Experimental Mild Traumatic Brain Injury Induces Functional Alteration of the Developing Hippocampus

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Yu Z, Morrison B. Experimental mild traumatic brain injury induces functional alteration of the developing hippocampus. J Neurophysiol 103: 499–510, 2010. First published November 18, 2009; doi:10.1152/jn.00775.2009. It is estimated that ~1.5 million Americans suffer a traumatic brain injury (TBI) every year, of which ~80% are considered mild injuries. Because symptoms caused by mild TBI last less than half an hour by definition and apparently resolve without treatment, the study of mild TBI is often neglected resulting in a significant knowledge gap for this wide-spread problem. In this work, we studied functional (electrophysiological) alterations of the neonatal/juvenile hippocampus after experimental mild TBI. Our previous work reported significant cell death after in vitro injury >10% biaxial deformation. Here we report that biaxial deformation as low as 5% affected neuronal function during the first week after in vitro mild injury of hippocampal slice cultures. These results suggest that even very mild mechanical events may lead to a quantifiable neuronal network dysfunction. Furthermore, our results highlight that safe limits of mechanical deformation or tolerance criteria may be specific to a particular outcome measure and that neuronal function is a more sensitive measure of injury than cell death. In addition, the age of the tissue at injury was found to be an important factor affecting posttraumatic deficits in electrophysiological function, indicating a relationship between developmental status and vulnerability to mild injury. Our findings suggest that mild pediatric TBI could result in functional deficits that are more serious than currently appreciated.

INTRODUCTION

Traumatic brain injury (TBI) is the major cause of pediatric death (Bishop 2006; National Center for Injury Prevention and Control 2005). Children who survive TBI may develop chronic cognitive and behavioral disturbances (Christensen et al. 2009; Fay et al. 1994; Levin et al. 2002). Despite a long history of clinical research into pediatric TBI (Bruce et al. 1979; Raphaley et al. 1980), the pathological mechanisms of TBI in childhood are still coming to light (Jankowitz and Adelson 2006). Although pediatric TBI patients are usually treated as miniature adult TBI patients, growing evidence suggests that the pathology and sequelae of TBI in the pediatric population differ from those in the adult TBI population (Cronin 2001). Plasticity might arguably be higher in the developing brain, which would suggest a better prognosis, yet children seem to have worse posttraumatic outcomes that may persevere throughout their lifetimes (Barlow et al. 2005; Levin 2003).

Most TBI that children suffer are mild with a loss of consciousness lasting <30 min (Annegers et al. 1998). Mild pediatric TBI is believed to cause no long-term neurocognitive impairment that occurs to varying degrees following moderate or severe injuries (Anderson et al. 2005; Nadebaum et al. 2007). A recent study reported that the rate of mild pediatric TBI hospitalizations decreased 63% from 1991 to 2005, while nonfatal hospitalization rates for moderate and severe pediatric TBI remained unchanged over the same period (Bowman et al. 2008), suggesting that children with mild TBI may not be receiving required medical attention. Although the acute presentation may appear mild, the need for medical attention may be greater in the pediatric population for two reasons. First, children are more likely to be vulnerable to the consequences of TBI than adults (Annegers and Coan 2000; Beaulieu 2002), thus a mild injury that may not induce functional deficits in the adult population could cause severe deficits in the pediatric population. Second, although the symptoms at the time of injury may be mild, the effect could have long-lasting consequences because the pediatric brain is in a highly plastic stage of development (Annegers et al. 1998; Giza and Prins 2006). Recent reports suggest that mild TBI does have lasting cognitive and developmental effects for children injured younger than 2 yr old compared with age-matched uninjured children (Christensen et al. 2009; Keenan et al. 2007).

Studying the acute response to mild experimental TBI in pediatric tissue may provide important insights into the initiated pathobiology. The acute response of the developing brain to injury may initiate multiple processes that can perturb normal development, e.g., alterations in molecular signals and neural connectivity, abnormal plasticity and neuronal network dysfunction (Ikonomidou et al. 1999; Pohl et al. 1999). Although previous studies have reported that mild human TBI caused lasting functional problems (Evans 1992), it is generally believed that mild pediatric TBI produces virtually no clinically significant long-term deficits in intellectual and neuropsychological functioning (Fay et al. 1993). In experimental models of mild TBI, no significant cell death has been reported in young animals, which is in contrast to the typical cell death reported for experimental moderate and severe TBI (Raghupathi 2004; Raghupathi et al. 2000). In an in vitro model of TBI using cortical and hippocampal brain slice cultures, moderate mechanical stimuli in excess of 10% biaxial deformation were required to induce significant cell death (Cater et al. 2006; Elkin and Morrison 2007). However, cellular or network electrophysiological function may be altered even in the absence of gross behavioral changes during the acute period after mild injury (Dikranian et al. 2008). Clinically subtle symptoms in the acute posttraumatic period that could be overlooked may lead to diagnosis of cerebral palsy, learning disabilities, and complex behavioral disorders later in childhood (Barlow et al. 2005; Christensen et al. 2009).

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Electrophysiological activity of the brain as a functional outcome has been studied after experimental TBI, yielding insights into the causes of postinjury neuropathology (Reeves et al. 2000; Santhakumar et al. 2001). This literature was recently reviewed (Cohen et al. 2007). In contrast to studies on moderate and severe TBI, there have been relatively few studies on neuroelectrophysiological changes after mild TBI (Ahmed et al. 2002; Kao et al. 2004; Schwarzbach et al. 2006; Weber et al. 1999) despite the fact that mild injuries account for up to 80% of TBI (Kraus and Nourjah 1988). In the present study, we used microelectrode arrays (MEAs) to record and analyze the posttraumatic electrophysiological activity of the hippocampus. MEAs make possible simultaneous multisite recording and have been used to decipher brain function and principles of neural signal processing, both in vivo and in vitro (Buzsaki 2004; Wirth and Luscher 2004; Yu et al. 2007), increasing our understanding of disease-related dysfunction of the brain (Sanchez et al. 2006). We have taken advantage of the benefits of MEAs to study the long-range connectivity of neural networks in hippocampal slice cultures after injury to gain additional insight into the injury-induced alterations in neuronal information processing. Moreover, we used the MEA to monitor posttraumatic neural activity from multiple anatomic subregions of the hippocampus (CA1, CA3, and DG), which have different local mechanical properties (Elkin et al. 2007) and thus may have different functional responses to mechanical stimuli.

