

Traumatic Brain Injury Increases Cortical Glutamate Network Activity by Compromising GABAergic Control

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Traumatic brain injury (TBI) is a major risk factor for developing pharmaco-resistant epilepsy. Although disruptions in brain circuitry are associated with TBI, the precise mechanisms by which brain injury leads to epileptiform network activity is unknown. Using controlled cortical impact (CCI) as a model of TBI, we examined how cortical excitability and glutamatergic signaling was altered following injury. We optically mapped cortical glutamate signaling using FRET-based glutamate biosensors, while simultaneously recording cortical field potentials in acute brain slices 2–4 weeks following CCI. Cortical electrical stimulation evoked polyphasic, epileptiform field potentials and disrupted the input–output relationship in deep layers of CCI-injured cortex. High-speed glutamate biosensor imaging showed that glutamate signaling was significantly increased in the injured cortex. Elevated glutamate responses correlated with epileptiform activity, were highest directly adjacent to the injury, and spread via deep cortical layers. Immunoreactivity for markers of GABAergic interneurons were significantly decreased throughout CCI cortex. Lastly, spontaneous inhibitory postsynaptic current frequency decreased and spontaneous excitatory postsynaptic current increased after CCI injury. Our results suggest that specific cortical neuronal microcircuits may initiate and facilitate the spread of epileptiform activity following TBI. Increased glutamatergic signaling due to loss of GABAergic control may provide a mechanism by which TBI can give rise to post-traumatic epilepsy.

Keywords: cortical hyperexcitability, epilepsy, glutamate signaling, network reorganization, traumatic brain injury

Introduction

Traumatic brain injury (TBI) leads to development of post-traumatic epilepsy (PTE) in ~8–33% of civilian head injuries and ~50% in military populations (Salazar et al. 1985; Annegers et al. 1998; Asikainen et al. 1999; Lowenstein 2009). TBI itself can be subdivided into primary insults, characterized by physical damage resulting from blunt force; and secondary insults, including increased glutamate release, inflammation, oxidative stress, metabolic dysregulation, and altered neuronal connectivity (Andriessen et al. 2010; Khan et al. 2011; Readnower et al. 2011; Ward et al. 2011; d'Avila et al. 2012). In most patients, an extended latent period lasting months or even years exists between the initial injury and the first spontaneous seizure. Animal models of TBI also show a latent period which lasts months. During this time multiple molecular, cellular, and network level changes occur. Determining which changes specifically contribute to epileptogenesis is of distinct clinical importance and may allow novel therapeutic intervention.

TBI has been effectively modeled in rodents using controlled cortical impact (CCI) (Lighthall et al. 1989; Mannix et al. 2011; Walker et al. 2012). This model of TBI induces spontaneous behavioral and electrographic seizures in 9–36% of animals, similar to the incidence of PTE in humans (Hunt et al. 2009; Statler et al. 2009; Bolkvadze and Pitkanen 2012). CCI induces cortical and hippocampal neurodegeneration resulting in major tissue loss in the cortex, often extending into the underlying hippocampus (Colicos et al. 1996; Fox et al. 1998; Kochanek et al. 2006; Mchedlishvili et al. 2010; Yang et al. 2010; Zhou et al. 2012). In hippocampus, mossy fiber sprouting following CCI correlates with increased epileptiform activity and severity of cortical damage, providing a specific change in connectivity which may underlie epileptogenesis (Hunt et al. 2009, 2012). In the cortex, however, less is known regarding circuit-level changes following CCI. We do know that surviving cortical tissue exhibits spontaneous epileptiform activity (Yang et al. 2010). Furthermore, intracortical stimulation shows reorganization of motor cortex 7 weeks after CCI (Nishibe et al. 2010) and alterations in hippocampal/entorhinal cortex circuitry 4 weeks postinjury (Card et al. 2005). The specific synaptic and network level changes in the cortex that contribute to increased excitability have not been identified.

Although multiple factors may lead to pathological network changes following CCI, we hypothesize that increased glutamate signaling in the cortex may occur during the latent period. Enhancing excitatory glutamatergic systems would have obvious proconvulsive effects. Extracellular glutamate levels are known to increase in rat cortex within the first hour after CCI (Nilsson et al. 1994; Folkersma et al. 2011) and in humans 24-h post-TBI (Chamoun et al. 2010). The question of how glutamatergic systems are altered beyond the initial injury is not known. Here, we investigated whether CCI alters cortical glutamate network function 2–4 weeks following CCI by combining electrophysiological recording and high-speed glutamate biosensor imaging in acute cortical brain slices (Dulla et al. 2008, 2012; Tani et al. 2010). We show that glutamate signaling is enhanced in specific cortical regions and that GABAergic control of glutamate systems is altered, which may contribute to cortical hyperexcitability. Our findings provide novel insight into how injured cortical networks function and suggest specific cortical connections which may be involved in post-traumatic epileptogenesis.

Materials and Methods

Animals

TBI was induced by CCI as previously reported (Lighthall et al. 1989; Mannix et al. 2011; Walker et al. 2012). Ten-week-old male C57BL/6

mice were anesthetized with isoflurane in O₂ (4% induction 2% maintenance) and placed in a Kopf stereotaxic frame. A core temperature of 37 °C was maintained using a computer-controlled infrared heating pad fitted with a rectal temperature probe (Kent Scientific). Following a midline incision, a 5-mm craniotomy was performed over the left sensorimotor cortex, lateral to the sagittal suture between bregma and lambda, ensuring no damage to the underlying dura. The craniotomy site was cooled via constant irrigation with sterile saline during drilling. The cortical lesion was induced using a pneumatic impactor (TBI0310, Precision Systems) fitted with a 3-mm-diameter tip at a speed of 3.5 m/s, with a dwell time of 400 ms and to a depth of 1 mm. These CCI parameters are consistent with a severe injury (Hunt et al. 2009). Excess bleeding was flushed away with room temperature sterile saline prior to suturing. To prevent pressure-induced damage to the lesion, the bone flap was not replaced. Sham mice received anesthesia and a craniotomy. Naïve animals did not undergo any surgical procedures. Although there is sometimes a small percentage of mortality associated with CCI (<5%), none of our animals died as a result of the injury. Animals used in these experiments were not monitored for clinical or subclinical seizure activity. Procedures followed all guidelines of Tufts University School of Medicine's Institutional Animal Care and Use Committee.

Production of FRET Glutamate Biosensor

Biosensor production was performed as previously described (Dulla et al. 2008). Briefly, BL21(DE3) bacteria were transformed by heat shock with pRSET-FLI181E-1 plasmids and streaked on an LB plate containing ampicillin (100 µg/mL). Bacteria were then incubated overnight at 37 °C and a single colony was picked and grown in 1 L LB with ampicillin (100 µg/mL) for 3–4 days at 21 °C with rapid shaking (300 rpm) in the dark. Cells were harvested by centrifugation, resuspended in extraction buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.2), and lysed using CellLytic B reagent (Sigma). FRET sensor was then purified by Talon His-affinity chromatography (Clontech). Binding to the resin was performed in batch at 4 °C, washed in a column with extraction buffer, and eluted with extraction buffer containing 150 mM imidazole.

Preparation of Brain Slices

Ten-week-old C57BL/6 mice were anesthetized (50 mg/kg pentobarbital i.p.) and decapitated as previously reported (Dulla et al. 2008, 2012). Brains were rapidly removed and placed in chilled (4 °C) low-Ca²⁺, low-Na⁺ slicing solution consisting of the following (in mM): 234 sucrose, 11 glucose, 24 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, and 0.5 CaCl₂, equilibrated with a mixture of 95% O₂ and 5% CO₂. The brain was glued to the slicing stage of a Leica VT1200S vibrotome and coronal slices containing sensorimotor cortex were cut (400 µm). The slices were then incubated for 1 h in 32 °C oxygenated artificial cerebral spinal fluid (aCSF) consisting of the following (in mM): 126 NaCl, 26 NaHCO₃, 3 KCl, 1.25 NaH₂PO₄, 2 MgCl₂, 2 CaCl₂, and 10 glucose, and then kept at room temperature until use.

Field Recordings

Slices were placed in an interface chamber maintained at 34 °C, superfused with oxygenated aCSF at 2 mL/min, and ascending cortical inputs were stimulated with a tungsten concentric bipolar electrode at the layer VI–white matter boundary. Electrical stimulation consisted of 8–25 µA, 100-µs pulses at 30 s intervals delivered by a stimulus isolator (World Precision Instruments). Glass micropipettes (resistance $\approx$ 1 M Ω) were filled with aCSF and placed in layer V of the cortex directly above the stimulation electrode. Electrophysiological data were recorded with an Axon Multiclamp 700A amplifier and Digidata 1322A digitizer (sampling rate = 20 kHz) with pClamp software (Molecular Devices). Threshold stimulation intensity was identified as the minimum amount of current required to elicit a detectable cortical field potential response ($\geq$ 0.05 mV). Threshold current stimulation values were doubled to achieve 2 $\times$ threshold stimulation values. GABAzine (GBZ 10 µM) was added to aCSF and washed in while the synaptic activity was evoked at 2 $\times$ threshold stimulation values. 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic

acid (CPP 10 µM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX 20 µM) was added to aCSF and washed in while stimulating at threshold.

