Regionally localized recurrent excitation in the dentate gyrus of a cortical contusion model of posttraumatic epilepsy

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Abstract

Posttraumatic epilepsy is a frequent consequence of brain trauma, but relatively little is known about how neuronal circuits are chronically altered after closed head injury. We examined whether local recurrent excitatory synaptic connections form between dentate granule cells in mice 8-12 weeks after cortical contusion injury. Mice were monitored for behavioral seizures shortly after brain injury and up to 10 weeks post-injury. Injury-induced seizures were observed in 15% of mice, and spontaneous seizures were observed weeks later in 40% of mice. Timm’s staining revealed mossy fiber sprouting into the inner molecular layer of the dorsal dentate gyrus ipsilateral to the injury in 95% of mice, but not contralateral to the injury or in uninjured controls. Whole-cell patch-clamp recordings were made from granule cells in isolated hippocampal brain slices. Cells in slices with posttraumatic mossy fiber sprouting had an increased EPSC frequency compared to cells in slices without sprouting from injured and control animals (p<0.001). When perfused with Mg\(^{2+}\)-free ACSF containing 100µM picrotoxin these cells had spontaneous bursts of EPSCs and action potentials. Focal glutamate photostimulation of the granule cell layer evoked a burst of EPSCs and action potentials indicative of recurrent excitatory connections in granule cells of slices with mossy fiber sprouting. In granule cells of slices without sprouting from injured animals and controls, spontaneous or photostimulation-evoked epileptiform activity was never observed. These results suggest that a new regionally localized excitatory network forms between dentate granule cells near the injury site within weeks after cortical contusion head injury.
Introduction

Traumatic brain injury (TBI) is accompanied by a long-lasting increase in the risk for developing posttraumatic epilepsy (PTE) (Caveness et al., 1979; Annegers et al., 1998; Englander et al., 2003). However, treatment options for preventing or suppressing chronic seizures after head injury are limited and have been largely unsuccessful (Temkin, 2009). Understanding basic mechanisms of posttraumatic epileptogenesis after experimental TBI, in comparison to other preclinical models, will provide important information necessary for developing cellular based therapeutic approaches for PTE.

In patients and experimental models of temporal lobe epilepsy, the generation of epileptic activity is associated with axon sprouting and reorganization of neuronal circuitry (Taufk and Nadler, 1985; Sutula et al., 1989; Dudek and Spitz, 1997; Buckmaster et al., 2002; Shibley and Smith, 2002; Hunt et al., 2009). The dentate gyrus, which is particularly susceptible to injury, often undergoes structural reorganization, and it is a widely used model system for studying altered synaptic circuitry in epilepsy. Several studies suggest that dentate granule cells, which are not normally interconnected, sprout axon collaterals into the inner molecular layer (i.e., mossy fiber sprouting) to form functional recurrent excitatory connections with nearby granule cells during epileptogenesis and may contribute to network synchronization (Cronin and Dudek, 1988; Cronin et al., 1992; Wuarin and Dudek, 1996, 2001; Lynch and Sutula, 2000; Winokur et al., 2004; Hunt et al., 2009). Mossy fiber sprouting has been reported weeks to months after experimental traumatic brain injury (TBI) in rodents (Santhakumar et al., 2001; Golarai et al., 2001; Kharatishvili et al., 2006; Hunt et al., 2009) and in temporal lobe epilepsy patients with a history of head injury (Swartz et al., 2006), but the
The degree of mossy fiber sprouting after experimental TBI is qualitatively less than the robust, bilateral sprouting observed weeks after experimental status epilepticus. While less widespread axon reorganization is a more typical representation of the clinical setting, computational models have suggested that synchronous network activity may only occur if robust recurrent synaptic connections are present (Traub and Wong, 1981; 1982). Studies using extracellular field recordings to examine network excitability in the dentate gyrus after experimental head injury have not consistently demonstrated epileptiform activity after TBI (Reeves et al., 1997; Santhumakar et al., 2001; Golarai et al., 2001; Hunt et al., 2009). Moreover, Santhakumar and colleagues (2001) reported a recovery within a month after fluid percussion injury from an early increase in extracellular excitability of the granule cell layer that may be related to mossy fiber sprouting. Therefore, mossy fiber sprouting may play a different functional role in the dentate gyrus after mechanical injury than in pharmacologically induced temporal lobe epilepsy models. The persistence of recurrent excitatory connections between granule cells after TBI has not been well established. Understanding how synaptic circuit reorganization may contribute to seizures or chronic changes in excitability after TBI should help to elucidate the importance of these cellular mechanisms in PTE.

Here, we performed controlled cortical impact (CCI) injury to test the hypothesis that mossy fiber sprouting forms an excitatory feedback circuit between granule cells after head injury. We specifically focused on three main questions: (1) can an increase in excitatory synaptic input onto individual granule cells be detected after injury; (2) do
granule cells in slices with posttraumatic mossy fiber sprouting exhibit spontaneous
epileptiform activity; and (3) can excitatory synaptic events be elicited by local glutamate
photostimulation at distant locations within the granule cell layer?

Methods

Animals. Seven to eight week old adult male CD-1 mice (Harlan) weighing 28-35g were
housed under a normal 12h/12h light/dark cycle. Water and food were available ad
libitum. Mice were housed for a minimum of 7d prior to experimentation, and all
procedures were first approved by the University of Kentucky Animal Care and Use
Committee.