No previous study has quantified changes in hippocampal electrophysiological function in response to precisely controlled and verified mechanical stimuli simulating TBI. Previous finite element model and in vivo studies have suggested that the threshold for strain-induced mild TBI is >10% strain deformation (Bain and Meaney 2000; Goldsmith 2001). To fill this significant gap in knowledge, organotypic hippocampal slices from postnatal rat pups were cultured and injured by either 5 or 10% biaxial deformation to induce mild injury, using our well-developed in vitro model of TBI (Morrison et al. 2006). We studied the posttraumatic, functional alterations within the hippocampus, which is believed to play an important role in learning and memory (Jarrard 1993) and has been demonstrated quantitatively to be more sensitive to tissue age (developmental state) at injury on posttraumatic, functional outcomes and the temporal development of functional impairment after mild TBI in the developing hippocampus in vitro. We found that electrophysiological function of the immature hippocampus was disrupted after 5 and 10% deformation, which previously was found to induce little cell death (Cater et al. 2006). The degree of dysfunction was correlated with the applied strain. The age of the tissue at injury was found to be an important factor affecting posttraumatic, functional outcome with an age-related window with reduced vulnerability to TBI. Our results indicate that developmental age affects functional outcome during the first week after experimental, in vitro TBI.

**Methods**

**Organotypic hippocampal slice cultures**

All animal procedures were approved by the Columbia University IACUC. The hippocampus of a postnatal day 9–10 (P9-10) rat pup was removed aseptically and cut into sections 400 μm thick with a McIlwain tissue chopper (Brinkmann Instruments). Slices were transferred to a precoated Biopore CM membrane (BGCM00010, Milli-pore) attached to a silicone membrane (Speciality Manufacturing) using polydimethylsiloxane (PDMS; 3140, Dow Corning) as shown in Fig. 1A. Membranes were precoated overnight with a mixture of poly-l-lysine (320 μg/ml, Sigma) and laminin (80 μg/ml, Invitrogen) and then rinsed with culture medium. The Biopore CM/silicone membrane complex was assembled into a metal well so that the membrane complex could be stretched to a prescribed strain using our injury device (see following text). The culture technique has been previously described in detail (Morrison et al. 2006). Slice cultures were incubated in 5 μg/ml propidium iodide to stain dead or injured cells to verify culture health (Morrison et al. 2006). Propidium iodide (PI) is cell impermeable and nonfluorescent. On binding nuclear DNA after entering through a damaged plasma membrane, PI becomes intensely fluorescent. Unhealthy cultures were eliminated from the study.

**Mild injury induced by biaxial stretch**

The primary cause of injury during TBI is believed to be rapid deformation of the brain. To help understand the pathology of TBI, we have previously developed an in vitro TBI model (Morrison et al. 2003, 2006), which is capable of generating biaxial deformation of brain tissues to mimic traumatic injury and to initiate injury cascades which occur in vivo after TBI. In this model, brain tissue was cultured on a highly stretchable silicone membrane and was injured by precisely controlled biaxial stretch and rate of stretch. The mechanical strain or severity of the stretch can be accurately controlled to generate mild to severe damage to the tissue. The strain was developed in a ramp at a constant strain rate to a maximum strain and then relaxed at the same strain rate. At the maximum strain, the strain was held for 5–10 ms before relaxation as standardized in our TBI model (Morrison et al. 2006). This mechanical loading produces a transient strain field in the tissue, which mimics the in vivo tissue deformations during a blow to the head, a fall, or rapid deceleration.

In this study, hippocampal slice cultures were injured after a prescribed period of culture and returned to the incubator until the desired time point for recording. The cultures were injured at 5 and 10% biaxial deformation to induce mild injuries (Morrison et al. 2006). The induced strain within the membrane complex and the cultured tissue was verified from images of the stretch event (Motion Pro 2000; DEL Imaging, CT; 1,280 × 408 pixels at 1,000 frame/s). Deformation was quantified by calculating Lagrangian strain from the videos

\[
E_{\text{Lagrangian}} = \frac{1}{2} (A_{\text{max}}/A_{0} - 1) \quad (1)
\]

where the total area of the hippocampal tissue before stretch (A₀) and during stretch at the maximum strain (A₁max) was measured with ImageJ software (Abramoff et al. 2004). The actual rate of stretch was 5.0 ± 1.6/s for all injury groups. A previous study reported that stretch-induced cell death in the hippocampus was dependent on tissue strain but not strain rate (Cater et al. 2006). Therefore the effect of strain rate was not systematically studied in this work.

**Electrophysiology**

 Cultures were maintained in an incubator (5% CO₂, 37°C) before use, and at the desired time point post injury, the neuronal activity was...
recorded. Prior to recording, the Biopore CM membranes were cut and the adherent culture inverted onto precoated MEAs (with polylysine/laminin as in the preceding text) and held in place with a nylon mesh (Fig. 1B). Cultures were perfused with artificial cerebrospinal fluid (ACSF containing, in mM: 125 NaCl, 3.5 KCl, 26 NaHCO₃, 1.2 KH₂PO₄, 1.3 MgCl₂, 2.4 CaCl₂, and 10 glucose; pH = 7.40), which was bubbled with 95% O₂-5% CO₂ and prewarmed to 37°C. A commercial MEA amplifier and data acquisition system (MEA1060-Inv-BC, MultiChannel Systems) was used for electrophysiology recording. Neural signals were recorded at 20 kHz with a 5-kHz analog, anti-aliasing filter. A programmable stimulator (STG2004, MultiChannel Systems) generated constant current, biphasic stimuli (a positive phase first for 100 μs followed by a negative phase for 100 μs) to evoke responses. Individual electrodes were segregated as to their anatomical location into three groups, CA1, CA3, and DG, for further analysis (Fig. 1C).