Patch Clamp Recordings

Slices were placed in the recording chamber of an Olympus BX51 microscope with continual superfusion of oxygenated aCSF maintained at 32 °C. Layer V pyramidal neurons were visually identified with infrared differential interference contrast microscopy and whole-cell patch-clamp recordings were made with a borosilicate glass electrode (3–5 M Ω) filled with (in mM): 140 CsMs, 10 HEPES, 5 NaCl, 0.2 EGTA, 5 QX314, 1.8 MgATP, 0.3 NaGTP, and pH 7.25. Recording electrode was placed approximately 200 µm from the site of injury. Data were collected using an axon Multiclamp 700B amplifier, Digidata 1440A digitizer and pClamp software. Spontaneous EPSCs and IPSCs were recorded at a holding potential of –70 and 0 mV, respectively. Recordings were analyzed using Clampfit (Axon Instruments, Inc.) and Mini Analysis (Synaptosoft). Only recordings with an access resistance that varied <20% were accepted for analysis.

Biosensor Imaging

Collection of glutamate biosensor data was done as previously described (Dulla et al. 2008). Slices were placed in a 0.4 µm, 30-mm-diameter cell culture inserts (Millicell), and all excess aCSF surrounding the slices was carefully removed with a pipette. A 50-µL aliquot of purified FRET-based glutamate biosensor was applied directly onto the surface of the slice. The cell culture insert was then covered and placed in an oxygenated interface incubation chamber for 5 min at 34 °C. Slices were then placed into the recording chamber of an Olympus BX51WI microscope with continual superfusion of aCSF for simultaneous imaging with an Olympus $\times$ 4 objective. Excitation with 440-nm wavelength light was used. A Neuro-CCD camera (RedShirt Imaging) was used to collect 40 $\times$ 80 pixel imaging data at 1000 Hz (1 ms exposure time/frame). Each imaging experiment consisted of collecting 5 movies each containing 1500 frames with a 30-s interstimulus interval (ISI). Each captured 1500-ms movie contained a 90-ms period of dark noise (camera signal before shutter opening) captured before fluorescence was turned on. Fluorescence was turned on for 1210 ms, and then turned off for 200 ms. Slices were stimulated 250 ms after fluorescence was turned on. Emission signals first passed through a 455-nm DCLP dichroic mirror to eliminate excitation fluorescence and were then separated into 2 channels using a Photometrics Dual-View or Optosplit two-channel imaging system to isolate cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) signals. Ratiometric images of CFP/YFP signals were created and analyzed as described below.

Evoked Field Potential Data Analysis

Electrophysiological data were analyzed using pClamp (Axon Instruments) and MATLAB software. Traces were recorded at threshold stimulation, the minimum stimulation required to elicit a response, at 2 $\times$ threshold, and after the addition of 10 µM GABAzine at 2 $\times$ threshold. Amplitude was defined as the maximum negative voltage change following electrical stimulation, while duration was defined as the amount of time following stimulation for the response to return to baseline. Area under the curve was used to determine the integrated network activity and was calculated by integrating the extracellular field potential during the first 250 ms following initial stimulation. Coastline analysis was used to determine high-frequency activity by summing the distance between each point taken every 50 µs over a 250-ms time window. We determined slice viability by increasing stimulus intensity 2-fold above threshold. In the vast majority of sham injured slices, a 2-fold increase in stimulation elicited a relatively similar (3-fold) increase in field excitatory postsynaptic potential (fEPSP) amplitude. In a subset of slices, a modest increase in stimulation resulted in a massive (20–45-fold) increase in fEPSP amplitude. This type of nonlinear increase in response was seen relatively infrequently (2 of 16 sham-injured slices, and 2 of 12 CCI slices), and these responses were excluded from statistical analysis.

Glutamate Imaging Data Analysis

Imaging data were analyzed using customized MATLAB software. Raw imaging data were first split into CFP and YFP and the ratio of the 2 fluorophores was computed. An average prestimulation ratio image was then made by averaging the 40 ms of imaging data prior to the stimulus. The prestimulation image was then subtracted from all images resulting in a Δ FRET image. A Gaussian filter (3×3 matrix, 0.5 Gaussian value) followed by a 5-point moving average temporal filter was applied to all Δ FRET images. Processed Δ FRET images were then converted into Δ FRET_{signal}/ Δ FRET_{noise} data, pixel by pixel, by dividing all time-points by the standard deviation (SD) of Δ FRET during the prestimulus time period. Imaging data were then analyzed to determine the peak amplitude of the signal and the time at which the signal reached its maximum value. The number of pixels above 2.5 SDs of the noise was calculated and divided by the total area of the cortex to analyze spatial activation of cortical networks. For spatio-temporal analysis, individual images were created by binning 5 ms of imaging data, and subsequent frames were subtracted to generate Δ signal/noise images. For regional temporal analysis, activation was defined as the time at which the Δ signal/noise value was greater than $5 \times$ the SD the prestimulus Δ signal/noise. Individual glutamate signal recordings were analyzed by drawing a region of interest (ROI) $\sim 200 \mu\text{m}$ adjacent to the CCI lesion and plotting the Δ FRET_{signal}/ Δ FRET_{noise} traces over time. ROI analysis included setting the FRET_{signal}/FRET_{noise} baseline to zero for simple comparison. For cortical subregion analysis, we considered the area adjacent to the injury to be the portion of intact cortex $\sim 200 \mu\text{m}$ from the lesion. Proximal and distal areas were considered to be ~ 400 and $\sim 600 \mu\text{m}$ from the lesion, respectively. We considered superficial layers to be the area of cortex $\sim 300 \mu\text{m}$ below the pial surface, while deep layers were $\sim 300 \mu\text{m}$ above the white matter. Each pixel was $\sim 17.4 \mu\text{m}^2$.

Immunohistochemistry

Sham- and CCI-injured mice were transcardially perfused with 4% paraformaldehyde. Fixed brains were sectioned at $40 \mu\text{m}$ using a Thermo Fisher Microm HM 525 cryostat. Brain sections were blocked using blocking buffer (5% normal goat serum, 1% bovine serum albumin, in PBS) for 1 h at room temperature. Parvalbumin (1:5000, Swant) and NeuN (1:1000, Millipore) antibodies were diluted in PBS with 2% Triton-X 100 and 5% blocking buffer. Cortical sections were incubated with diluted primary antibodies overnight at 4°C . Secondary antibodies (goat anti-rabbit Cy3.5, goat anti-mouse FITC, Jackson Labs) were diluted in PBS with 5% blocking buffer and added to cortical sections for 2 h at room temperature. Slices were mounted using Vectashield (Vector Labs) and imaged with a Nikon Eclipse E800 upright epifluorescence microscope.

Cell Quantification

Parvalbumin (+) and somatostatin (+) cells were quantified with MATLAB software using the Image Processing Toolbox. Briefly, images were normalized to a 10 000 color-intensity range to control for signal variation resulting from differences in exposure times. We then blurred the images 100 times using a Gaussian filter and subtracted the blurred images from the original to create a background-subtracted image; negative values were set to zero. Next, we applied a median filter and thresholded images at median intensity. Cell counting was then performed using MATLAB object recognition tools. Cell numbers were divided by the area of the ROI which yielded the number of positive cells/ 10 mm^2 . Cortical subregions were determined as in Glutamate Imaging Data Analysis section.

Western Blot Analysis

Expression of GLT-1 and GLAST was analyzed via western blot. Brains from sham- and CCI-injured mice were removed and sliced ($400 \mu\text{m}$) using a Leica VT1200S vibratome as described in "Preparation of Brain Slices" section. Neocortex from sham and CCI slices ($\sim 3 \text{ mm}$ from site of injury) was dissected. Tissue was collected from the white matter/cortex boundary to the cortical surface, encompassing all cortical layers. Tissue samples from 3 to 4 slices per animal were combined

and homogenized using RIPA buffer [150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0] and protease inhibitor cocktail. Samples were then centrifuged at 13 200 rpm for 15 min at 4°C . Protein ($15 \mu\text{g}$) was loaded onto a 10% gel and analyzed via SDS-polyacrylamide gel electrophoresis. Proteins were detected using a rabbit polyclonal antibody against GLT-1 (1:1000, Abcam) and a rabbit polyclonal antibody against GLAST residues 522–541, batch Ab#314 (Holmseth et al. 2009). Mouse monoclonal anti- β -actin (1:2000, Sigma) antibody was used to confirm equal loading. Protein bands were visualized using enhanced chemiluminescence and imaged with a LAS-3000 imaging system. Quantification of protein expression was conducted using ImageJ (NIH) and expressed in relation to β -actin expression.

Statistical Analysis

For comparison between 2 experimental groups, Student's *t*-test or χ^2 analyses were used. For 3 or more experimental groups, a one-way ANOVA with the Tukey post hoc test was used. Values of $P < 0.05$ were considered statistically significant.

