of $[\text{Ca}^{2+}]_i$ from a glutamate-induced free peak $[\text{Ca}^{2+}]_i$ in sham and TBI neurons. The majority of Ca^{2+} efflux in neurons after an increase in $[\text{Ca}^{2+}]_i$ is regulated by activation of the plasmalemmal $\text{Na}^+ \pm \text{Ca}^{2+}$ exchanger (Blaustein & Lederer, 1999; Annunziato *et al.*, 2004). Thus, inhibiting the $\text{Na}^+ \pm \text{Ca}^{2+}$ exchanger blocks a large majority of neuronal Ca^{2+} efflux. An effective method to inhibit the $\text{Na}^+ \pm \text{Ca}^{2+}$ exchanger is to replace extracellular Na^+ with the impermeant *N*-methyl-D-gluconate to eliminate the Na^+ electrochemical gradient driving Ca^{2+} extrusion and thus shutting off the efflux of Ca^{2+} due to the $\text{Na}^+ \text{--} \text{Ca}^{2+}$ exchanger. This technique has been effectively utilized by several laboratories to inhibit Ca^{2+} efflux in isolated neurons (Goldman *et al.*, 1994; Gleason *et al.*, 1995; White & Reynolds, 1995; Sidky & Baimbridge, 1997). We replaced extracellular Na^+ only during the glutamate washout so it did not affect the free Ca^{2+} load produced by glutamate stimulation. As shown in Fig. 9D, substitution of *N*-methyl-D-gluconate for Na^+ ($n = 5$ animals) following glutamate stimulation did not significantly prevent the delayed $[\text{Ca}^{2+}]_i$ recovery in TBI neurons in comparison with sham neurons. Although both TBI and sham neurons recovered more slowly with inhibition of the $\text{Na}^+ \text{--} \text{Ca}^{2+}$ exchanger, the impaired Ca^{2+} homeostatic mechanisms accounting for the delayed TBI recovery was still significantly different from sham neurons (Fig. 9D). This indicated that TBI largely did not cause alterations in Ca^{2+} homeostasis by altering the activity of the $\text{Na}^+ \text{--} \text{Ca}^{2+}$ exchanger and its effect on Ca^{2+} efflux.

Discussion

TBI is a common cause of morbidity and mortality (Ghajar, 2000). Survivors of TBI often suffer with difficulties in attention, memory and learning (Capruso & Levin, 1992). Functional deficits have been observed in the absence of significant neuronal death and have been observed to reverse in the chronic setting after injury (Lyeth *et al.*, 1990; Delahunty *et al.*, 1995; Zohar *et al.*, 2003). These findings suggest that some alteration in the physiology of traumatized, surviving neurons must exist to underlie these common post-traumatic impairments associated with TBI. In this study, we have demonstrated that TBI in the rat moderate central fluid percussion injury model causes long-lasting elevations in hippocampal neuronal $[\text{Ca}^{2+}]_i$ levels and alterations in Ca^{2+} homeostasis. Traumatized neurons demonstrated a Ca^{2+} plateau of elevated $[\text{Ca}^{2+}]_i$ for as long as 1 week after TBI and $[\text{Ca}^{2+}]_i$ levels returned to baseline by 30 days after TBI. However, alterations in Ca^{2+} homeostasis did not return to control levels even as long as 1 month after moderate TBI. These long-lasting changes in $[\text{Ca}^{2+}]_i$ levels and Ca^{2+} homeostasis were not the result of dying neurons and represent a persistent neuronal plasticity in Ca^{2+} dynamics in injured but surviving neurons. The results indicate that these long-term alterations in Ca^{2+} dynamics contribute to the pathophysiology of TBI and may

represent a molecular basis for mediating some of the persistent plasticity changes associated with the chronic disability from TBI, including cognitive disorders and post-traumatic epilepsy (Capruso & Levin, 1992; McAllister & Arciniegas, 2002; Corrigan *et al.*, 2004).