Stimulus response (S/R) curves were generated for each culture. Bipolar, biphasic stimuli of varying magnitudes (0–300 μA in 10-μA steps) were applied to the tissue through electrodes located in the mossy fibers of the hilus. Tissue response was recorded from all channels simultaneously, and the amplitude of the response quantified as peak-to-peak amplitude of evoked field potentials (Fig. 1D). In addition, the evoked response amplitude at each electrode was plotted as a function of stimulus intensity (Fig. 2), known as a S/R curve, and then fit to a sigmoid function for statistical comparison of parameters

$$R(S) = R_0 + \frac{R_{\text{max}}}{1 + e^{m(S - S_0)}}$$

where $R_0$ was the background signal artifact, $R_{\text{max}}$ was the maximum response, $S_0$ was the current which produced a half-maximal response, $S$ was the intensity of stimulus, and $m$ was proportional to the
slope of the linear region of the sigmoid. The $I_{50}$ and $R_{max}$ values of the S/R curves from CA1, CA3, and DG regions were analyzed separately. Data are presented as means $\pm$ SE in figures.

Paired-pulse facilitation/depression (PPF/D) ratios were also generated by delivering two successive stimuli of the same intensity ($I_{50}$) with an interstimulus interval of 40 ms. Paired-pulse ratios of field potential amplitudes were calculated by

$$\text{Ratio} = \frac{\text{Amplitude}_{\text{2nd Response}}}{\text{Amplitude}_{\text{1st Response}}}$$

where $\text{Amplitude}_{\text{1st Response}}$ and $\text{Amplitude}_{\text{2nd Response}}$ represented the amplitudes of the field potentials evoked by the two successive stimuli. A ratio $>1$ indicated PPF, whereas a ratio $<1$ indicated PPD.

**Experimental design and statistical analysis**

In this study of pediatric brain injury, age-matched uninjured cultures were used as control groups for functional comparisons with injured cultures based on the $R_{max}$ and $I_{50}$ parameters for each region (CA1, CA3, and DG). For each experimental group, 6–10 slice cultures were generated from a minimum of three rat pups. The following experiments were implemented and analyzed.

1) The development of electrophysiological function over time at 0, 1, 2, and 3 wk in vitro was studied by recording neural activity from the cultures at 2, 8, 18, and 22 days in vitro (DIV), respectively. The effect of culture age was analyzed with ANOVA followed by Bonferroni post hoc tests to determine significant differences between groups.

2) The effect of tissue deformation level was analyzed with ANOVA followed by Bonferroni post hoc tests to determine significant differences in dysfunction among regions (CA1, CA3, and DG). The development of posttraumatic dysfunction during the acute period post mild injury (within the 1st week post injury) was studied in slice cultures that were injured at 16 DIV by 5% biaxial strain. Two time points post injury were chosen at 2 and 6 DPI, and the posttraumatic alteration of neural activity was analyzed with ANOVA to determine differences in function between the two time points post injury.

3) The functional outcome of tissue injured at different developmental states of 0, 1, and 2 wk in vitro was studied by recording neural activity at 2 DPI from cultures injured at 2, 8, and 16 DIV, respectively. Mild injury was generated by applying 5% biaxial strain to hippocampal slice cultures. ANOVA followed by Bonferroni post hoc tests was applied to determine significant differences between injured and age-matched tissue when injured at different culture ages. In addition, the posttraumatic alteration of neural activity was analyzed by ANOVA followed by Bonferroni post hoc tests to determine significant differences in dysfunction among regions (CA1, CA3, and DG). The effect of tissue deformation level was analyzed with ANOVA followed by Bonferroni post hoc tests to determine significant differences in dysfunction when cultures were injured at different ages.

Data in all figures are presented as means $\pm$ SE, with significance denoted as follows: *$P < 0.05$, **$P < 0.01$.

Posttraumatic changes in short-term synaptic plasticity were studied by comparing cumulative probability distributions (CPD) of PPF/D ratios for each region (CA1, CA3, and DG). CPD were calculated from all the recording sites within the corresponding anatomical region of all the tested cultures. Briefly, the CPD indicates the probability that the PPF/D ratio takes on a value less than or equal to an indicated number. The two-sample Kolmogorov-Smirnov test.
(KS test) was used to determine if the CPD from injured cultures were statistically different from age-matched uninjured cultures.