Drugs and Reagents

All salts and glucose for buffers were obtained from Sigma-Aldrich. GABazine was obtained from Tocris Bioscience; CPP and DNQX were purchased from Abcam and dissolved in DMSO at 1000 $\times$ stock solutions. A final concentration of $10 \mu\text{M}$ GBZ, $10 \mu\text{M}$ CPP, and $20 \mu\text{M}$ DNQX was used for all experiments by diluting in aCSF.

Results

Epileptiform Activity is Increased After Traumatic Brain Injury

CCI is a precise and repeatable method of reliably inducing TBI (Lighthall et al. 1989; Mannix et al. 2011; Walker et al. 2012). We utilized a CCI protocol which has previously been shown to lead to spontaneous seizures in 36% of mice 6–10 weeks postinjury (Hunt et al. 2009). Using this protocol, 10-week-old male C57BL/6 mice underwent CCI and acute cortical slices were prepared 2–4 weeks after injury, prior to the reported onset of seizures. To investigate the effects of TBI on cortical excitability, we first examined extracellular field potentials for epileptiform activity. Epileptiform activity was defined as fEPSPs which contained high-frequency activity, as well as increased peak amplitude and duration (Fig. 1*B*). The frequency of epileptiform activity was calculated both as the percentage of slices that showed any epileptiform activity and as the percentage of fEPSPs that were epileptiform per slice. Brain slices from CCI-injured cortex frequently generated at least one epileptiform event when stimulated at threshold (10 of 12, 83%) while sham injured slices had no epileptiform fEPSPs. A significantly lower input stimulation was necessary to achieve a threshold response in CCI-injured cortex ($9.3 \pm 0.08 \mu\text{A}$) compared with sham slices ($11.7 \pm 0.06 \mu\text{A}$, $P < 0.05$). The likelihood of evoking an epileptiform event was then quantified on a slice-by-slice basis by counting the number of fEPSPs with epileptiform activity divided by the total number of fEPSPs recorded in that slice. The percent of fEPSPs showing epileptiform activity was significantly greater in CCI injured cortical slices ($57.3 \pm 10.0\%$) compared with sham-injured cortex (Fig. 1*C*), consistent with previous studies (Yang et al. 2010). Contralateral cortical slices were not significantly different from sham cortex suggesting that disruptions in cortical network activity following TBI are largely maintained within the injured hemisphere.

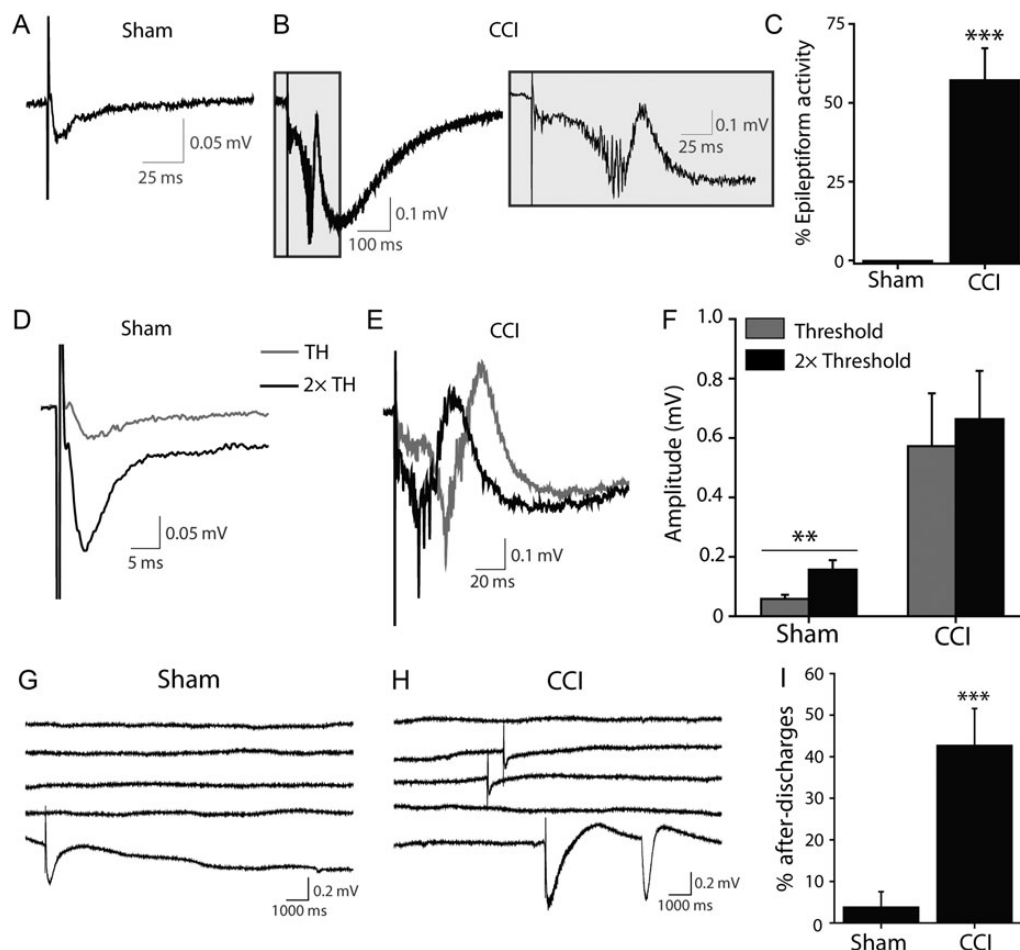


Figure 1. Cortical electrophysiological activity is increased following TBI. Representative traces of evoked field potentials from sham- (A) and CCI-injured cortex (B). Inset in (B), outlined by gray box, highlights epileptiform activity occurring during the first 175 ms in CCI-injured cortex. Cortical field potential responses were collected from a recording electrode placed in layer V with a stimulating electrode at the layer VI–white matter interface at an ISI of 30 s. Traces from CCI-injured cortex show increased high-frequency epileptiform activity with increased fEPSP amplitude and duration. (C) Quantification of % epileptiform activity, the number of recordings showing epileptiform activity was divided by total number of recordings per slice, *** $P < 0.001$, χ^2 test. Representative evoked field responses at threshold (gray traces) and 2 $\times$ threshold stimulation (black traces) for sham (D), and CCI-injured cortex (E). (F) Average peak amplitude of field responses showing a significant difference in sham-injured cortex between threshold and 2 $\times$ threshold stimulation, ** $P < 0.01$, bars represent mean $\pm$ SEM, pair-sample t -test, $n = 16$ sham, 12 CCI slices. Five representative cortical field potential recordings from sham (G) and CCI-injured cortex (H) showing after-discharges occurring after initial evoked stimulus. (I) Quantification of % after-discharges, the number of recordings showing after-discharges was divided by total number of recordings per slice, *** $P < 0.001$, bars represent mean $\pm$ SEM, two-sample t -test, $n = 16$ sham, 12 CCI slices.

Table 1
Quantification of electrophysiological activity parameters at threshold stimulation

Threshold stimulation	Sham	CCI
Amplitude (mV)	0.06 ± 0.01	$0.57 \pm 0.18^{**}$
Duration (s)	0.13 ± 0.01	$1.44 \pm 0.52^{**}$
Area (mV ms)	3.64 ± 0.68	$40.98 \pm 12.29^{**}$
Coastline ($\Delta mV/ms$)	$1.9E-6 \pm 1.3E-6$	$6.0E-5 \pm 1.9E-5^{**}$

fEPSP amplitude, duration, area, and coastline from CCI-injured cortex stimulated at threshold stimulation are significantly increased compared with sham controls, ** $P < 0.01$, values represent mean $\pm$ SEM, two-sample t -test, $n = 12$ –16 slices.

To perform more quantitative analysis, fEPSPs were analyzed based on amplitude, duration, area, and coastline. fEPSPs from sham-injured cortex were electrophysiologically normal, as indicated by small amplitudes (0.06 ± 0.01 mV) and brief (0.13 ± 0.01 s) fEPSPs (Fig. 1A) at threshold stimulation. In the CCI-injured cortex, fEPSPs were significantly increased in amplitude (0.57 ± 0.18 mV, $P < 0.01$) and duration (1.44 ± 0.52 s, $P < 0.01$) at threshold stimulation compared with sham-injured

cortex (Table 1). We also investigated the fEPSP area and the amount of high-frequency activity as measured by coastline analysis. fEPSPs from the CCI-injured cortex were significantly larger in area (40.98 ± 12.29 mV ms, $P < 0.01$) and had a significantly higher coastline index ($6.0E-5 \pm 1.9E-5$ $\Delta mV/ms$, $P < 0.01$), indicative of more high-frequency activity, compared with sham-injured cortex (Table 1).

Cortical Input–Output Relationships are Altered Following Traumatic Brain Injury

An increase in stimulation intensity will normally elicit a corresponding increase in fEPSP amplitude, generally referred to as the input–output relationship. In order to evaluate the cortical input–output relationship following TBI, we measured evoked field responses in slices from control and CCI injured mice at threshold and 2 $\times$ threshold stimulus intensities 2–4 weeks postinjury. In sham cortex, increasing the stimulation intensity significantly increased the average peak amplitude (Fig. 1D,F, $P < 0.05$). In the CCI-injured cortex, however, increasing

stimulation intensity did not significantly increase fEPSP amplitude (Fig. 1*E,F*). We then compared the ratio of fEPSP amplitude evoked at 2× versus 1× threshold stimulation. In sham-injured cortex, this ratio was 3.0 ± 0.5 (Fig. 1*D*) while, in the CCI-injured cortex, this ratio was significantly lower (1.2 ± 0.4 , $P < 0.05$ compared with sham-injured cortex) (Fig. 1*E*). This disruption in the input–output relationship in the CCI-injured cortex suggests that following TBI, cortical networks generate all-or-none epileptiform discharges and are largely incapable of generating controlled, scaled responses to input.