Delayed neuronal death is associated with massive elevations in neuronal $[Ca^{2+}]_i$ (Limbrick *et al.*, 1995) and typically occurs 1–3 days after injury (Kirino, 2000). In prior studies, the moderate central fluid percussion injury model in the rat was shown not to cause significant hippocampal neuronal death (Lyeth *et al.*, 1990; Delahunty *et al.*, 1995). In the present study, CA3 hippocampal neurons were counted 7 days post-injury and no statistical differences were observed between sham-operated and TBI animals, confirming that neuronal death in the CA3 region of the hippocampus did not occur at detectable levels in experiments performed under our conditions. In addition, the distribution of $[Ca^{2+}]_i$ 7 days post-TBI did not reveal neurons with the exceedingly high $[Ca^{2+}]_i$ associated with delayed neuronal death (Limbrick *et al.*, 1995), but rather a moderate ‘right shift’ of the distribution towards higher concentrations. In the more severe, lateral fluid percussion model of TBI, marked accumulations in neuronal $[Ca^{2+}]_i$ were shown to be associated with regions of gross tissue damage and demonstrate that more severe injury can cause neuronal loss due to apoptosis and cell death (Fineman *et al.*, 1993). We choose the moderate central fluid percussion injury model in the rat (Lyeth *et al.*, 1990; Delahunty *et al.*, 1995), as it is associated with the development of significant morbidity and chronic post-TBI effects, but avoids the development of neuronal loss seen with the more severe injury models (Fineman *et al.*, 1993). The data in this study indicate that the elevations in $[Ca^{2+}]_i$ after TBI were not occurring in a state of irreversible Ca^{2+} overload and delayed neuronal death, but rather were occurring in surviving neurons in a state of altered Ca^{2+} homeostasis. This is consistent with our previous observations from brain injury due to status epilepticus (Pal *et al.*, 1999, 2000; Raza *et al.*, 2001, 2004) and glutamate excitotoxicity (Sun *et al.*, 2002, 2004) under conditions where the insult injures but does not kill neurons. Together, these studies indicate that injured but surviving neurons manifest long-term plasticity changes in Ca^{2+} homeostasis from TBI, glutamate excitotoxicity and status epilepticus. These persistent changes in Ca^{2+} homeostasis after brain injury may account for many of the commonly shared morbidities in survivors of brain injury (Delorenzo *et al.*, 2005).

The ability of neurons to handle a given load of free $[Ca^{2+}]_i$ following glutamate stimulation is a widely used method to test alterations in Ca^{2+} homeostatic mechanisms (Raza *et al.*, 2001). The acutely dissociated neuronal preparation gives the advantage of faster solution exchange and more control of extracellular milieu in the absence of confounding factors from glia. However, one might contend that glutamate stimulation would elicit different free $[Ca^{2+}]_i$ peak levels at various time-points following TBI. To address this issue, we quantified the peak Ca^{2+} level upon glutamate stimulation. The peak $[Ca^{2+}]_i$ ratio and $[Ca^{2+}]_i$ values achieved at 1, 7 and 30 days after glutamate stimulation were essentially identical between the sham and TBI neurons, suggesting that glutamate stimulation is producing similar Ca^{2+} loads that are not significantly different.

Energy deficits after TBI may alter neurotransmission and cellular transport, and, one might argue, in this case affect ionic homeostasis thereby contributing to metabolic dysfunction and cell death. Our results demonstrate that apoptotic or necrotic cell death was not a major factor in either sham or TBI acutely isolated neurons. Furthermore studies from the VCU neurotrauma research group and others using the fluid percussion model or the cortical contusion model have shown that ATP levels are either not affected (Vink *et al.*, 1987) or decline (Holloway *et al.*, 2007; Zhou *et al.*, 2007) or even increase (Vink *et al.*, 1994) immediately and up to 1–3 h after moderate TBI. However, by 24 h post-TBI, ATP levels recover to those of sham-injury group to maintain a steady metabolic state (Ahmed *et al.*, 2000; Marklund *et al.*, 2006). Thus, the energy-requiring secondary injury cascades that occur early following injury do not challenge the neurons to the extent of ATP depletion and could not be contributing to

the alterations in $[Ca^{2+}]_i$ levels or Ca^{2+} homeostatic mechanisms observed at the longer time points in this study.

It is also important to elucidate the molecular mechanisms mediating the altered Ca^{2+} levels in neurons following TBI. Calcium homeostasis in neurons is a complex, balanced system of influx, efflux, sequestration and extrusion. The Ca^{2+} signal can be amplified in neurons by altering Ca^{2+} influx and efflux mechanisms and by the involvement of Ca^{2+} -induced Ca^{2+} release from intracellular Ca^{2+} stores by either NMDA or metabotropic receptors. In addition, the involvement of the sarco/endoplasmic reticulum calcium ATPase has been suggested in the induction of persistent plasticity changes in long-term depression. Other systems including Ca^{2+} buffering systems such as calbindin, calretinin and parvalbumin, and mitochondrial Ca^{2+} uptake or release may also play roles in altering the homeostatic mechanisms (reviewed in DeLorenzo *et al.*, 2005). A comprehensive evaluation of the multiple potentially altered mechanisms of Ca^{2+} control following TBI is beyond the scope of this investigation; however, we have provided an initial characterization of the mechanisms involved.