RESULTS

Development of electrophysiological function in vitro

Postnatal hippocampal slices were cultured, and their electrophysiological function was recorded at different days in vitro corresponding to different developmental ages (Fig. 3). In the CA1 region, the $R_{\text{max}}$ value was $768 \pm 82$ (SE) $\mu$V at 2 DIV and then decreased to $610 \pm 33$ $\mu$V at 8 DIV, after which the $R_{\text{max}}$ value increased to $693 \pm 52$ $\mu$V at 18 DIV and $730 \pm 67$ $\mu$V at 22 DIV; this was near to that at 2 DIV. Similarly in the CA3 and DG regions, $R_{\text{max}}$ values decreased from 2 to 8 DIV and then increased during the period between 8 and 22 DIV. These results indicated that the maximum evoked response of hippocampal circuits developed during the first 3 wk in vitro. However, the three anatomic regions displayed different temporal patterns. The CA1 region showed no significant change in $R_{\text{max}}$ or $R_{\text{max}}$ was statistically not dependent on DIV in the CA1 region ($P = 0.130$) during the first week of culture despite a 20% decrease to the minimal value at 8 DIV. By contrast, the CA3 and DG regions showed larger variation in functional development. As shown in Fig. 3A, in the CA3 region, $R_{\text{max}}$ decreased $-62\%$, from $1,111 \pm 179$ $\mu$V at 2 DIV, to the minimal value at 8 DIV and then increased to $1,694 \pm 140$ $\mu$V at 22 DIV, which was $-52\%$ larger than that at 2 DIV. In the DG region, $R_{\text{max}}$ decreased $-80\%$, from $2,124 \pm 163$ $\mu$V at 2 DIV, to the minimal value at 8 DIV and then increased to $923 \pm 73$ $\mu$V at 22 DIV, which was $-57\%$ less than that at 2 DIV.

The value of $I_{50}$ was used to evaluate the excitability of hippocampal circuits (Fig. 3B). In the CA1 region, $I_{50}$ increased $-100\%$, from $39 \pm 2$ $\mu$A at 2 DIV to $79 \pm 2$ $\mu$A at 8 DIV and remained elevated at $80 \pm 2$ $\mu$A until 18 DIV and finally decreased to $45 \pm 2$ $\mu$A at 22 DIV, which was $-15\%$ larger than that at 2 DIV. Similarly for the CA3 and DG regions, $I_{50}$ values increased from 2 to 8 DIV, changed little at 18 DIV, and then decreased at 22 DIV to the level of that at 2 DIV. In the CA3 region, $I_{50}$ increased $-94\%$, from $37 \pm 3$ $\mu$A at 2 DIV to $73 \pm 3$ $\mu$A at 8 DIV and then $72 \pm 4$ $\mu$A at 18 DIV and finally decreased to $42 \pm 2$ $\mu$A at 22 DIV; this was not significantly different from that at 2 DIV. In the DG region, $I_{50}$ increased $-9\%$, from $35 \pm 2$ $\mu$A at 2 DIV to $82 \pm 3$ $\mu$A at 8 DIV and then $81 \pm 5$ $\mu$A at 18 DIV and finally decreased to $37 \pm 2$ $\mu$A at 22 DIV, which was not significantly different from that at 2 DIV. These results indicated that the excitability of hippocampal circuits changed and developed during the first 3 wk in vitro. Moreover, all three anatomic regions displayed a similar pattern for the development of excitability.

Changes in electrophysiological function after in vitro mild injury

The electrophysiological function of hippocampal slice cultures after 5 and 10% biaxial strain was recorded at 4 DPI (Fig. 4). After 5% strain injury, the $R_{\text{max}}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly decreased in the CA1 region by 46 ± 6%, in the CA3 region by 46 ± 7% and in the DG region by 34 ± 8% (Fig. 4A). After 10% strain injury, the $R_{\text{max}}$ value from injured cultures was significantly decreased in the CA1 region by 53 ± 14%, in the CA3 region by 69 ± 19% and in the DG region by 49 ± 14%. Taken together, the maximal evoked response of the hippocampal circuitry was significantly decreased in all three regions after mild injury. The CA3 region appeared to be the most vulnerable to injury at 10% strain level, whereas the DG region appeared to be the least vulnerable to injury-induced deficits in $R_{\text{max}}$ at 5% strain level; however, these differences did not reach statistical significance. Moreover, 10% strain injury resulted in larger posttraumatic alteration in $R_{\text{max}}$, than 5% strain injury, although not significantly, suggesting that deficits in response magnitude might be correlated with tissue strain.

After 5% strain injury, the $I_{50}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly increased in the CA1 region by 70 ± 4%, in the CA3 region by 88 ± 7%, and in the DG region by 87 ± 9% (Fig. 4B). After 10% strain injury, the $I_{50}$ value from injured cultures was increased in the CA1 region by 96 ± 9%, in the CA3 region by 122 ± 17%, and in the DG region by 114 ± 18%. These results indicated that the excitability of hippocampal circuits was significantly decreased in all three regions after mild injury. Overall, the CA3 region was significantly the most vulnerable to injury while the CA1 region was the least. Moreover, 10% strain injury resulted in significantly larger posttraumatic alterations in $I_{50}$ than 5% strain injury, suggest-
ing that deficits in excitability were correlated with injury severity.

Development of electrophysiological dysfunction after in vitro mild injury

Hippocampal slice cultures were injured at 16 DIV by 5% biaxial strain, and their neural activity was recorded at 2 and 6 DPI (Fig. 5). In the CA1 region, the $R_{\text{max}}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly decreased 55 ± 9% at 2 DPI and 43 ± 8% at 6 DPI. In the CA3 region, the $R_{\text{max}}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly decreased 59 ± 12% at 2 DPI and 53 ± 8% at 6 DPI. In the DG region, the $R_{\text{max}}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly decreased 39 ± 9% at 2 DPI and 34 ± 10% at 6 DPI. The posttraumatic deficit in $R_{\text{max}}$ at 6 DPI was significantly different from that at 2 DPI in the CA1 region but not in the CA3 or DG regions.

The excitability of injured hippocampal slices was evaluated by the $I_{50}$ value (Fig. 5B). In the CA1 region, the $I_{50}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly increased 56 ± 4% at 2 DPI and was further increased 130 ± 6% at 6 DPI. In the CA3 region, the $I_{50}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly increased 69 ± 8% at 2 DPI and 142 ± 10% at 6 DPI. In the DG region, the $I_{50}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly increased 45 ± 7% at 2 DPI and 168 ± 12% at 6 DPI. The posttraumatic increase in $I_{50}$ was significantly increased in all three regions from 2 to 6 DPI. These results indicated that the injury-induced reduction in excitability continued to worsen during the acute period after mild injury in contrast to the improving trend for $R_{\text{max}}$.