After-Discharges Increase in Brain Slices Following Traumatic Brain Injury

To further investigate changes in cortical network function following TBI, we measured after-discharges from sham- and CCI-injured cortex at threshold stimulation 2–4 weeks postinjury. Our stimulus paradigm used a 30 s interstimulus interval. After-discharges were defined as field potentials with a minimum amplitude of 0.05 mV which occurred after the initial stimulus-evoked field potential had returned to baseline. After-discharges were seen in 6% (1 of 16) of sham-injured slices when compared with 83% (10 of 12) of CCI injured cortical slices (Fig. 1*G,H*). The average after-discharge per slice was quantified by counting the number of fEPSPs with an after-discharge divided by the total number of fEPSPs recorded. These values were then combined for all slices within each treatment group to obtain the percentage after-discharge value. CCI injured slices showed a significant increase in percentage after-discharges ($42.7 \pm 8.9\%$, $P < 0.01$, Fig. 1*I*) compared with sham-injured cortex. These results suggest that cortical networks are more prone to generate recurrent activity following stimulus-evoked activity.

TBI-Induced Epileptiform Activity is NMDA Receptor Dependent

In order to investigate whether cortical hyperexcitability was glutamate receptor dependent, we pharmacologically blocked NMDA and AMPA receptors in epileptiform brain slices from mice 2–4 weeks after CCI injury. CPP (10 μ M) and DNQX (20 μ M) were bath applied to cortical slices to sequentially block NMDA and AMPA receptors, respectively. Polyphasic epileptiform activity (Fig. 2*A*) was inhibited after ~ 5 min of CPP wash-in (Fig. 2*B*). The remaining fEPSP was completely eliminated ~ 5 min after DNQX wash-in (Fig. 3*C*). Quantification of fEPSP amplitude showed a significant decrease after CPP and DNQX wash-in compared with control aCSF (Fig. 2*D*). fEPSP amplitude in DNQX was significantly decreased compared with CPP. fEPSP area and coastline were also significantly decreased after CPP and DNQX wash-in compared with control aCSF (Fig. 2*E,F*). These data suggest that TBI-induced epileptiform activity is NMDA receptor dependent and that cortical hyperexcitability is unable to propagate throughout the injured cortex using AMPA receptors alone.

Glutamate Network Signaling Increases 2–4 Weeks After Traumatic Brain Injury

Previous studies have reported increases in extracellular glutamate levels in rats immediately following TBI (~ 1 h) (Folkersma et al. 2011) and in human epileptic foci (Cavus et al. 2005; Pan et al. 2008). We hypothesized that network dysfunction during the latent period following TBI may be associated with increased glutamate network signaling. In order to assess the spatiotemporal properties of cortical glutamate network signaling during this time window, we imaged stimulus-evoked changes in extracellular glutamate levels using high-speed glutamate biosensor imaging. The glutamate biosensor is a FRET-

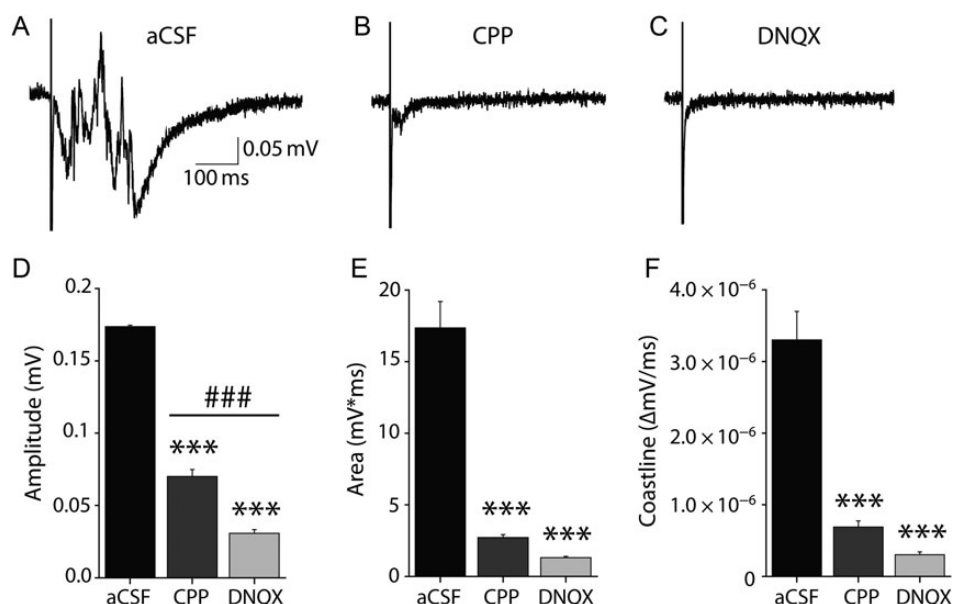


Figure 2. Epileptiform activity after TBI is NMDA receptor dependent. Representative cortical field potential recordings from CCI injured slice showing epileptiform activity (A), ~ 5 min after perfusion of CPP (B), and DNQX (C). Epileptiform activity is eliminated after blocking NMDA receptors with CPP. Cortical field potential is completely abolished after AMPA receptor blockade with DNQX. (D) Quantification of cortical field potential amplitude showing a significant decrease after CPP and DNQX perfusion compared with aCSF; amplitude from DNQX is significantly decreased compared with CPP. (E) Quantification of cortical field potential area during the first 250 ms after evoked stimulus shows a significant decrease in CPP and DNQX compared with aCSF. (F) Quantification of cortical field potential coastline during the first 250 ms after evoked stimulus shows a significant decrease in CPP and DNQX compared with aCSF; *** $P < 0.05$ compared with aCSF; ### $P < 0.05$ between CPP and DNQX, bars represent mean $\pm$ SEM, $n = 8$ slices.

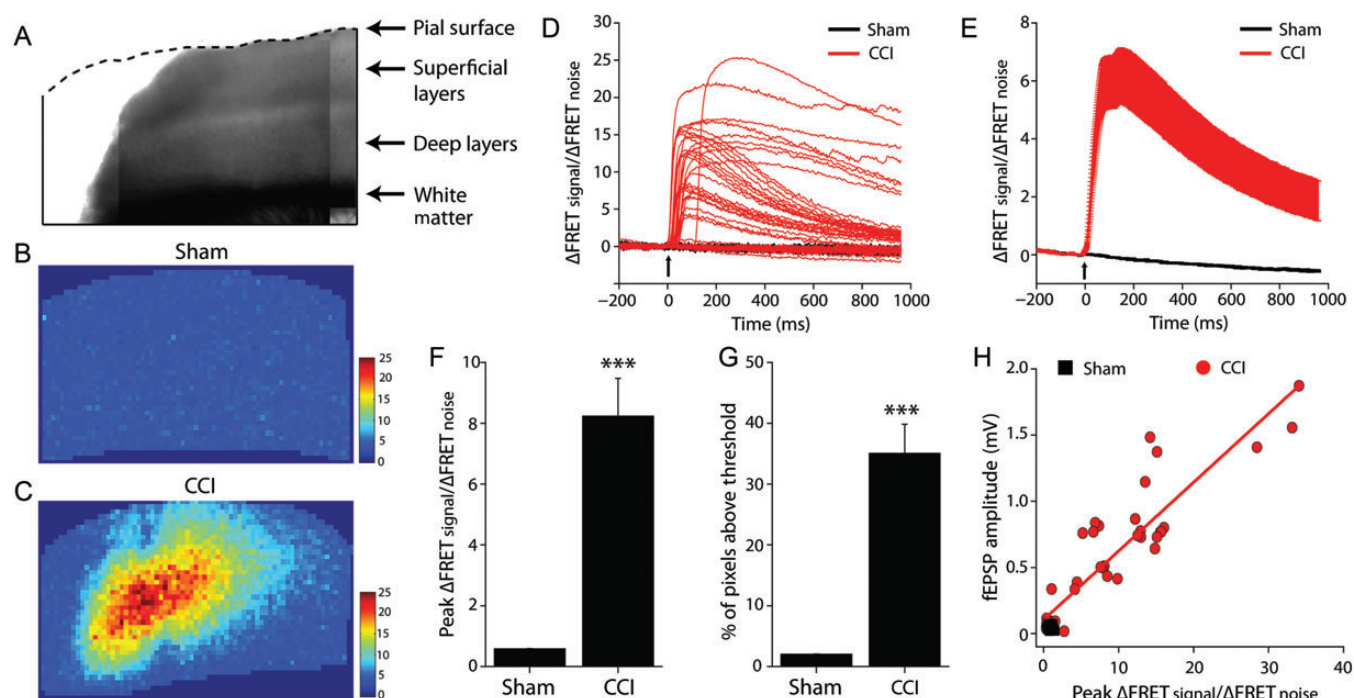


Figure 3. Glutamate signaling is increased in CCI-injured cortex at threshold stimulation. (A) Representative bright field image of a CCI injured cortical slice demonstrating the massive loss of cortical tissue (left) 2–4 weeks post-TBI. Arrows point to pial surface (dotted line), superficial cortical layers, deep cortical layers, and white matter. Representative, pseudo-colored glutamate images showing the peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) for each pixel from sham- (B) and CCI-injured cortex (C). (D) Individual $\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$ traces over time for all sham (black) and CCI injured (red) recordings. (E) Averaged $\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$ traces over time for all sham (black) and CCI injured (red) recordings. Black arrows indicate time of stimulation. (F) Quantification of peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) showing a significant increase in CCI-injured cortex compared with sham. (G) Quantification of activated cortical area, data are expressed as percentage of pixels above 2.5 SDs of the noise. *** $P < 0.001$ compared with sham, bars represent mean $\pm$ SEM, two-sample t -test. (H) Correlation of fEPSP amplitude (mV) versus peak $\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$ from sham- and CCI-injured cortex ($R^2 = 0.83$), $n = 75$ sham, 50 CCI recordings.