Head injury. Thirty-three mice were subjected to a severe unilateral cortical contusion by
controlled cortical impact (CCI) injury as previously described (Scheff et al., 1997; Hunt
et al, 2009). Briefly, mice were anesthetized by 2% isoflurane inhalation and placed in a
stereotaxic frame. The skull was exposed by a midline incision, and a 4mm craniotomy
was made lateral to the sagittal suture and centered between bregma and lambda. The
skull cap was removed without damage to the exposed underlying dura. The contusion
device consisted of a computer controlled, pneumatically driven impactor fitted with a
beveled stainless steel tip 3mm in diameter (Precision Systems and Instrumentation,
Fairfax, VA). Brain injury was delivered using this device to compress the cortex to a
depth of 1.0mm at a velocity of 3.5m/sec and 400ms duration. Surgiseal (Johnson &
Johnson, Arlington, TX) was placed over the dura after injury, the incision sutured, and
the animal was allowed to recover.
Seizure monitoring. Injured animals were monitored for immediate seizures (i.e., injury-induced) during a 90 minute interval that began ~90 minutes after CCI injury. We chose this time for practical reasons (i.e., to allow animals to fully recover from anesthesia and surgery) and based on a previous report that behavioral manifestations are not observed until at least 1h post-CCI (Kochanek et al., 2006). Control and injured mice were subsequently observed 4-6 hr/wk for spontaneous seizures during random 1–2h intervals until 10 weeks post-injury. Observation periods occurred during the light phase of the light/dark cycle, and seizures were rated from 1 to 5, with 5 being the most severe, according to a modified Racine scale (Racine, 1972; Shibley and Smith, 2002). To minimize subjectivity in seizure assessment, category one seizures (i.e. facial automatisms, increased grooming behaviors) were excluded from analysis. Posttraumatic seizures were classified based on the time post-injury in which they occurred: immediate, first 90 minute monitoring session; early, 1 - 7d post-injury; and late, > 7d after injury.

Slice preparation. Mice were deeply anesthetized by isoflurane inhalation and decapitated. The brain was removed and immersed in ice cold (2–4 °C) oxygenated artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 3 KCl, 1.3 CaCl₂, 26 NaHCO₃, 1.3 MgCl₂, 1.25 NaH₂PO₄ and equilibrated with 95% O₂–5% CO₂ (pH 7.2–7.4) with an osmalality of 290-305 mOsm/kg. Brains were blocked, glued to a sectioning stage, and 400 μm thick coronal slices were cut in cold, oxygenated ACSF using a vibrating microtome (Vibratome Series 1000; Technical Products International, St. Louis, MO). The hippocampus was isolated from all surrounding tissue, making sure to completely remove the entorhinal cortex. Slices were transferred to a storage chamber
containing oxygenated ACSF at 34–36 °C and their order maintained so that the location relative to the injury within each hippocampus was known. Some experiments were performed in Mg\(^{2+}\)-free ACSF containing 100µM picrotoxin (PTX) (see results, Figures 4-7).

Whole-cell patch-clamp electrophysiology. After an equilibration period of at least 1h, slices were transferred to a recording chamber on an upright, fixed-stage microscope equipped with infrared, differential interference contrast optics (Olympus BX50WI). Whole-cell patch-clamp recordings were performed on visually identified granule cells. Patch pipettes were pulled from borosilicate glass (1.65 mm outer diameter and 0.45 mm wall thickness, King Precision Glass, Claremont, CA) with a P-87 puller (Sutter Instruments). The intracellular solution contained (in mM): 140 K\(^+\) gluconate, 1 NaCl, 5 EGTA, 10 HEPES, 1 MgCl\(_2\), 1 CaCl\(_2\), 3 KOH, 2–4 ATP, and 0.2% biocytin (pH 7.15 – 7.3). Open tip resistance was 4-7 MΩ. Recordings were obtained with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 2-5 kHz, digitized at 88 kHz (Neuro-corder; Cygnus Technology, Delaware Water Gap, PA), and recorded to pClamp 10.2 (Clampfit, Axon Instruments). Once in whole-cell configuration, cells were voltage-clamped for ~5 min at -70mV (i.e., near resting membrane potential) to allow equilibration of intracellular and recording pipette contents. Resting membrane potential was determined by temporarily removing the voltage clamp and monitoring voltage. Spontaneous and evoked excitatory postsynaptic currents (sEPSCs and eEPSCs) were examined at a holding potential of –70 mV. Current-clamp (i.e., voltage) recordings were performed at resting membrane potential. Series resistance was monitored throughout the
recordings, and data was only used for analysis if the series resistance remained less than 25 MΩ and changed by ≤ 20% during the recordings.

Glutamate photostimulation. Slices were perfused with γ-(carboxy-2-nitrobenzyl) ester, trifluoroacetic acid salt (i.e., CNB-caged glutamate, 250µM; Molecular Probes, Eugene, OR) added to recirculating Mg²⁺-free ACSF and 100µM PTX. Brief pulses of fluorescent light (30ms exposure, UV filter, Chroma Technology, Rockingham, VT) were directed into the slice through the 40x objective. The objective was initially positioned to “uncage” glutamate directly over the recorded cell. This consistently resulted in a large inward current in voltage-clamp or a large depolarization in current clamp with three to six superimposed action potentials in all cells (n=13). The objective was then moved away from the recorded cell by manually moving the microscope until a direct inward current after stimulation was no longer observed to establish the effective radius of stimulation (<50 µm). Photostimulation was applied to sites along the entire extent of the granule cell layer and in the hilus and CA3 region. A series of at least five stimuli were applied per stimulation location at 0.1Hz stimulation frequency.