Critical developmental period in vitro for reduced vulnerability to injury

Hippocampal slice cultures were injured at 2, 8, and 16 DIV, and the neural activity of injured hippocampal slices at 2 DPI was recorded (Fig. 6). In the CA1 region, the $R_{\text{max}}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly increased 56 ± 4% at 2 DPI and was further increased 130 ± 6% at 6 DPI. In the CA3 region, the $R_{\text{max}}$ value from injured cultures, compared with that from age-matched uninjured cultures, was significantly increased 45 ± 7% at 2 DPI and 168 ± 12% at 6 DPI. The posttraumatic increase in $I_{50}$ was significantly increased in all three regions from 2 to 6 DPI. These results indicated that the injury-induced reduction in excitability continued to worsen during the acute period after mild injury in contrast to the improving trend for $R_{\text{max}}$. 

FIG. 5. Percent change in evoked field potentials from cultures injured by 5 or 10% biaxial strains measured at 4 days post injury (DPI) compared with age-matched uninjured cultures. Cultures were injured at 16 DIV. A: percent change in $R_{\text{max}}$ as a function of strain level. The $R_{\text{max}}$ value showed larger decreases after 10% biaxial strain than after 5% strain. B: percent changes in $I_{50}$ as a function of strain level. The $I_{50}$ value showed larger increases after 10% biaxial strain. Inset: full comparisons were made among the 3 groups (0, 5, and 10% biaxial strain) for each region and comparisons were made among the 3 regions (CA1, CA3, and DG) at each injury severity with significance level indicated for each paired comparison (*$P < 0.05$; **$P < 0.01$).

FIG. 6. Percent change in evoked field potentials from cultures injured by 5 or 10% biaxial strains measured at 4 days post injury (DPI) compared with age-matched uninjured cultures. Cultures were injured at 16 DIV. A: percent change in $R_{\text{max}}$ as a function of days post injury. $R_{\text{max}}$ decreased significantly in all 3 regions after injury at both 2 and 6 DPI. The deficit in $R_{\text{max}}$ was reduced at 6 DPI, recovering to some extent. B: percent change in $I_{50}$ as a function of days post injury. The $I_{50}$ value increased significantly in all 3 regions after injury at both 2 and 6 DPI. The $I_{50}$ value showed further increase at 6 DPI, more than that at 2 DPI. Inset: injured cultures were compared with age-matched uninjured cultures in all 3 regions at both 2 and 6 DPI with significance indicated for each paired comparison ($*P < 0.05$; **$P < 0.01$). Moreover the percentage change of injured cultures over age-matched uninjured cultures at 2 DPI was compared with that at 6 DPI in all 3 regions with significance levels indicated for each paired comparison ($*P < 0.05$; **$P < 0.01$).
injured cultures, decreased 70 ± 27% when injured at 2 DIV, decreased 25 ± 5% when injured at 8 DIV, and decreased 56 ± 9% when injured at 16 DIV. In the CA3 region, the $R_{\text{max}}$ value from injured cultures decreased 81 ± 25% when injured at 2 DIV and decreased 59 ± 12% when injured at 16 DIV; however, there was no significant difference between injured and age-matched cultures when injured at 8 DIV. In the DG region, the $R_{\text{max}}$ value from injured cultures was decreased 81 ± 9% when injured at 2 DIV and decreased 39 ± 9% when injured at 16 DIV; however, there was no significant difference between injured and age-matched cultures when injured at 8 DIV. As shown in Fig. 6, in all three regions, there was no significant difference in the percentage change of $R_{\text{max}}$ caused by the injury at 2 and 16 DIV. These results indicated that the posttraumatic changes in evoked response magnitude depended on tissue age in vitro and that 8 DIV cultures might be more resistant to injury than the other ages tested.

Similarly, the posttraumatic changes in excitability depended on tissue age in vitro (Fig. 6B). In the CA1 region, the $I_{E0}$ value from injured cultures, compared with that from age-matched uninjured cultures, increased 29 ± 12% when injured at 2 DIV, decreased 7 ± 2% when injured at 8 DIV, and increased 56 ± 4% when injured at 16 DIV. In the CA3 region, the $I_{E0}$ value was increased 38 ± 13% when injured at 2 DIV and increased 69 ± 8% when injured at 16 DIV; however, there was no significant difference between injured and age-matched cultures when injured at 8 DIV. In the DG region, the $I_{E0}$ value increased 66 ± 11% when injured at 2 DIV and increased 45 ± 7% when injured at 16 DIV; however, there was no significant difference between injured and age-matched cultures when injured at 8 DIV. Overall, no significant changes in excitability were found in the CA3 and DG regions when cultures were injured at 8 DIV; and significantly smaller changes were found in all the three regions when cultures were injured at 8 DIV compared with cultures injured at 2 or 16 DIV. These results indicated that the postransformative changes in excitability depended on tissue age in vitro and that 8 DIV cultures might be more resistant to injury than the other ages tested. There could be a critical developmental window of reduced vulnerability to injury at around 8 DIV.

### Posttraumatic changes in short-term plasticity

The effect of injury on short-term plasticity was tested with a paired pulse paradigm to determine whether synaptic mechanisms were involved in the posttraumatic deficits in electrophysiological function. Paired-pulse responses recorded from injured and age-matched uninjured cultures were compared as shown in Fig. 7. The paired-pulse activity of uninjured cultures was studied by region. As shown in Fig. 7A, each of the three regions exhibited significantly different CPD from the other two regions at 9 DIV. However, at 12 DIV, the CPD for the CA3 region was not significantly different from either CA1 or DG regions, although there was still a significant difference between the CPD for CA1 and DG.