Our results determined that the majority of Ca^{2+} entering TBI and sham neurons after a glutamate stimulus was mediated by Ca^{2+} entry through NMDA-activated and voltage-gated Ca^{2+} channels and that TBI did not affect this free peak $[Ca^{2+}]_i$ entry during glutamate stimulation. In addition, the analyses of free peak $[Ca^{2+}]_i$ levels and the Ca^{2+} decay curves in the presence and absence of pharmacological manipulations of Ca^{2+} entry and efflux mechanisms following glutamate stimulation indicated that the major mechanisms mediating Ca^{2+} influx and efflux were not responsible for the observed alterations in Ca^{2+} homeostatic mechanisms in TBI neurons. Thus, other mechanisms mediating Ca^{2+} homeostasis that are independent of influx and efflux mechanisms must be involved. Indeed, in the acute setting of TBI, disturbances in endoplasmic reticulum Ca^{2+} signaling have been characterized (Weber *et al.*, 2001). Further studies are needed to evaluate the altered mechanisms involved in mediating the changes in Ca^{2+} dynamics associated with TBI.

As Ca^{2+} is a major signaling molecule in neurons, alterations in Ca^{2+} homeostasis, although below the threshold of excitotoxicity, could have significant effects on neuronal physiology. Calcium regulates numerous enzyme systems and has effects on gene transcription (West *et al.*, 2001). Prolonged elevations in $[Ca^{2+}]_i$ that do not cause neuronal cell death have been demonstrated to cause alterations in neuronal excitability (Sun *et al.*, 2002; Delorenzo *et al.*, 2005) and long-term changes in gene expression (Morris *et al.*, 1999, 2000). Many changes in the expression of transcription factors have been documented in the acute setting of TBI (Hayes *et al.*, 1995; Raghupathi & McIntosh, 1996; Morrison *et al.*, 2000). The findings in this paper present the first direct evidence that the long-lasting changes in Ca^{2+} homeostasis following TBI are a possible candidate for providing a persistent second messenger signal that may underlie the chronic changes in gene expression observed following TBI.

As a common cause of acquired epilepsy in younger adults (Hauser & Hesdorffer, 1990), TBI may produce lasting alterations to neuronal Ca^{2+} signaling that may contribute to the enhanced ability of TBI animals to develop epilepsy (Coulter *et al.*, 1996). This paper provides the first direct evidence that TBI can cause a long-lasting alteration in Ca^{2+} levels in neurons that survive TBI under conditions that leave several major Ca^{2+} influx and efflux mechanisms intact. The results indicate that this long-lasting Ca^{2+} plateau of elevated $[Ca^{2+}]_i$ levels and altered Ca^{2+} homeostatic mechanisms may contribute to the pathophysiology of TBI. A greater understanding of the mechanisms of altered Ca^{2+} homeostasis in traumatized, surviving neurons in TBI may offer novel therapeutic interventions that may contribute to the treatment and relief of some of the morbidity associated with TBI.

Acknowledgements

We thank our research colleagues Drs Robert Blair and Dawn Carter for their critical suggestions. We also thank Dr Viswanathan Ramakrishnan for his help with statistics. We thank Elisa Attkisson for her help with the acute neuronal isolation procedure. This study was supported by National Institute of Neurological Disorders and Stroke Grants RO1NS051505 and RO1NS052529, and award UO1NS058213 from the National Institutes of Health CounterACT Program through the National Institute of Neurological Disorders and Stroke to R.J.D. The contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of the Federal Government. In addition, the Milton L. Markel Alzheimer's Disease Research Fund and the Sophie and Nathan Gumenick Neuroscience Research Fund also funded this work.

Abbreviations

[Ca²⁺]_i, intracellular calcium concentration; TBI, traumatic brain injury; NMDA, *N*-methyl-D-aspartate..

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FIG. 1.

Basal neuronal $[Ca^{2+}]_i$ in TBI CA3 hippocampal neurons 1, 7 and 30 days after moderate central fluid percussion injury. (A) Acutely isolated CA3 hippocampal neurons 1 day post-injury manifested significant ($P < 0.001$) elevations in $[Ca^{2+}]_i$ compared with both age-matched control and sham neurons. (B) Elevations in basal neuronal $[Ca^{2+}]_i$ persisted 7 days following TBI. Injured neurons manifested significant ($P < 0.001$) elevations in $[Ca^{2+}]_i$ compared with both age-matched control and sham neurons. (C) Basal neuronal $[Ca^{2+}]_i$ normalized 30 days after moderate central fluid percussion injury. Thirty days post-injury, acutely isolated CA3 hippocampal neurons did not manifest significant differences in $[Ca^{2+}]_i$ compared with age-matched control and sham neurons ($P = 0.0514$). * $P < 0.05$, ANOVA and Tukey *post-hoc* test, n

= 7 animals for TBI, five animals each for sham and control condition with a minimum 5–7 neurons per animal for all the time points studied. Data are represented as mean $\pm$ SEM.