Timm’s histochemistry. Coronal hippocampal slices used for recordings were placed in 0.37% sodium sulfide solution in 0.1M NaHPO₄ for 20 min, followed by 4% paraformaldehyde in 0.15M phosphate buffer overnight to fix the slices. Slices were then rinsed three times with phosphate buffered saline (PBS; 0.01 M; pH 7.4) and placed in a 30% sucrose solution in PBS overnight or until they sank for cryoprotection. The slices were sectioned at 20µm on a cryostat, rinsed, mounted on charged slides (Superfrost Plus;
Fisher Scientific), and dried overnight. Sections were treated according to previous protocols using Timm’s stain to reveal mossy fibers and Nissl counterstained with cresyl violet to visualize cell bodies (Tauck and Nadler, 1985; Shibley and Smith, 2002; Hunt et al., 2009). To semi-quantitatively assess the regional distribution of mossy fiber sprouting after CCI, sections from the ipsilateral and contralateral hemispheres were examined by an investigator who was blind to the electrophysiological outcomes. Scores for sprouting were assigned based on the following scale of Tauck and Nadler (1985): 0, little to no Timm granules in the granule cell layer; 1, mild staining in the granule cell layer but not the inner molecular layer; 2, moderate continuous staining through the granule cell layer with discontinuous, punctuate staining in the inner molecular layer; and 3, continuous band of dense staining throughout the inner molecular layer. At least three sections from each slice were examined and the median score reported if variability between sections existed. Because Timm’s staining between the blades of the granule cell layer was almost always asymmetric, Timm scores were assigned based on the region of the granule cell layer in which the recorded cell was obtained (i.e., inner blade, apex, or outer blade). Regions of the dentate gyrus with Timm scores >1 were considered to have an abnormal degree of mossy fiber sprouting (Patrylo and Dudek, 1998; Shibley and Smith, 2002; Hunt et al., 2009). The degree of structural damage to the dentate gyrus was analyzed at the injury site in the same Timm scored sections by switching the filter from bright field to fluorescence. Cresyl violet emits red fluorescence under a TRITC filter (Alvarez-Buylla et al., 1990).
Data analysis and statistics. Data analysis was performed using pClamp 10.2 (Clampfit, Axon Instruments), MiniAnalysis 6.0 (Synaptosoft, Leonia, NJ), and Instat (GraphPad software, San Diego, CA) programs. A two minute sample recording per cell was used for measuring sEPSC frequency and amplitude. Events characterized by a typical fast rising phase and exponential decay phase were manually detected using MiniAnalysis, and only currents with amplitudes greater than three times the root mean square (RMS) noise level were included for analysis. Event frequency and mean amplitude were averaged across neurons (i.e., n= neurons) and groups were compared by one-way ANOVA followed by a Tukey’s post hoc test. Spearman rank correlation was used for the nonparametric assessment of the relationship between Timm score and EPSC frequency. To analyze differences in spontaneous epileptiform burst responses, two-tailed Fisher’s exact test was used to compare cells in slices with mossy fiber sprouting versus slices without sprouting. For photostimulation experiments, EPSC frequency was analyzed every 100ms for 500ms prior to and after each stimulation trial and averaged for each stimulus location (i.e., n= stimulation sites). A response at a given location was considered to be positive (i.e., a local synaptic connection was evoked) if the following criteria were satisfied: 1) The number of EPSCs in at least one of the first three 100ms segments after stimulation was greater than the mean number of events per 100ms prior to stimulation + 3SD. 2) A response was observed in at least 3 trials with relatively consistent delay between stimulation and response, demonstrating that the response was repeatable. Responses with < 10ms latency between photostimulation and response were considered to be direct (i.e., due to postsynaptic activation of the recorded neuron) and were excluded from further analysis such that it would interfere with detection of synaptic responses.
(Calloway and Katz, 1993; Schubert et al., 2001). All stimulus locations in which the selection criteria were not met were considered to have a negative response. The number of eEPSCs for each stimulation site was calculated by subtracting the number of EPSCs in the first 300ms before stimulation from the number of EPSCs in the first 300ms after stimulation. We chose this relatively short time window to limit confounding effects of polysynaptic activation and because we previously found that field potential bursts indicative of recurrent excitatory connections between granule cells occur within the first 300ms after an initial antidromically evoked population spike (Hunt et al., 2009). Data are expressed as mean ± SD, and significance was set at P < 0.05.

Results

Posttraumatic seizures

Mice were monitored for behavioral seizures from the time of injury until 10 weeks post-injury. During the first 90 minute monitoring period following injury, 5 of 33 mice (15%) were observed to have at least one injury-induced seizure. The majority of these mice had only one or two seizures; one mouse had three category 3-5 seizures during this period. One mouse (3%) that did not have immediate seizures had a category 3 seizure at 3d post-injury (i.e., early seizure). Spontaneous category 2-4 seizures were observed in 12 of 30 mice (40%), and category 3-4 seizures were observed in 4 of 30 mice (13%) (Figure 1). The average latency from injury to first observed spontaneous seizure was 6.5 ± 1.3 weeks after injury. This latent period is considered to be an
estimate, since we did not monitor continuously. Of the 5 mice that had immediate seizures, 3 (60%) were observed to have spontaneous seizures.