Slice cultures were injured within the critical developmental period that was identified above at 8 DIV by a 5% biaxial strain, and neural activity was recorded at 1 or 4 DPI to generate CPD for paired-pulse ratios (Fig. 7B). For the CA1 and CA3 regions, the CPD for injured cultures was significantly shifted left relative to that for age-matched uninjured cultures at 1 DPI ($P = 0.001$ for both regions), indicating an overall decrease in paired pulse ratios. However, the CPD for injured cultures was significantly shifted right relative to that for age-matched, uninjured cultures at 4 DPI ($P = 0.039$ for CA1 and $P = 0.035$ for CA3). These results suggested that paired-pulse ratios in the CA1 and CA3 regions decreased at 1 DPI, but recovered and even increased to be larger than uninjured level at 4 DPI. In contrast, the CPD for DG responses for injured and uninjured cultures were not significantly dif...
different at either 1 DPI \((P = 0.102)\) or 4 DPI \((P = 0.601)\). Although the temporal changes in CPD were not identical for all three regions, in general, short-term plasticity was initially impaired by injury events and then recovered, to some extent, during the acute recovery phase over 4 days.

**DISCUSSION**

In this work, we studied the neural activity of organotypic hippocampal slice cultures during development in vitro and explored how in vitro mild TBI induced developmental deficits during the acute period after injury. These studies begin to fill a critical gap in understanding quantitatively the effects of mild mechanical stimuli on the function of the developing hippocampus and are the first to demonstrate that very mild mechanical deformation, as low as 5%, can significantly affect hippocampal function. These effects lasted for days after injury, while some spontaneous recovery was measured over the first week postinjury. Our results suggest that what is usually considered mild TBI may in fact have more serious repercussions than previously recognized and may only be evident with sensitive electrophysiological measures.

The hippocampal slice cultures used in our study were isolated from P9 to 10 rats during a period of in vivo process outgrowth and synaptogenesis (Gahwiler 1984; Sharonova and Khaspekov 1982). Previous studies have demonstrated that the in vitro development of organotypic hippocampal slice cultures proceeded with a time course that compared well with that in vivo (Buchs et al. 1993; Muller et al. 1993). The developmental changes in organotypic cultures are not simply a consequence of explanation but reflect some of the developmental processes in vivo. Moreover, the highly organized and laminar arrangement of synaptic pathways makes the organotypic hippocampal slice a convenient in vitro model for studying electrophysiological function (Gahwiler et al. 1997; Sundstrom et al. 2005). The neural circuits of the hippocampus continue to develop in vitro (Collin et al. 1997; Gahwiler 1984), and it has been demonstrated that in vitro neural cultures maintained not only the capacity to propagate neural signals but also a remarkable degree of functional organization resembling neural circuits in vivo (Gahwiler et al. 1997; Kiernan and Pettit 1971), although altered synaptic connections and development processes (Collin et al. 1997) have been previously reported. Therefore the organotypic hippocampal slice culture is a good simplified model to study the TBI-induced functional alteration during development.

Stimulus/response curves were generated and then fit to a sigmoid function for hippocampal slice cultures, and the curve-fitting parameters, \(R_{\max}\) and \(I_{50}\), were determined to quantify electrophysiological function. These parameters are determined by various characteristics of electrophysiological function, including the density of excitable neuronal cells and synapses, the efficacy of synaptic transmission or the balance of modulation between excitatory and inhibitory circuits. However, the maximum response, \(R_{\max}\), was found to closely correlate with synaptic contact density during development (Buchs et al. 1993; Muller et al. 1993), suggesting that the value of \(R_{\max}\) represented a good approximation of the total number of functional synaptic contacts. \(I_{50}\) was the stimulus intensity to evoke a half-maximal response, reflecting the synaptic excitability of neural circuitry. Because the densities and the distribution of spine, shaft, and sessile synapses varies in the hippocampus (Buchs et al. 1993), the total number of

![FIG. 7. Plots of cumulative probability distributions (CPD) for paired-pulse ratios presented by region.](image-url)
synaptic contacts, represented by $R_{\text{max}}$, is different from location to location even within the same anatomic regions of CA1, CA3, or DG. Therefore the average of the $R_{\text{max}}$ measured from multiple locations, rather than from one chosen location, may be more representative of the overall density and distribution of the synaptic contacts within one anatomic region. A major advantage of MEA technology is that stimulus/response curves can be generated from multiple electrodes within multiple anatomic locations at the same time (Cater et al. 2007; Shimono et al. 2002) to provide an unbiased estimation of the overall density of synaptic contacts in CA1, CA3, and DG regions as in the current study.

Children who suffer mild TBI usually present transient symptoms that recover spontaneously, such as a loss of consciousness lasting <30 min (Annegers et al. 1998). It is generally believed that mild pediatric TBI causes no severe cell loss that might result in long-term deficits in brain function but produces only a temporary functional disturbance (Fay et al. 1993). In experimental models of mild TBI, no overt cell death has been reported in young animals, and no significant cell death has been reported during the acute period following in vitro experimental mild injury corresponding to <10% biaxial deformation (Cater et al. 2006; Elkin and Morrison 2007). However, our current results demonstrate that the cellular/network function in the hippocampus was altered during the first week after in vitro, experimental mild injury. The synaptic transmission and excitability, quantified by $R_{\text{max}}$ and $I_{50}$, were found to be significantly impaired after injury of 10% or even as little as 5% biaxial deformation. Such a low mechanical threshold to impairment of hippocampal function has not been reported previously.