FIG. 2.

Distribution of $[Ca^{2+}]_i$ levels for sham and TBI hippocampal neurons 1, 7 and 30 days after moderate central fluid percussion injury. (A) Sham neurons demonstrated a normal distribution for $[Ca^{2+}]_i$ levels with approximately 88% of neurons exhibiting $[Ca^{2+}]_i$ levels less than 200 nM. In contrast 1-day TBI neurons demonstrated a rightward shift towards higher $[Ca^{2+}]_i$ levels with only 35% neurons exhibiting $[Ca^{2+}]_i$ levels less than 200 nM. (B) Seven days following TBI, neurons still exhibited a rightward shift towards higher $[Ca^{2+}]_i$ levels compared with sham neurons. (C) Thirty - days following TBI there was no shift towards higher $[Ca^{2+}]_i$ levels. Approximately 75 and 85% of sham and TBI neurons, respectively, exhibited $[Ca^{2+}]_i$ levels less than 200 nM. The distributions of $[Ca^{2+}]_i$ levels was significantly different at days 1 and 7

post-TBI ($P < 0.0001$ two-way $_{ANOVA}$ and Kolmogorov–Smirnov test) but not at day 30 following TBI (two-way $_{ANOVA}$, $P = 0.26$ and Kolmogorov–Smirnov test, $P = 0.35$; for each condition a total of eight animals were utilized yielding a population of greater than 100 neurons for each distribution analysed).

FIG. 3.

Inability of TBI CA3 hippocampal neurons to restore basal neuronal $[Ca^{2+}]_i$ following excitatory stimulation. A $[Ca^{2+}]_i$ load was produced by a glutamate exposure (50 μM , 4 min) and the ability to restore preglutamate Ca^{2+} levels was measured using the low-affinity Ca^{2+} indicator, Fura-FF. Glutamate stimulation in both sham and TBI dissociated neurons at all the time points following injury produced a Ca^{2+} load, as evidenced by the sudden increase in Fura-FF ratio. Upon removal of glutamate, sham neurons promptly restored Fura-FF ratio to prestimulation levels. However, TBI neurons [(A) 1 day, (B) 7 days and (C) 30 days] failed to restore Fura-FF ratio to preglutamate levels despite glutamate washout. The ratio remained elevated throughout the experiment, indicating a chronic inability of TBI neurons to handle

Ca²⁺ loads. All the time points for TBI neurons following the glutamate washout were significantly ($P < 0.01$, Student's t -test) different compared with sham neurons. Asterisks were omitted for clarity. $n = 5$ animals for each condition with a minimum of five neurons per animal. Data represented as mean $\pm$ SEM.

FIG. 4.

Peak free $[Ca^{2+}]_i$ levels in sham and TBI neurons in response to glutamate-induced $[Ca^{2+}]_i$ loads were not different. Peak free $[Ca^{2+}]_i$ values were determined in response to glutamate stimulation ($50 \mu M$, 4 min) with Fura-FF at 1, 7 and 30 days following TBI. The peak $[Ca^{2+}]_i$ levels between sham and TBI neurons were not statistically different for any of the days following TBI ($P = 0.1$, t -test, $n = 5$ animals with a minimum of six neurons per animal per time point studied).

FIG. 5.

Pseudocolor images of representative sham and TBI neurons (1 day and 30 days post injury) before, during, and after the 50 μM glutamate exposures. Basal $[\text{Ca}^{2+}]_i$ ratios were low in the sham and 30-day post-TBI neurons but were higher in 1-day TBI neurons. The ratio rose rapidly in all the neurons during the glutamate exposure. Twenty minutes after the excitatory stimulus, the TBI neurons (both at 1 day and 30 days) exhibited high $[\text{Ca}^{2+}]_i$ levels, as evidenced by elevated Fura-FF ratio, while the neurons from the sham-operated animals had already recovered. The top panels show the phase-bright photomicrographs of the representative sham and TBI neurons. The data shown were the representative of five animals with over 20 neurons sampled per condition.

FIG. 6.