based fluorophore which consists of a glutamate binding protein coupled to CFP and YFP. Upon binding to glutamate, a conformational change occurs which leads to a decrease in FRET efficiency. This biosensor allowed us to monitor changes in stimulus-evoked extracellular glutamate signaling in the cortex. Slices from sham- and CCI-injured cortex were loaded with biosensor and imaged at threshold stimulation. Figure 3 shows pseudo-colored pixel peak glutamate biosensor images, which depict the peak glutamate biosensor signal at each pixel regardless of the time that the peak occurred. CCI-injured cortex (Fig. 3C) showed a marked increase in peak glutamate signal after threshold stimulation compared with cortical slices from sham controls (Fig. 3B). In the CCI-injured cortex, threshold stimulation evoked large glutamate biosensor signals in 58% (29 of 50) of evoked responses (Fig. 3D). Averages of the glutamate biosensor traces from both treatment groups revealed a large increase in glutamate signaling immediately following stimulation in CCI injured slices while virtually no glutamate signal was seen in sham injured control slices (Fig. 3E). We quantified the differences in the peak glutamate signal between both conditions by calculating the maximum amplitude of each glutamate transient. The peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) from the CCI-injured cortex (8.23 ± 2.12) was significantly increased compared with sham controls (0.58 ± 0.02 , $P < 0.001$) indicating increased evoked glutamate network signaling in the CCI-injured cortex at threshold stimulation (Fig. 3F). Peak glutamate signal from naïve (0.69 ± 0.02) and contralateral slices (0.61 ± 0.06) were not significantly different compared with sham-injured cortex. Additionally, we quantified

the area of activated cortex as the percentage of pixels above threshold ($>2.5 \times$ prestimulus noise) for each treatment condition, and divided this value by the number of pixels within the cortex. The percentage of pixels which crossed this threshold after an evoked stimulus from the CCI-injured cortex ($35.06 \pm 9.78\%$) was significantly higher than sham controls ($2.03 \pm 0.05\%$, $P < 0.001$) (Fig. 3G). Percentage of pixels above threshold from naïve ($2.25 \pm 0.07\%$) and contralateral slices ($2.47 \pm 0.38\%$) were not significantly different from sham-injured cortex. We next plotted the peak fEPSP amplitude versus the peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) from both sham- and CCI-injured cortex. A positive correlation was found in CCI-injured cortex ($R^2 = 0.83$) (Fig. 3H), suggesting that an increase in electrical excitability corresponds directly with increased glutamate signaling after TBI. Taken together, these data suggest that during the latent period following TBI, electrical stimulation of ascending cortical fibers evokes an increase in glutamate network signaling over a large cortical area causing a significant disruption in network function.

Electrical Activity and Glutamate Signaling in Disinhibited Cortical Slices are Indistinguishable Between CCI and Control Animals

To determine the nature of excitatory neurotransmission in isolation, we recorded cortical fEPSPs during the pharmacological blockade of GABA_A receptors with GABA_Azine (10 μM), a GABA_A receptor antagonist (Mienville and Vicini 1987). Stimulus-evoked glutamatergic signaling was examined ~10–15 min after GABA_Azine wash-in from sham and CCI slices 2–4

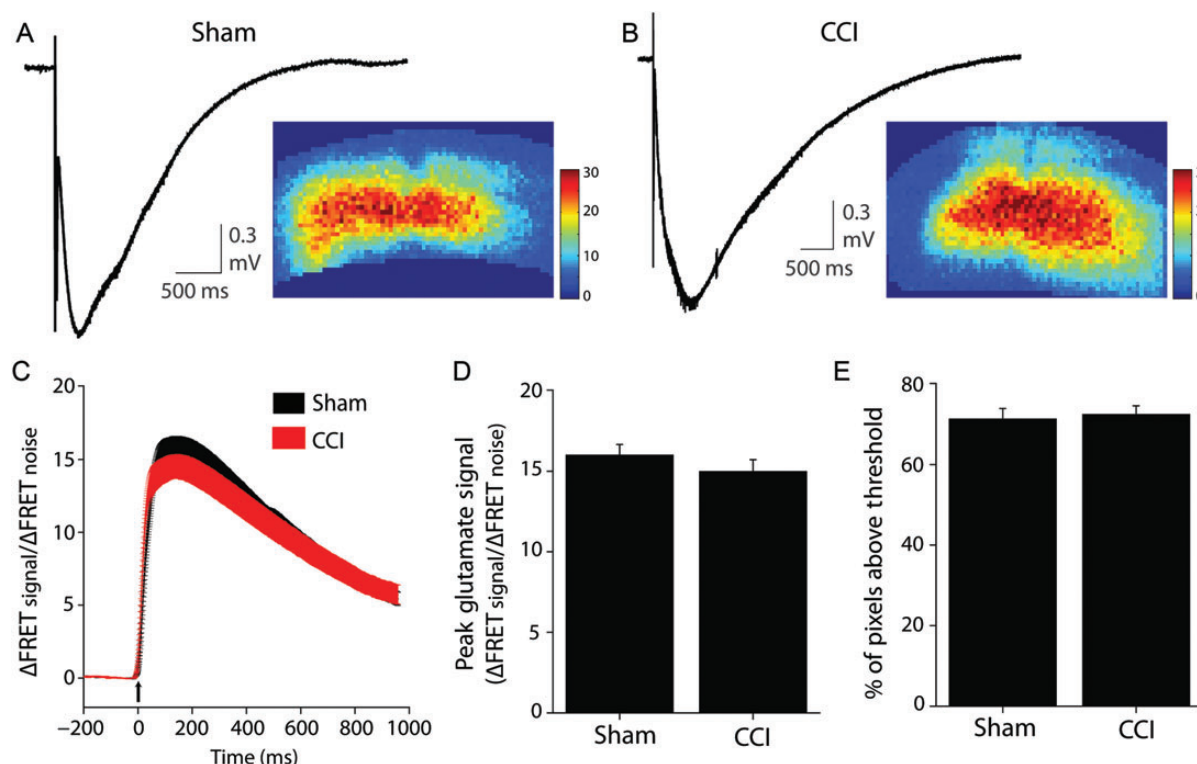


Figure 4. Disinhibited slices from sham and CCI cortex show similar electrophysiological parameters and glutamate signaling. Representative evoked field responses from sham- (A) and CCI-injured cortex (B) show similar increases in electrophysiological parameters after 10–15 min local perfusion of 10 μM GBZ. Representative, pseudo-colored glutamate signal images showing the peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) from sham- (A, inset) and CCI-injured cortex (B, inset) after 10–15 min local perfusion of 10 μM GBZ. (C) Averaged $\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$ traces over time for sham (black) and CCI injured (red) recordings. Black arrow indicates time of stimulation. (D) Quantification of peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) showing no difference between sham- and CCI-injured cortical slices. (E) Quantification of activated cortical area, data are expressed as percentage of pixels above 2.5 SDs of the noise; bars represent mean $\pm$ SEM, $n = 71$ sham, 50 CCI recordings.

Table 2
Quantification of electrophysiological activity parameters in disinhibited slices

GABAzine	Sham	CCI
Amplitude (mV)	1.35 ± 0.12	1.24 ± 0.07
Duration (s)	6.38 ± 0.61	7.81 ± 0.86
Area (mV ms)	213.2 ± 24.2	163.5 ± 12.4
Coastline ($\Delta\text{mV}/\text{ms}$)	$1.6\text{E}-4 \pm 2.4\text{E}-5$	$2.0\text{E}-4 \pm 2.3\text{E}-5$

fEPSP amplitude, duration, area, and coastline from CCI-injured cortex were stimulated at $2\times$ threshold stimulation during bath application of 10 μM GBZ. There is no significant difference between sham and CCI slices for any of the electrophysiological parameters tested, values represent mean $\pm$ SEM, two-sample t -test, $n = 12$ –16 slices.

weeks post-injury. By blocking inhibitory neurotransmission with GABAzine, we evoked a fEPSP which was high amplitude, polyphasic, and prolonged in duration. Under these conditions, evoked fEPSPs were virtually indistinguishable between sham- and CCI-injured slices (Fig. 4A,B). fEPSP amplitude, duration, area, and coastline after bath application of GABAzine were statistically similar between sham and CCI brain slices (Table 2). This suggests that the electrical activity evoked in the absence of GABA_A receptor-mediated inhibition is quite similar in both sham- and CCI-injured cortex.