279  *Injury-induced mossy fiber reorganization*

Coronal brain sections of the dorsal hippocampus used for recordings in 21 mice were examined for gross anatomical damage in the hippocampus and mossy fiber sprouting at the injury site. Damage through the entire depth of the cortex directly below the impact site and hippocampal distortion were observed in all injured mice examined 8-12 weeks post-TBI, as previously described (Tong et al., 2002; Hall et al., 2005; Saatman et al., 2006). In 20 of 21 mice (95%), the cortical cavitation at the injury site extended into the hippocampus and a separation of the dorsal and ventral blades of the granule cell layer was visible. Mossy fiber sprouting was observed in sections ipsilateral to the injury in all 20 mice. One mouse had hippocampal and granule cell layer distortion, but cavitation was restricted to the cortex. Mossy fiber sprouting was not present in this mouse. Figure 2 shows the range of lesion to the dentate gyrus and degree of mossy fiber sprouting under the injury. No gross structural damage was observed in any section contralateral to the injury (n= 21) or in control mice (n= 15 animals), and these sections were also devoid of abnormal mossy fiber organization (i.e., all Timm’s scores ≤ 1).

294  *Excitatory input to dentate granule cells*

To analyze whether excitatory synaptic input to dentate granule cells was increased after CCI injury, whole-cell voltage-clamp recordings of sEPSCs were obtained from granule cells in four treatment groups based on post hoc identification of mossy
fiber sprouting: 1) ipsilateral slices with mossy fiber sprouting (n= 23 cells in 20 slices from 13 animals), 2) ipsilateral slices without mossy fiber sprouting (n= 22 cells in 18 slices from 14 animals), contralateral slices (n= 9 cells in 8 slices from 7 animals), and uninjured controls (n= 10 cells in 9 slices from 6 animals). Recordings were made from cells in the apex or outer blade regions of the granule cell layer where Timm’s staining was typically most robust. The average resting membrane potential for these cells was: controls, -75 ± 3.8 mV; contralateral, -76 ± 2.9 mV; ipsilateral with no MFS, -75 ± 5.3 mV; and ipsilateral with MFS, -79 ± 4.4 mV. These values are consistent with previously reported ranges of -65mV to -85mV (Fricke and Prince, 1984; Staley et al., 1992; Wuarin and Dudek, 1996), and one-way ANOVA found no difference between groups (F(3,64)=2.03, P >0.05).

Spontaneous EPSCs were recorded at a holding potential of -70mV. At this potential, outward currents were not typically present. Application of 1mM kynurenic acid blocked inward events, indicating that these currents were mediated by glutamate receptors. Representative recordings for each group are shown in Figure 3A-D. As shown in table 1, EPSC kinetics (i.e., 10-90% rise time and decay time constant) were comparable to previous reports (Keller et al., 1991; Staley and Mody, 1992), and significant differences were not observed between groups (P>0.05). A significant difference in the mean event frequency between treatment groups was detected by one-way ANOVA (control: 0.64 ± 0.3Hz, contralateral: 0.66 ± 0.4Hz, ipsilateral without MFS: 0.65 ± 0.4Hz, ipsilateral with MFS: 2.2 ± 0.9Hz; F(3,64)= 27.1, P<0.001; Figure 3E). Post hoc comparisons revealed that granule cells from ipsilateral slices with mossy fiber sprouting had a significantly higher frequency of sEPSCs versus other groups. To
determine whether EPSC frequency was associated with the density of Timm’s staining, a Timm score was obtained for each slice in which a recording was obtained. The average sEPSC frequency for each cell in slices ipsilateral to the injury was plotted as a function of Timm score (Figure 3F), and a Spearman’s rank correlation analysis indicated a significant positive relationship between EPSC frequency and Timm’s score ($r_s = 0.82$, $P < 0.001$). This is consistent with previous reports that suggest a relationship between EPSC frequency and degree of mossy fiber sprouting in kainate treated rats (Wuarin and Dudek, 2001). The mean amplitude of sEPSCs for each treatment group was: control, $-7.4 \pm 3.0\text{pA}$; contralateral, $-6.8 \pm 1.7\text{pA}$; ipsilateral without MFS, $-7.1 \pm 1.7\text{pA}$; and ipsilateral with MFS, $-9.4 \pm 5.4\text{pA}$. One-way ANOVA did not indicate significant differences between groups ($F_{(3, 64)} = 1.9$, $P > 0.05$; Figure 3G).