Impaired calcium buffering capacity of dissociated neurons following TBI. Basal neuronal $[Ca^{2+}]_i$ was measured before and after glutamate stimulation. The percentage of $[Ca^{2+}]_i$ buffered by neurons from sham-operated and TBI neurons 1, 7 and 30 days following injury was then calculated. TBI neurons demonstrated a chronic alteration in Ca^{2+} buffering capacity at all the time points tested following injury. $*P < 0.05$, ANOVA and Tukey *post-hoc* test. Data are represented as mean $\pm$ SEM ($n = 5$ animals for each time point studied with a minimum of 5–7 neurons per animal).

FIG. 7.

Moderate central fluid percussion injury did not cause neuronal death 7 days post-TBI. (A) TBI did not cause a significant difference in the number of cresyl-violet-stained CA3 hippocampal neurons per field observed 7 days after TBI in sham or TBI-isolated neurons ($P = 0.326$, Student's t -test, $n = 4$ animals; individual counts from at least four serial sections at least 80 μm apart were made and averaged as neurons per region for each animal). (B) Representative images of live and dead neurons. Viable neurons were stained with fluorescein diacetate (green), excluding propidium iodide (red). These viable neurons included in the study had a smooth surface and a pyramidal-like morphology with processes. Non-viable neurons, excluded in the study, were stained red. Non-viable red-staining neurons were swollen or

circular and the surface was uneven and irregular. (C) Percentage cell death in dissociated neurons was also quantified using two different assays: fluorescein diacetate–propidium iodide and annexin–propidium iodide two-dye method. No significant increase in neuronal death was observed at 7 days post TBI using both the methods ($P = 0.67$ for fluorescein diacetate–propidium iodide and $P = 0.81$ for annexin–propidium iodide technique, $n = 5$ animals for each condition with a minimum of seven neurons per animal), a time point when $[Ca^{2+}]_i$ was still elevated and delayed cell death, if any, would be prominent. These results demonstrate that the isolation procedure did not significantly affect the viability of the neurons and that sham and TBI neurons had similar viability profiles. The percentage of dead neurons in control and injured preparations was less than 5% using both the dye methods, demonstrating that the moderate TBI injury did not produce cell death in a large number of CA3 neurons and that the neurons manifesting altered Ca^{2+} homeostasis were viable neurons manifesting long-lasting plasticity changes in their ability to handle $[Ca^{2+}]_i$. Data are represented by mean $\pm$ SEM.

FIG. 8.

Peak free $[Ca^{2+}]_i$ levels in sham and TBI neurons in response to glutamate-induced $[Ca^{2+}]_i$ load in the presence of low extracellular Ca^{2+} or pharmacological manipulations of Ca^{2+} entry. The initial rise in free $[Ca^{2+}]_i$ upon glutamate stimulation was not different between TBI and control neurons ($P = 0.7$, t -test). Peak free $[Ca^{2+}]_i$ was significantly depressed upon reduction of extracellular Ca^{2+} to 0.2 mM. Glutamate stimulation in the presence of nifedipine (5 μM), or MK-801 (10 μM) also significantly reduced peak $[Ca^{2+}]_i$. These two routes of Ca^{2+} entry essentially accounted for all of the glutamate-induced Ca^{2+} load. No significant differences in peak $[Ca^{2+}]_i$ between sham and TBI neurons were observed in the presence of any of the pharmacological manipulations of Ca^{2+} entry ($*P < 0.001$, one-way ANOVA, *post-hoc* Tukey test, $n = 5$ animals per condition with a minimum of six neurons per animal). Data are represented as mean $\pm$ SEM.

FIG. 9.

$[Ca^{2+}]_i$ decay curves for the sham and TBI neurons postglutamate exposure in the presence of (A) low extracellular Ca^{2+} (0.2 mM), (B) the L -type Ca^{2+} channel blocker nifedipine (5 μM), (C) the NMDA channel inhibitor MK-801 (10 μM) and (D) low extracellular Na^+ (equimolar replacement with N -methyl-D-glucuronate). Individual decay curves were generated by calculating the mean free $[Ca^{2+}]_i$ levels at 15-s intervals and then normalized the starting value to a maximum value of 1.0 using SigmaPlot. The resultant decay curves from the highest point after the glutamate-induced $[Ca^{2+}]_i$ increase (50 μM for 4 min) are displayed. Standard errors and statistical significance between sham (circles) and TBI (triangles) neurons were determined every 5 min. TBI neurons showed a significant delayed decline in $[Ca^{2+}]_i$ levels compared with

the sham neurons as displayed by the asterisks at 5-min time points for each condition in A–D (* $P < 0.01$, Student's t -test, $n = 5$ animals per condition with a minimum of 5–7 neurons per animal).