Similar to our increase in fEPSP amplitude, duration, area, and coastline in both sham- and CCI-injured cortex after bath application of GBZ, glutamate signaling was equally increased in sham- and CCI-injured slices. Pseudo-colored pixel peak glutamate biosensor images from sham (Fig. 4A, inset) and CCI

cortex (Fig. 4B, inset) show largely equivalent activation of glutamatergic signaling in the presence of GABAzine. Average $\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$ traces over time from sham- and CCI-injured cortex revealed similar increases in glutamate signaling from disinhibited slices in both treatment groups immediately following stimulation (Fig. 4C). The peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) was not statistically different between sham (16.84 ± 1.24) and CCI injured (17.26 ± 1.26) cortical slices (Fig. 4D). The percentage of pixels 2.5 SDs above the noise after an evoked stimulus from sham ($71.30 \pm 5.13\%$) and CCI-injured cortex ($72.43 \pm 3.84\%$) was not statistically different (Fig. 4E), suggesting that an equal area of activation occurs during disinhibition in the sham and injured cortex. These data suggest that, in the absence of GABA_A receptor-mediated inhibition, glutamatergic signaling is largely similar between CCI and sham-injured brain slices.

Glutamate Signaling is Enhanced in Deep Cortical Layers Near the Site of Injury Following Traumatic Brain Injury

In order to further understand how glutamate signaling is altered during the 2- to 4-week period after injury, we analyzed glutamate biosensor responses in subregions of CCI injured cortical slices. We subdivided CCI injured slices into the following subregions: 1) adjacent to injury, 2) proximal superficial layers, 3) proximal deep layers, 4) distal superficial layers, and 5) distal deep layers (Fig. 5A). ROIs were drawn over each cortical subregion, peak glutamate signal was calculated, and all peak values were normalized to proximal superficial layers for interslice

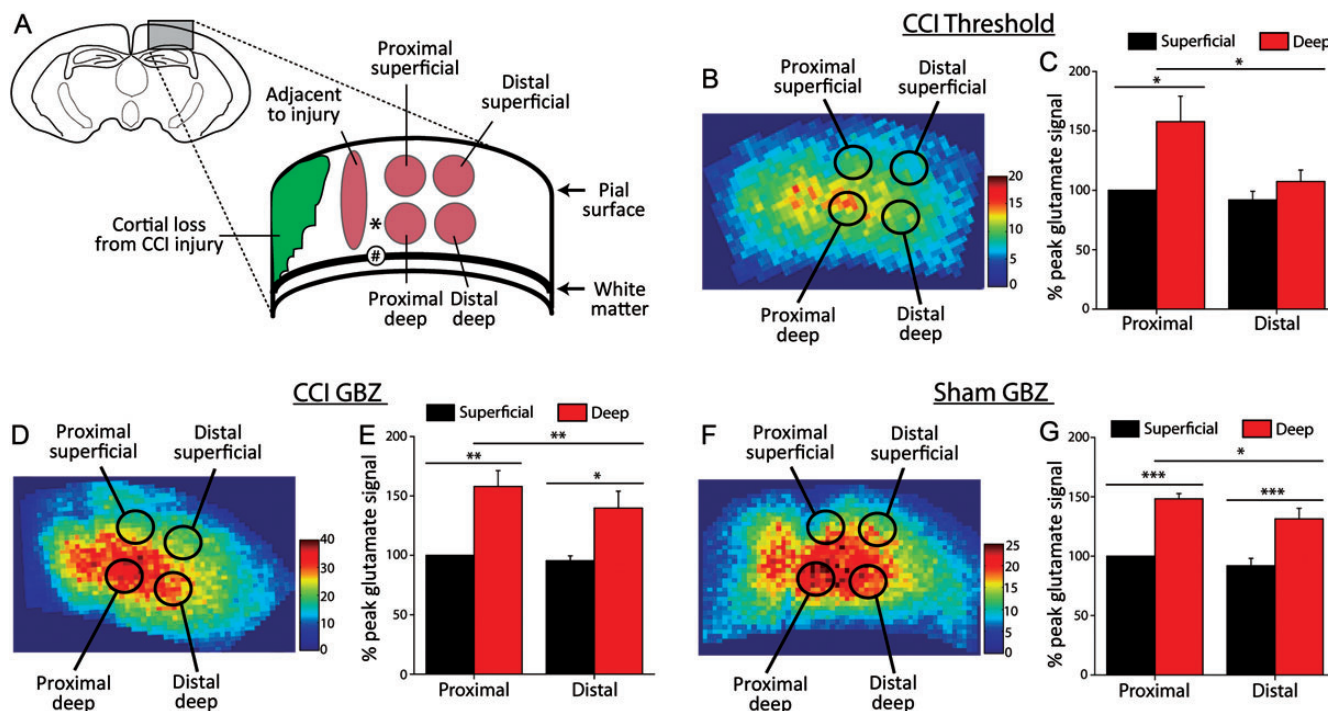


Figure 5. Glutamate signaling is enhanced in proximal deep cortical layers following traumatic brain injury. (A) Illustration showing cortical subregions for glutamate signal analysis, # indicates position of stimulating electrode, * indicates position of recording electrode. Representative, pseudo-colored glutamate images showing the peak glutamate signal ($\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) from CCI-injured cortex at threshold stimulation (B), CCI-injured cortex in GBZ (D), and sham-injured cortex in GBZ (F). Circles indicate layer-specific cortical subregions relative to the area adjacent to the injury. Quantification of layer-specific peak glutamate signaling in CCI-injured cortex at threshold stimulation (C), proximal deep layers are significantly different from proximal superficial layers and distal deep layers. In disinhibited (10 μM GBZ) CCI-injured cortex (E) and disinhibited sham-injured cortex (G), proximal deep layers are significantly increased compared with distal deep layers; cortical deep layers are significantly increased from superficial layers both proximally and distally, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, paired t -test, bars represent mean $\pm$ SEM, $n = 15$ sham, 10 CCI slices.

comparisons. Peak glutamate signal from CCI-injured cortex was the highest in deep layers proximal to the injury site (Fig. 5B). No significant difference in peak glutamate signal was detected between the area adjacent to the injury ($8.23 \pm 2.12 \Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$) and proximal deep layers ($9.16 \pm 2.57 \Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$). Peak glutamate signaling was significantly increased in proximal deep layers ($157.7 \pm 21.6\%$) compared with proximal superficial layers ($P < 0.05$) and distal deep layers ($107.3 \pm 9.7\%$) at threshold stimulation ($P < 0.05$) (Fig. 5C). This suggests that the highest level of glutamate signaling in the injured cortex occurs immediately adjacent to the lesion as well as in deep cortical layers proximal to the site of injury. A similar layer-specific pattern emerged in pharmacologically disinhibited CCI injured cortical slices, where glutamate signaling was significantly increased in proximal deep layers ($157.9 \pm 13.3\%$) compared with proximal superficial layers ($P < 0.01$) and distal deep layers ($139.8 \pm 14.1\%$) ($P < 0.01$). Additionally, however, glutamate signaling in distal deep layers was significantly increased compared with distal superficial layers ($95.4 \pm 4.1\%$) ($P < 0.05$) (Fig. 5D,E). Interestingly, disinhibited cortex from sham injured animals demonstrated the same pattern of cortical activation as disinhibited CCI injured cortical slices. Peak glutamate signaling was significantly increased in proximal deep layers ($148.3 \pm 4.4\%$) compared with proximal superficial layers ($P < 0.01$) and distal deep layers ($131.4 \pm 8.9\%$) ($P < 0.05$). Distal deep layers were significantly increased compared with distal superficial layers ($91.9 \pm 6.2\%$) ($P < 0.01$) (Fig. 5F,G). These data suggest that cortical glutamate increases in a layer-specific manner, and that deep layers, proximal to the

lesion, exhibit greater glutamate signaling following TBI. Additionally, these data suggest that glutamate networks remain largely intact following TBI.