**Spontaneous epileptiform burst activity in slices with MFS**

When surgically isolated from afferent inputs, dentate granule cells normally do not fire spontaneous bursts of action potentials at resting membrane potential, even when inhibition is depressed (Fricke and Prince, 1984; Cronin et al., 1992, Wuarin and Dudek, 1996). However, in conditions of reduced inhibition and increased excitability, spontaneous burst responses can be observed in slices from kainate-treated rats with mossy fiber sprouting, but not in controls (Cronin et al., 1992). We tested whether spontaneous epileptiform burst activity occurred in granule cells after injury. Slices were perfused with Mg$^{2+}$-free ACSF containing 100μM PTX to unmask NMDA receptor-mediated excitatory synapses and block GABA$\_A$-mediated inhibition. Recordings were first obtained from granule cells in voltage-clamp at a holding potential of -70mV.
Spontaneous bursts of repetitive or compound EPSCs were never observed in 42 granule cells in slices from injured and control slices without mossy fiber sprouting (n= 5, control; n= 14, contralateral; and n= 23, ipsilateral without mossy fiber sprouting). In contrast, 34 of 51 cells (67%) in 34 slices with mossy fiber sprouting from 19 injured animals had spontaneous bursts of large amplitude compound EPSCs (Figure 4). This difference was found to be significant (P<0.001; Fisher’s exact test). Some of the cells examined for EPSCs were also examined for spontaneous epileptiform bursts of action potentials by switching to current-clamp mode and recording activity at resting membrane potential. Action potentials were not observed in any of 17 cells from slices without mossy fiber sprouting from injured and control animals (n=5, control; n= 5, contralateral; and n=7, ipsilateral without mossy fiber sprouting). In contrast, 65% of cells in slices with mossy fiber sprouting displayed bursts of action potentials (n= 17 of 26, Figure 5). This difference was statistically significant (P<0.001; Fisher’s exact test). Action potential bursts were only observed in cells that also had bursts of EPSCs. Spontaneous burst activity was therefore only observed in granule cells of slices with mossy fiber sprouting from injured animals and was never observed in slices without mossy fiber sprouting from control or injured animals.

Excitatory synaptic connections between granule cells after CCI

Localized glutamate photostimulation has been used to elicit responses that reflect synaptic connections between granule cells in slices with mossy fiber sprouting after pilocarpine or kainate treatment (Wuarin and Dudek 1996, 2001; Lynch and Sutula, 2000; Winokur et al., 2004). We used glutamate photolysis to test the hypothesis that
synaptic connections between granule cells can be evoked in slices with mossy fiber sprouting ipsilateral to the injury and not in the contralateral dentate gyrus where mossy fiber sprouting was absent. The presence (or absence) of synaptic connections was assessed by examining two postsynaptic responses: 1) photolysis-evoked EPSCs were first recorded at a holding potential of -70mV and 2) photolysis-evoked action potentials were recorded at resting membrane potential. Stimuli applied directly to the recorded cell evoked a large amplitude inward current with occasional superimposed sodium currents (voltage-clamp) or a large depolarization with superimposed action potentials (current-clamp), confirming that the stimulation parameters were capable of evoking action potentials in the recorded cells (Figure 6A,B).

An increase in evoked synaptic activity was not observed in cells from slices contralateral to the injury (Figure 6); i.e., the average number of EPSCs in any given 100ms segment after stimulation was not greater than the average number of EPSCs before stimulation + 3SD at any stimulation site contralateral to the injury (n= 42 stimulus locations in 5 cells from 3 animals). In current-clamp recordings, photostimulation at the same locations did not evoke EPSPs or action potentials at any site, except when applied directly to the recorded neuron. Therefore, glutamate photostimulation failed to elicit a synaptically-mediated response at any location along the granule cell layer or in the hilus/CA3 region in slices contralateral to the injury.

In contrast, photostimulation of the granule cell layer evoked a synaptic response and an increase in activity in 6 of 8 cells (75%) from 8 ipsilateral slices with mossy fiber sprouting (n= 66 stimulus locations, 5 animals; Figure 7). A total of 18 stimulation locations (1 to 6 locations per cell) were determined to give a positive response to
photostimulation. Figures 8 and 9 show the variability of evoked responses in the granule cell layer ipsilateral to the injury site. A frequency histogram was constructed to show the distribution of the average number of eEPSCs for locations that had a positive response to photostimulation (Figure 8E). In current-clamp mode, EPSPs were consistently evoked and often reached action potential threshold (66%). Photostimulation evoked action potentials in nearly all trials (96%) at locations that had \( \geq 4 \) eEPSCs in voltage-clamp mode. Only 24\% of stimulations evoked action potentials at locations that had \(< 4\) eEPSCs. The remaining stimulus locations (n= 48), including all 17 locations in the hilus and CA3, had no response to photostimulation. An increase in the number of EPSCs after stimulation was not observed and EPSPs were not evoked by photostimulation at these locations.

Discussion

Cellular mechanisms underlying the increased risk of epilepsy after head injury are not well understood. Here, we examined local network interactions in dentate granule cells from mice with posttraumatic mossy fiber sprouting and seizures. The goal of this study was to test the hypothesis that regionally localized mossy fiber sprouting after experimental cortical contusion injury was sufficient to produce a recurrent excitatory circuit in granule cells. An increase in excitatory input to individual granule cells were detected in slices with posttraumatic mossy fiber sprouting. In conditions of increased excitation and decreased inhibition, these cells displayed spontaneous epileptiform burst activity. Responses that reflect synaptic connections between granule cells were activated
by glutamate photostimulation at some locations of the granule cell layer but never in the hilus or CA3. These data are consistent with the development of a local recurrent excitatory circuit (Traub and Wong, 1981; 1982; Miles et al., 1986; Miles and Wong, 1987; Lynch and Sutula, 2000; Wuarin and Dudek, 2001; Winokur et al., 2004).