Previous studies found a significant reduction in both NMDA and AMPA currents in hippocampal slices after in vivo mild experimental TBI (Schwarzbach et al. 2006) and an increase in GABA-elicited currents after in vitro mild experimental TBI (Kao et al. 2004), although the latter study used much larger strains than we used. These alterations of glutamate and GABA receptors following mild TBI may cause an imbalance between excitatory and inhibitory synaptic modulation within hippocampal circuitry, leading to posttraumatic depression of synaptic transmission. In our study, there was no significant difference in the impairment of synaptic transmission between the two injury severities; but the decrease in excitability did correlate with injury severity, being larger after 10% deformation as compared with after 5% deformation. Moreover, the posttraumatic alteration of electrophysiological function developed during the first week after injury. The deficit in synaptic transmission recovered during the period from 2 to 6 DPI, to some extent, although the recovery was not significantly different between the injury severities. However, the deficit in excitability significantly worsened during the same 4-day period. Our findings of the acute response to mild experimental TBI in pediatric tissue may give important insights into how posttraumatic pathology develops during the acute period after mild injury.

Previous in vivo and in vitro studies suggested that synaptic development in the hippocampus continues during the first 3–4 wk after birth and becomes stable after that period (Muller et al. 1993; Pokorny and Yamamoto 1981a,b; Xiang et al. 2000). We harvested slice cultures from P9 to 10 rats, which were cultured in vitro for an additional 3 wk spanning a period when synaptic connections are undergoing substantial developmental changes. Our results indicated that $R_{\text{max}}$ and $I_{50}$ developed along a U-shaped curve during the first 3 wk in culture with a transient decrease in both maximum evoked responses and excitability around 8 DIV. Similar results have been reported by other groups (Muller et al. 1993; Xiang et al. 2000), suggesting a transient synaptic depression in hippocampal slice cultures during the first several days after dissection followed by recovery. The transient depression in neural activity during the first week of culture is mainly caused by the reduced density of synapses immediately after dissection that then increases to age-matched in vivo levels after a couple of weeks in culture (Buchs et al. 1993). Cell bodies and processes were transected and damaged during the dissection procedure (Fiala et al. 2003), such that some neurons and neuroglial died. In response, neurites and synapses may also undergo degenerative changes (Kiernan and Pettit 1971). Previous studies have found that around half of the axons in hippocampus cultures degenerate during the first 4 DIV; however, degenerated cells were rarely seen after 6 DIV, such that slices contained only relatively healthy-looking cells and little basophilic granular debris from dead cells by 6 DIV (Kiernan and Pettit 1971). These results demonstrated that the electrophysiological function of neonatal hippocampal slice cultures continue developing in vitro, highlighting the absolute need for age-matched controls to quantify trauma-induced effects.

Although the developing brain exhibits enhanced plasticity compared with the adult brain (Muller et al. 1993; Pokorny and Yamamoto 1981a,b), paradoxically, the developing brain has been found to be more vulnerable to apoptotic neuronal death after TBI (Bittigau et al. 1999). One interesting finding in the field of pediatric TBI is a discontinuity between age and outcome after severe TBI in infants, children and adolescents (Levin et al. 1992). In a Traumatic Coma Data Bank report, children between the ages of 5 and 10 had the best outcomes, in fact better than both adolescents and adults, indicating a critical developmental period with less vulnerability to TBI (Levin et al. 1992). The “optimal” age window appears to vary in different species. A critical developmental period with optimal postsinsult recovery at around P30 has been observed in cats (Villablanca et al. 1998) and at around P17 day in rats (Fineman et al. 2000; Raghupathi and Huh 2007). Similarly in our study, a critical time window of in vitro development was found at around 8 DIV, during which hippocampal slice cultures were less vulnerable to injury responding with reduced deficits in the maximal evoked response and excitability. The cultures for our study were dissected from P9- to 10-day rat pups with an in vitro development period of 8 days for a combined age of 17–18 days. Our results are consistent with the previous findings because slice cultures of the CNS have been reported to continue the developmental process in vitro similarly to the in vivo brain (Collin et al. 1997; Kiernan and Pettit 1971), although temporal deviations from the in vivo developmental process have been reported (Collin et al. 1997).

Because neuronal processes were transected during the preparation of the slice cultures, which then went on to degenerate (Fiala et al. 2003), the influence of this neuronal degeneration must be considered together with intrinsic neuronal plasticity when studying the development of hippocampal slice cultures and their ability to resist mechanical injury. On one hand, as reviewed by Prins and Hovda (2003), a critical developmental window may exist during the period of greatest neuronal
plasticity and therefore potential recovery, which could alleviate posttraumatic degeneration of neural circuits and restore hippocampal organization and development. Consistent with the in vivo findings identifying a critical developmental period with maximal neuronal plasticity and optimal posts insult recovery at P17 (Fineman et al. 2000; Raghupathi and Huh 2007), the window of greatest intrinsic neuronal plasticity is expected during the period of postnatal 15–24 days; thus 8 DIV for the cultures which were harvested from P9- to 10-day rat pups might be expected to be within the period of greatest intrinsic neuronal plasticity as well. On the other hand, taking into account the external effect of dissection and in vitro culture, the degeneration due to dissection may be complete by 8 DIV, as dissection-induced, degenerated neuronal cells were rarely seen after 6 DIV in other studies (Kiernan and Pettit 1971). Therefore the slice cultures may be maximally resistant to mechanical injury around 8 DIV due to a combination of maximal intrinsic neuronal plasticity and minimal dissection-induced degeneration. Such a developmental window of reduced vulnerability is consistent with our findings that the cultures at 8 DIV were more resistant to injury than those at 2 and 16 DIV.