Spatiotemporal Analysis of Glutamate Signaling Shows Consistent Patterns of Activation in the Injured and Disinhibited Brain

To further understand the properties of altered glutamate network function following TBI, we analyzed the temporal parameters of glutamate signal onset. ROIs were drawn over cortical subregions as in Figure 5 and compared within each slice. Figure 6A shows time-integrated $\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$ images from 5 ms of binned imaging data in CCI-injured cortex at threshold stimulation. Values reported for local activation are expressed as milliseconds after activation of the injury site (or isotopic area in shams) using nontime binned data. Thus, these values define the relative progression of glutamergic signaling through the cortical network once activity has been initiated. Using this approach, we found that proximal deep cortical areas activated significantly faster (4.8 ± 1.2 ms) compared with proximal superficial layers (9.8 ± 1.7 ms) ($P < 0.01$) and distal deep layers (12.0 ± 1.9 ms) ($P < 0.01$) 2–4 weeks post-injury. The onset of glutamate signal in distal superficial layers (16.4 ± 2.8 ms) was significantly slower compared with proximal superficial layers ($P < 0.01$) and distal deep layers ($P < 0.05$) (Fig. 6B). The same temporal pattern of glutamate signal onset can be seen in pharmacologically disinhibited CCI injured slices (Fig. 6C,D) and in disinhibited slices from sham-injured cortex (Fig. 6E,F). The sequential order of

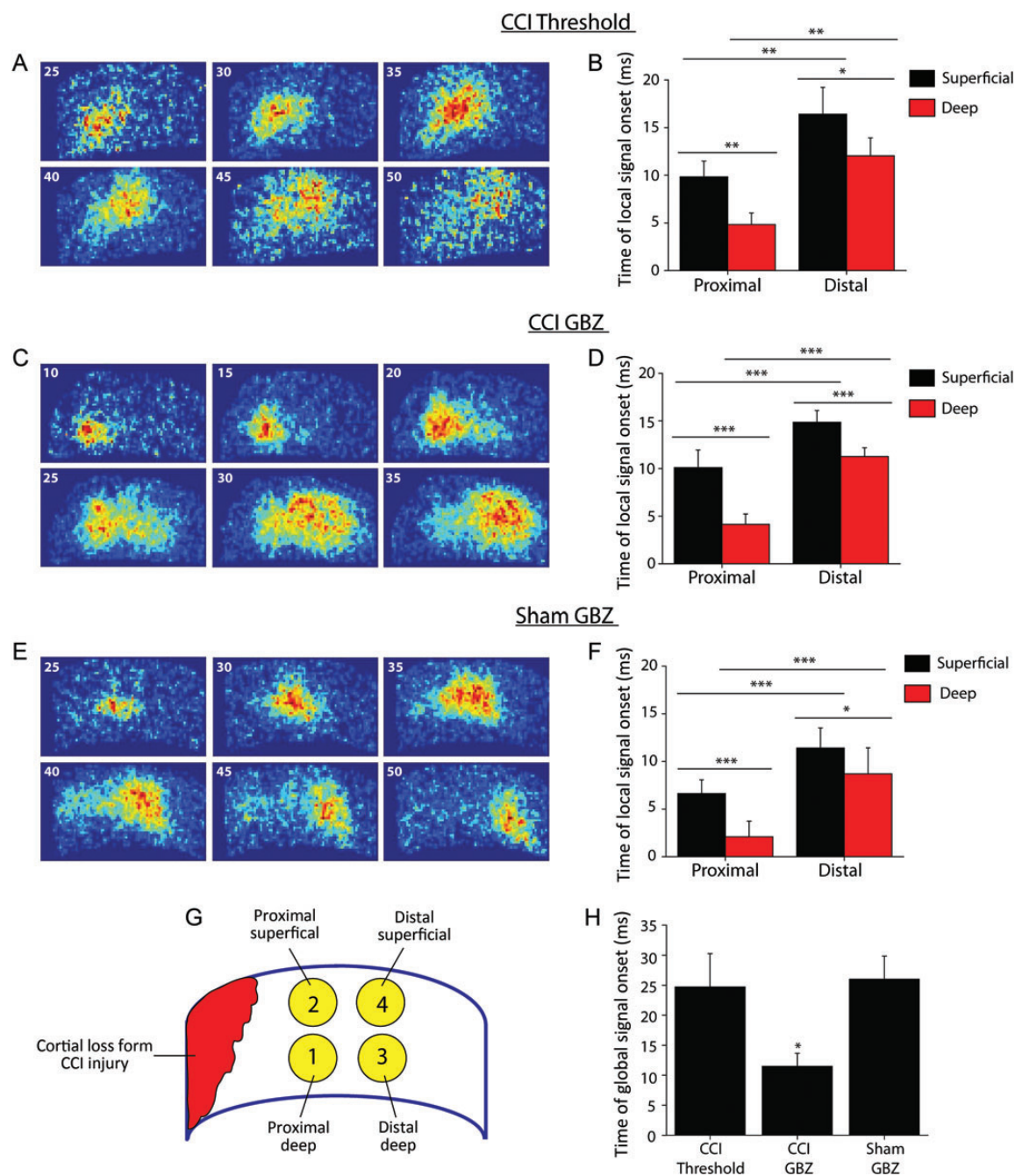


Figure 6. Temporal analysis of glutamate signaling in the cortex following TBI. (*A, C, E*) Time-integrated $\Delta\text{FRET}_{\text{signal}}/\Delta\text{FRET}_{\text{noise}}$ images from 5-ms binned increments illustrating regional specific onset of glutamate signal in CCI-injured cortex at threshold stimulation (*A*), disinhibited CCI-injured cortex in 10 μM GBZ (*C*), and disinhibited sham-injured cortex in 10 μM GBZ (*E*); post-stimulation time of each frame is indicated in white in upper left corner of each image. (*B, D, F*) Time of local signal onset from CCI-injured cortex at threshold stimulation (*B*), disinhibited CCI-injured cortex in 10 μM GBZ (*D*), and disinhibited sham-injured cortex in 10 μM GBZ (*F*). Time of signal onset was calculated as the average time in milliseconds at which pixels within a given cortical subregion crossed a threshold of 2.5 SDs above the noise. Superficial and deep areas proximal to the injury activate earlier than superficial and deep distal areas; deep layers are activated earlier than superficial layers both proximally and distally, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, paired-*t* test. (*G*) Diagram illustrating the sequential time of local signal onset within cortical subregions. (*H*) Time of global signal onset from whole slices showing CCI-injured cortex in GBZ activates earlier than CCI-injured cortex at threshold stimulation and sham-injured cortex in GBZ, $^{*}P < 0.05$, two-sample *t*-test, bars represent mean + SEM, $n = 15$ sham, 10 CCI slices.

cortical subregion signal onset is illustrated in Figure 6*G*. These findings suggest that the glutamate network from CCI-injured cortex activates with similar temporal parameters as in disinhibited cortex. This also suggests that even though a significant injury has occurred following CCI, the temporal sequence of cortical activation remains surprisingly similar.

We next analyzed the time of global signal onset by calculating the time at which glutamate signaling was detectable at any

region in the entire slice, and without using the time of glutamate signaling in the injury site as a point of temporal reference. Thus, this measure indicated the latency to the initiation of network activation. Using this analysis approach, we found that slices from disinhibited CCI-injured cortex activated significantly faster (11.5 ± 2.2 ms) compared with CCI-injured cortex at threshold stimulation (24.7 ± 5.5 ms, $P < 0.05$) and disinhibited sham-injured cortex (26.0 ± 3.9 ms, $P < 0.05$)

(Fig. 6H). The increased rate of activation of glutamate signaling in the CCI cortex when GABA_A receptor-mediated inhibition is absent suggests increased axon excitability. This is consistent with the decreased threshold stimulation intensity and the increased glutamate release seen in CCI slices. Alternatively, this could result from decreased glutamate reuptake capacity in the CCI-injured cortex.

Parvalbumin and Somatostatin Positive GABAergic Interneurons are Decreased Following TBI

The dysfunctional glutamate network in CCI-injured cortex exhibits similar spatiotemporal activation parameters as disinhibited cortex. In order to determine whether loss of GABAergic interneurons contributes to increased excitation 2–4 weeks after TBI, we first examined immunoreactivity for parvalbumin in sham- and CCI-injured cortex (Fig. 7A, left panel). Parvalbumin is an intracellular calcium binding protein used to identify subpopulations of GABAergic interneurons (Celio 1986; Cowan et al. 1990; Van Brederode et al. 1990). In the CCI-injured cortex, the density of parvalbumin positive (PV+) cells was significantly decreased (0.84 ± 0.17) compared with sham-injured cortex (2.22 ± 0.23 , $P < 0.01$) (Fig. 7B). This suggests that a global loss of PV+ GABAergic interneurons

occurs during the latent period following TBI. We then asked whether there were differences in PV+ cell density between deep and superficial cortical layers. The border between deep and superficial layers was roughly between layers 3 and 4 (superficial layers $\approx$ layers 1–3; deep layers $\approx$ layers 4–6). Within sham injured cortical slices, the number of PV+ cells from deep cortical layers (2.12 ± 0.22) was statistically greater than superficial layers (1.56 ± 0.16 , $P < 0.05$). This is consistent with previous observations showing a higher density of PV+ cells in mammalian cortical layer V (Glezer et al. 1993; Hof et al. 1999; Goldshmit et al. 2010). We therefore separately compared the number of PV+ cells between sham- and CCI-injured cortex within the deep layers from those of superficial layers and subdivided cortical regions as in Figure 5A. CCI-injured cortex showed a significant decrease in PV+ cell density in the area adjacent to the lesion, and throughout deep layers when compared with isotopic regions in sham-injured cortex ($P < 0.01$) (Fig. 7C). Similar findings were seen in superficial cortical layers ($P < 0.01$) (Fig. 7D). Furthermore, the area adjacent to the site of injury showed a significantly decreased number of PV+ cells compared with both deep (Fig. 7C, $P < 0.01$) and superficial layers (Fig. 7D, $P < 0.01$) within the CCI-injured cortex. In addition to PV staining, cortical slices