An important unresolved question regarding mossy fiber sprouting after TBI is whether it is associated with a relevant functional alteration in neuronal circuitry. A dominant hypothesis has been that mossy fiber sprouting after epileptogenic insult forms a positive feedback circuit between granule cells. Another hypothesis proposes that sprouted mossy fibers preferentially form synaptic connections with inhibitory interneurons, and may act to bolster recurrent inhibition (Sloviter, 1991, 1992; Kotti et al., 1997). Studies that have examined chronic changes in excitability after TBI have primarily used extracellular field recordings and have not consistently identified synchronous network activity indicative of a recurrent excitatory circuit. This may be because many of these experiments were not performed in the presence of GABA\textsubscript{A}-receptor antagonists (Reeves et al., 1997; Santhakumar et al., 2001), which is necessary for revealing recurrent excitatory circuits in the dentate gyrus (Cronin et al., 1992). However, Santhakumar et al (2001) reported that evoked population activity in the chronically injured dentate gyrus with mossy fiber sprouting after moderate fluid percussion injury was similar to controls, even in conditions of disinhibition. Because only minimal Timm granules are detected in the inner molecular layer months after moderate fluid percussion injury (Shumate et al., 1995; Santhakumar et al, 2001), the failure to detect an increase in recurrent excitability by extracellular recordings from granule cells may also be due to the paucity of new connections in that model. A basic
property of recurrent excitatory circuits is that there must be a sufficient number of excitatory interconnections between cells in order for activity to spread through the entire network (Traub and Wong, 1981, 1982; Miles and Wong, 1983). Other studies report that spontaneous electrographic seizures or mossy fiber sprouting do not develop until 7-12 months after severe fluid percussion injury in rats (Kharatishvili et al., 2006; 2007). On the other hand, we recently demonstrated spontaneous population activity indicative of synchronous network activation in slices with more robust mossy fiber sprouting after severe CCI (Hunt et al., 2009), a focal injury model.

In the present study, we provide relatively direct evidence for increased granule cell–granule cell connections after TBI. Several independent studies have indicated that recurrent excitatory circuits are normally masked by recurrent inhibitory circuits, even in animals with frequent spontaneous seizures, and can be revealed in conditions of reduced inhibition and/or increased excitation (Cronin et al., 1992; Wuarin and Dudek, 1996, 2001; Patrylo and Dudek, 1998; Lynch and Sutula, 2000; Smith and Dudek, 2001; 2002; Winokur et al., 2004; Hunt et al., 2009). Therefore, reorganized circuits are proposed to form the basis from which functional electrical discharges periodically arise in context of other abnormalities. We used Mg\(^{2+}\)-free ACSF containing 100µM PTX in the present study to unmask NMDA-mediated excitatory synapses and block GABA\(_A\)-mediated inhibition. These conditions were sufficient to reveal new excitatory connections in slices from injured animals with mossy fiber sprouting that were absent in control and injured slices without mossy fiber sprouting under identical recording conditions. It is unknown whether similar results would be obtained in the presence of PTX alone. Glutamate photostimulation is useful for identifying local synaptic connections, because it allows for
focal stimulation of cell bodies and dendrites without activating axons of passage (Calloway and Katz, 1993). Evoked responses to photostimulation in some areas of the granule cell layer - but not in other areas - suggests an underlying local circuitry. Moreover, we observed variation in these responses (i.e., mild to robust responses), indicating that not all regions of the granule cell layer contribute equally to the newly formed network. Despite reports that indicate mossy cell-granule cell connections can sometimes be observed in slice preparations (Buckmaster et al., 1992; Scharfman, 1995), we did not find evidence of a photolysis-evoked excitatory synaptic connection between the hilus or CA3 region and any of our recorded granule cells. This is not surprising due to the relatively low probability of finding connections between the hilus and granule cells in vitro (Scharfman, 1995).

The degree of neuronal circuit reorganization necessary to produce a seizure focus remains unresolved. It seems reasonable to suggest that in a network configuration consisting of the appropriate principle cells interconnected by regionally localized recurrent excitatory connections, activating these particular circuits under appropriate conditions (e.g., a transient failure of inhibition) may engage an entire population of neurons and contribute to seizure generation (Traub and Wong, 1981; Miles and Wong, 1983). Activating nearby areas without interconnections may not lead to synchronous network activation. The presence of regionally localized mossy fiber sprouting and recurrent excitation in mice with spontaneous seizures after CCI injury is consistent with this proposed mechanism. More extensive synaptic reorganization may thus increase the probability of seizure generation or seizure severity. However, this relationship is likely indirect (Buckmaster and Dudek, 1997). Development of seizure foci in other susceptible
brain regions, such as CA1 (Smith and Dudek 2001, 2002; Scheff et al., 2005) or neocortex (Salin et al., 1995), may also undergo synaptic reorganization independent of mossy fiber sprouting. Moreover, chronic injury-induced dysfunction of inhibitory circuitry may compromise the ability of these circuits to mask new excitatory connections. The net effect of new localized recurrent excitatory circuits in combination with altered synaptic inhibition after head injury may contribute to the generation of spontaneous seizures. Interestingly, we previously reported an interval-specific alteration in extracellular paired-pulse responses, an indirect measure of synaptic inhibition, selective for ipsilateral slices with mossy fiber sprouting (Hunt et al., 2009). Future studies aimed at more thoroughly investigating altered inhibition after CCI are necessary to resolve this issue.