It has been demonstrated that mild TBI can disrupt the fine structure of the hippocampus, contributing to the alteration of synaptic plasticity (Grady et al. 2003; Lowenstein et al. 1992). Short-term plasticity was studied in this work by analyzing paired-pulse paradigms, which provide insight into the functionality of synaptic transmission, particularly of the synaptic machinery (Thomson 2000). In our studies, as indicated by a leftward-shift of the CPD for injured cultures, paired-pulse ratios were significantly decreased with some PPF becoming PPD in CA1 and CA3 at 1 DPI. Previous studies have found that paired-pulse ratios in the CA1 region could be either suppressed (Cao et al. 2006) or elevated (Reeves et al. 2000) after experimental TBI with changes persistent for a week post injury (Reeves et al. 2000). The difference between previous findings and our results could be due to several possible reasons. First, different injury models and tissue age could lead to induction of different pathological cascades. Those studies utilized the fluid percussion injury model of TBI in adult animals, whereas we used a biaxial deformation, in vitro model with developing hippocampal slice cultures. Second, the paired-pulse ratio in the CA1 region was evoked by stimuli applied to mossy fibers, thus the CA1 responses in our study were disynaptic in nature. Previous work suggests that short-term plasticity of monosynaptic transmission may be different from that involving polysynaptic transmission (Sirvio et al. 1996). In addition, previous TBI studies performed single electrode, local recordings, whereas we took advantage of MEA recordings using as many as 60 electrodes simultaneously to examine function throughout the whole hippocampus. With our methodology, we were able to determine the cumulative distribution of paired-pulse ratios rather than single values. Our methods capture a much larger sample and provide a better representation of the true population and range of synaptic changes. Given our results and the nature of the distribution from PPD to PPF, it is understandable how the other studies could measure either PPF suppression or elevation in the same injury model due to low sample number. As shown in Fig. 7, PPF and PPD could occur simultaneously in the CA1 region, depending on different detailed anatomic locations, the culture age, and injury status.

An interesting finding of our study is that deficits in electrophysiological function after mild injury were region-specific. When injured at 8 DIV, the maximal evoked response from the DG region was not altered (~4.1% change), whereas the responses from the CA1 and CA3 regions were altered by >20% at 2 days after a 5% deformation injury (Fig. 6A). Moreover, synaptic function within the DG region was not significantly changed during the first 4 DPI, while short-term plasticity within the CA1 and CA3 regions was significantly decreased at 1 DPI, recovering by 4 DPI (Fig. 7). These results suggest that the DG was less functionally vulnerable to mild TBI than the CA1 or CA3 regions. This regional susceptibility is consistent with our previous report of less cell death in the DG at larger deformations compared with the CA1 and CA3 (Cater et al. 2006; Elkin et al. 2007). Additionally, this region-specific vulnerability to TBI may also reflect the different recovery capacities and rates of synaptic growth which vary within the same slice cultures from one area to the other depending on the type of cells and synaptic contacts (Buchs et al. 1993).

The main three regions of the hippocampus, CA1, CA3, and DG, are well organized in the hippocampus slice cultures, maintaining the tri-synaptic circuit (Amaral 1993; Amaral and Witter 1989). In our studies, stimuli were applied to mossy fibers, and as a result, the CA3 pyramidal neurons were activated through monosynaptic connections. In turn, the CA1 pyramidal neurons were activated through disynaptic connections, whereas the DG granule cells were activated through antidromic stimulation. Entorhinal cortex was not maintained in our cultures, therefore stimulation through mossy fibers was used as an alternative to activate the whole hippocampus. The S/R curves and paired-pulse ratios of polysynaptic responses have been used to study electrophysiological function of the hippocampus previously (Cater et al. 2007; Sirvio et al. 1996). By leveraging the advantages of the MEA technology, we were able to simultaneously record the neural activity of all three regions after stimuli were applied through the mossy fiber pathway, instead of focusing on the signal transmission in each region individually. Although propagation of signals through the trisynaptic circuit may approximate the natural spread of signals in vivo, the involvement of polysynaptic and antidromic responses of the three regions could contribute to the region-specific, electrophysiological function that we found (Sirvio et al. 1996). For instance, the unchanging paired-pulse ratio in the DG region after injury could be explained because the DG granule cells were more resistant to mechanical injuries than other regions or because the antidromic stimulation did not involve synaptic transmission. In addition, the contribution of recurrent connections including feedforward and feedback connections, which have been documented within hippocampal slice cultures, is difficult to determine (Amaral 1993; Amaral and Witter 1989). Therefore interpreting the holistic signal transmission through the hippocampus should be done with caution.

Hippocampal slice cultures were cultured and injured on a Biopore CM/silicone membrane complex and then were moved onto MEAs for recording. It was not possible to injure cultures on MEAs because they are rigid, being fabricated on glass, and cannot deform to induce injury. As such, we were unable to record from the same cultures before and after injury. Instead functional measures within injured cultures were compared with age-matched uninjured cultures as controls. Therefore different slice cultures were compared when quantifying changes in functional outcome after injury; this was a limitation of this study. Inherent variability in the organization of
neural circuits within individual slices combined with variation in slice preparation and in vitro development could result in a large variation in measured function. A substrate-embedded MEA compatible with the stretch injury model could overcome this limitation. To this end, we have been developing a stretchable microelectrode array (SMEA) with the capability of monitoring neural activity from the same locations within a given culture pre- and postinjury, allowing for normalization of posttraumatic changes to preinjury values (Graudejus et al. 2009; Yu et al. 2009).

In summary, we examined the neural activity of hippocampal cultures during development in vitro and explored how in vitro mild TBI induced region-specific functional deficits. Our results demonstrated that experimental mild TBI induced functional deficits in hippocampal slice cultures and that functional outcomes were dependent on injury severity. Posttraumatic outcome varied with tissue age (developmental state) at injury, and our results suggest that a critical developmental period may exist for optimal postinjury recovery in the slice cultures at around 8 DIV. After the injury event, electrophysiological function spontaneously recovered to a limited degree during the first week after mild injury. In addition, posttraumatic changes in function were region-dependent, and the DG was more resistant to injury than the CA1 or CA3 regions. Our work provides an important foundation for future studies of TBI during development.

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