The abnormal electrophysiological responses in granule cells after head injury were qualitatively less robust and less widespread than reports at similar time-points after pharmacologically-induced status epilepticus (Wuarin and Dudek 1996, 2001; Lynch and Sutula, 2000; Winokur et al., 2004). This may be due to less robust Timm staining in the inner molecular layer or less extensive synaptic network remodeling after head injury (i.e., only a portion of the dentate gyrus forms a recurrent excitatory circuit). Mechanisms other than mossy fiber sprouting and formation of new excitatory connections, such as altered glutamate receptor pharmacology (Meldrum et al., 1999) or altered ion channel function (Steinlein, 2004), could also contribute to the abnormal responses observed after injury and can not be ruled out. However, if these were sufficient features it would then be expected that epileptiform activity be present in the injured dentate gyrus independent of mossy fiber sprouting. We found a relationship between the degree of Timm granules
in the inner molecular layer and EPSC frequency as previously reported after kainate-treatment in rats (Wuarin and Dudek, 2001). Slices from injured animals that were devoid of mossy fiber sprouting in the inner molecular layer acted as internal controls, and responses in these slices were similar to those from uninjured controls, even in ipsilateral slices from animals with regionally localized sprouting in adjacent sections of the dentate gyrus. This supports the hypothesis that the presence of epileptiform activity is associated with mossy fiber spouting, rather than injury.

Structural damage and mossy fiber sprouting after CCI injury remains relatively localized to areas near the injury site, even in mice that have spontaneous seizures. This is different from induction paradigms that use status epilepticus and typically result in widespread damage that includes bilateral lesion of the hippocampus. Severe fluid percussion injury, which primarily produces a concussive injury, also results in a variable degree of bilateral damage and mossy fiber sprouting (Kharatishvili et al., 2006). The present results suggest that widespread damage and synaptic reorganization is not an obligatory component of recurrent circuit formation or spontaneous seizure generation, but at least some degree of synaptic reorganization appears to be requisite for seizure generation. We report here a gross anatomical description of cortical damage after CCI injury to provide a qualitative representation of the hippocampal damage observed in mice used in this study. Other reports have provided more detailed analyses of the degree of injury and cell loss produced by severe CCI (Tong et al., 2002; Hall et al., 2005; Saatman et al., 2006). In the present study, a lesion to the granule cell layer at the injury site and mossy fiber sprouting was observed in nearly all mice. While mossy fiber sprouting is more robust after severe versus moderate contusion injury (Hunt et al.,
2009), it is unlikely that a granule cell layer lesion is necessary to elicit mossy fiber sprouting after TBI (Kharatishvili et al., 2006).

Sprouting after CCI was typically most robust in sections just temporal to the injury site and became progressively less robust with increased distance away from the injury. In contrast, sprouting was generally absent in sections septal to the injury. While not always detected, granule cell loss has been reported in some temporal lobe epilepsy patients (Houser, 1990), and reductions in hippocampal volume have been reported after TBI (Bigler et al., 1997). Likewise, a recent study reported mossy fiber sprouting in at least a portion of the dentate gyrus - but not all areas - of resected tissue from all temporal lobe epilepsy patients with a history of head injury (Swartz et al., 2006). Therefore, localized mossy fiber sprouting is likely a relevant marker of TBI-induced epilepsy associated with mesial temporal lobe sclerosis.

Seizure detection was limited to infrequent behavioral monitoring, and many seizures likely went undetected. Moreover, non-convulsive seizures and other electrographic abnormalities (i.e., interictal spiking) cannot be accounted for. Statler et al. (2009) recently reported abnormal spontaneous electrographic activity and recurrent seizures in rats after CCI. Convulsive seizures were accompanied by behavioral manifestations similar to those described in the present study. Therefore, convulsive seizures associated with abnormal EEG activity are apparent in this model. While the monitoring method employed in the present study is widely used and allows for comparison with earlier reports using models of status-epilepticus (Racine et al., 1979; Cronin and Dudek, 1988; Sloviter, 1992; Buckmaster and Dudek, 1997; Patrylo and Dudek, 1998; Wuarin and Dudek, 2001; Shibley and Smith, 2002), additional studies that
use continuous EEG monitoring are necessary to provide more quantitative analyses of posttraumatic seizures. Regardless, spontaneous convulsive seizures are apparent in many mice after severe CCI injury.

The development of PTE in humans is highly variable and likely depends on injury characteristics such as severity, location, and injury type in addition to genetic background and acute and long-term treatment (Pitkanen and McIntosh, 2006). Therefore, it is impossible to experimentally reproduce all of the manifestations of human PTE. The lack of a reliable animal model in which PTE is easily and consistently reproducible has complicated studies aimed at elucidating cellular mechanisms of epileptogenesis after closed head TBI. The present study demonstrates that severe CCI injury is an advantageous model of PTE as many mice develop chronic seizures and localized synaptic circuit reorganization in the dentate gyrus.

Acknowledgements
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References


Figures

Figure 1. Mice develop injury-induced and spontaneous seizures after severe CCI injury. Cumulative probability plot of the first observed seizure after CCI injury (time 0). Seizure counts were reset after 1d and 7d to separate immediate, early, and spontaneous seizures.

Figure 2. Cavitation into the hippocampus and posttraumatic mossy fiber sprouting 8-12 weeks after severe CCI injury. A. Representative Nissl stain of the dentate gyrus contralateral to the injury site. B. Timm’s stain of the same section in A shows the absence of mossy fiber sprouting in the inner molecular layer. C, E, G. Representative Nissl stain images of the ipsilateral dentate gyrus at the injury site. Arrows indicate severe thinning of the granule cell layer. D, F, H. Timm’s stain of the same sections in C, E, G. Note the presence of moderate mossy fiber sprouting in all sections (arrows). Percentage of mice with each type of lesion/Timm stain at the injury site is indicated. Scale bar is 100µm.

Figure 3. Increased sEPSCs in slices of the ipsilateral dentate gyrus with mossy fiber sprouting (MFS). A-D. Representative whole-cell patch-clamp recordings of granule cells in slices from A) control, B) contralateral, C) ipsilateral without MFS, and D) ipsilateral with MFS. Boxed areas of each trace are enlarged below. E. Average EPSC frequency for cells in each treatment group. The numbers of cells are indicated in parentheses above each bar. F. EPSC frequency plotted as a function of Timm score. Solid lines represent
mean EPSC values and dotted lines represent ±SD. G. Average amplitude for cells in each of the four treatment groups. Error bars indicate mean ± SD. Asterisk (*) indicates P<0.001.

**Figure 4.** Spontaneous bursts of compound EPSCs in cells from slices with mossy fiber sprouting ipsilateral to the injury. Representative whole-cell voltage-clamp recordings of granule cells in A) a slice from an uninjured control mouse, B) a contralateral slice in an injured mouse, C) a slice ipsilateral to the injury without MFS, and D) a slice ipsilateral to the injury with MFS. Arrow indicates expanded portion of the trace in D. Slices were incubated with Mg²⁺-free ACSF and 100µM PTX; Vₘ = -70mV for all recordings.

**Figure 5.** Spontaneous epileptiform bursts of action potentials in cells of slices with mossy fiber sprouting ipsilateral to the injury. Representative whole-cell current-clamp recordings of granule cells in A) a slice from an uninjured control mouse, B) a contralateral slice in an injured mouse, C) a slice ipsilateral to the injury without mossy fiber sprouting (MFS), and D) a slice ipsilateral to the injury with MFS. Inset shows expanded sections of the underlined portions of the trace in D labeled 1 and 2. Resting membrane potential is indicated for each trace.

**Figure 6.** Granule cell - granule cell connections are not detected by glutamate photostimulation in cells from slices contralateral to the injury. A. Voltage-clamp recordings at -70mV from a granule cell. B. Current clamp recordings at resting membrane potential from the same cell as A. Dotted vertical lines indicate the time of
stimulation. Numbers to the left of each trace indicate corresponding stimulus position shown in C. C. Nissl stain image of the slice from which the recorded cell was obtained. Numbers correspond to the approximate locations along the granule cell layer that photostimulation was applied to give the numbered responses recorded in A and B. Stimulation position 3 (circled) is the approximate location of the recorded cell. Note that direct activation of the recorded cell induced an inward current and burst of action potentials. D. Timm’s stain image of the same section in C indicating no MFS into the inner molecular layer.

Figure 7. Granule cell - granule cell connections are detected by glutamate photostimulation in cells from slices ipsilateral to the injury with mossy fiber sprouting. A. Voltage-clamp recordings at -70mV from a granule cell. B. Current-clamp recordings at resting membrane potential from the same cell as A. Dotted vertical lines indicate the time of stimulation. C. Nissl stain image of the slice from which the recorded cell was obtained. Numbers correspond to the approximate location along the granule cell layer that photostimulation was applied to give the responses recorded in A and B. Stimulation site number 2 (circled) is the approximate location of the recorded cell. Note that direct photoactivation of the recorded cell induced an inward current and burst of action potentials (A2, B2). Activity induced in neurons at locations 3 and 4 resulted in synaptic responses (A3, A4) and action potentials (B3, B4) in the recorded granule cell. D. Timm’s stain image of the same section in C. Note: mossy fiber sprouting surrounds the position of the recorded cell.
Figure 8. Variation in eEPSC responses in granule cells from slices ipsilateral to the lesion. A-C. Representative responses in a single neuron at three different stimulation locations of the granule cell layer. Each response shows five consecutive overlapping responses, with three consecutive individual traces separately shown below. A. Photostimulation did not evoke a response. B. A mild response of 1-2 EPSCs was consistently evoked in each of 5 trials. C. A more robust response that consisted of 5-9 eEPSCs in each of 5 trials after photostimulation at a different site in the granule cell layer. D. Number of EPSCs per 100ms before and after stimulation for each representative response in A-C. Contralateral responses are averaged across all stimulation locations in 5 neurons. Arrows indicate the time of stimulation. E. Frequency histogram shows the distribution of the average number of eEPSCs at 18 stimulation sites that had a positive response.

Figure 9. Evoked EPSPs and action potentials in granule cells ipsilateral to CCI injury after photostimulation at distant locations in the granule cell layer. A-C. Representative responses in a single neuron at three different stimulation locations of the granule cell layer. Each response shows five consecutive overlapping responses. A. No response evoked by photostimulation. B. A mild response was evoked, with one of five responses reaching action potential threshold. Photostimulation at this location evoked an average of 3 EPSCs in voltage-clamp mode. C. A more robust evoked response that consisted of a small burst of action potentials in each of five trials. Photostimulation at this location evoked an average of 10 EPSCs in voltage-clamp mode. Insets show expanded portion of one trace. Arrows indicate the time of stimulation.
<table>
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<th></th>
<th>Avg Timm Score</th>
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<td>-79 ± 4.4</td>
<td>1.7 ± 0.3</td>
<td>7.3 ± 1.3</td>
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Values are mean ± SD