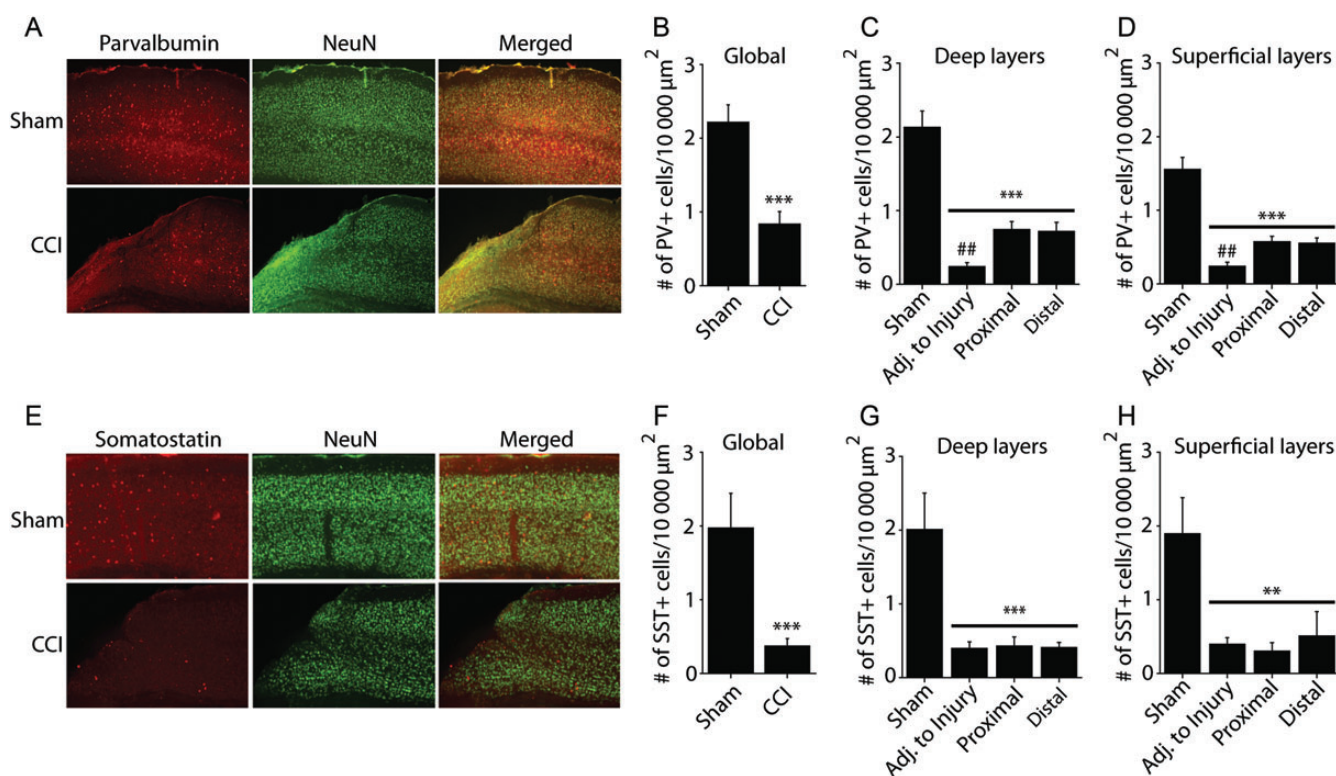


Figure 7. Number of parvalbumin (+) and somatostatin (+) GABAergic interneurons decrease following TBI. Representative images from PV (A) (red, left) and SST (E) (red, left) from sham- and CCI-injured cortical sections. NeuN images are shown in green and merged images are in yellow. Fewer PV+ and SST+ GABAergic interneurons are seen in CCI compared with sham-injured cortex. Global quantification of PV+ (B) and SST+ (F) cells per 10 000 μm^2 cortex from the entire cortical slice shows a significant decrease in cell number in CCI compared with sham-injured cortex, paired *t*-test. (C) Quantification of PV+ cells per 10 000 μm^2 in CCI injured deep cortical subregions showing a significant decrease in cell number in the area adjacent to the CCI lesion, proximal deep and distal deep layers compared with deep sham layers. The area adjacent to the injury was significantly decreased compared with proximal and distal superficial layers, **** $P < 0.001$ compared with sham, ## $P < 0.01$ compared with proximal and distal regions, one-way ANOVA, bars represent mean $\pm$ SEM, $n = 3$ sham, 4 CCI animals; 22 sham, 40 CCI sections. (D) Quantification of PV+ cells per 10 000 μm^2 in CCI injured superficial cortical subregions showing a decrease in cell number in the area adjacent to the CCI lesion, proximal superficial and distal superficial layers compared with deep cortical sham layers. The area adjacent to the injury was significantly decreased compared with proximal and distal superficial layers, **** $P < 0.001$ compared with sham, ## $P < 0.01$ compared with proximal and distal regions, one-way ANOVA, bars represent mean $\pm$ SEM, $n = 3$ sham, 4 CCI animals; 22 sham, 40 CCI sections. (G) Quantification of SST+ cells per 10 000 μm^2 in CCI injured deep cortical subregions showing a significant decrease in cell number in the area adjacent to the CCI lesion, proximal deep and distal deep layers compared with deep sham layers. (H) Quantification of SST+ cells per 10 000 μm^2 in CCI injured superficial cortical subregions showing a decrease in cell number in the area adjacent to the CCI lesion, proximal superficial, and distal superficial layers compared with deep cortical sham layers, *** $P < 0.01$, **** $P < 0.001$ compared with sham, one-way ANOVA, bars represent mean $\pm$ SEM, $n = 3$ animals, 16 sections.

were co-stained for NeuN in order to identify all neuronal cell types. No changes in NeuN-positive cells were detected between sham- (10.39 ± 0.56) and CCI-injured cortex (9.21 ± 0.53 cells/10 000 μm^2) (Fig. 7A, middle panel). This suggests that selective loss of GABAergic interneurons, rather than universal neuronal loss occurs during the latent period following TBI. These data suggest that loss of PV+ interneurons likely contributes to enhanced cortical glutamate network excitability following TBI.

We next examined immunoreactivity for somatostatin (Fig. 7E, left panel) in order to verify that the loss of GABAergic interneurons was not specific to PV+ interneurons. Somatostatin is a neuropeptide that labels a subsection of interneurons which do not overlap with PV+ interneurons in the neocortex (Rudy et al. 2011). The density of somatostatin-positive (SST+) cells was significantly decreased in CCI-injured cortex (0.38 ± 0.10) compared with sham-injured cortex (1.98 ± 0.47 , $P < 0.001$) (Fig. 7F). CCI-injured cortex showed a significant decrease in SST+ cell density in the area adjacent to the lesion, and throughout deep layers when compared with isotopic regions in sham-injured cortex ($P < 0.001$) (Fig. 7G). Similar findings were seen in superficial cortical layers ($P < 0.01$) (Fig. 7H). This suggests that global loss of multiple subtypes of GABAergic interneurons may be contributing to increased excitation 2–4 weeks following TBI.

Astrocytic Glutamate Transporters GLT-1 and GLAST are Unaltered 2–4 Weeks Following TBI

Protein expression of GLT-1 and GLAST, the 2 primary astrocytic glutamate transporters in the brain, has been shown to acutely decrease in rat cortex 6–72 h postinjury (Rao et al. 1998). In order to determine whether decreased expression of astrocytic glutamate transporters contributed to increased glutamate signaling, we compared protein expression of GLT-1 and GLAST in sham vs. CCI cortex 2–4 weeks post-injury. Western blot analysis of cortical lysates from CCI-injured animals showed no change in GLT-1 protein expression compared with sham-injured controls (Fig. 8A,B). GLAST protein expression was also not significantly altered in CCI-injured cortex compared with sham (Fig. 8C,D). These data suggest that astrocytic glutamate transporters are not downregulated in mice 2–4 weeks post CCI injury, and that deficiencies in astrocytic glutamate transporters likely do not contribute to increased excitation following TBI.

Spontaneous Excitatory Postsynaptic Currents Increase and Inhibitory Postsynaptic Currents Decrease Following TBI

Increased cortical glutamate network activity can be mediated by increased excitatory or decreased inhibitory connectivity. In order to differentiate between changes in inhibitory and excitatory systems 2–4 weeks postinjury, we obtained whole-cell recordings of spontaneous excitatory postsynaptic currents (sEPSCs) and spontaneous inhibitory postsynaptic currents (sIPSCs) from layer V of sham- and CCI-injured cortex. Holding single pyramidal neurons at a potential of -70 mV allowed us record sEPSCs while a holding potential of 0 mV was used to record sIPSCs. sEPSCs frequency was increased in CCI-injured cortex (gray trace) compared with sham cortex (black trace) (Fig. 9A). sEPSC amplitude was indistinguishable between sham- and CCI-injured cortex when average traces were superimposed

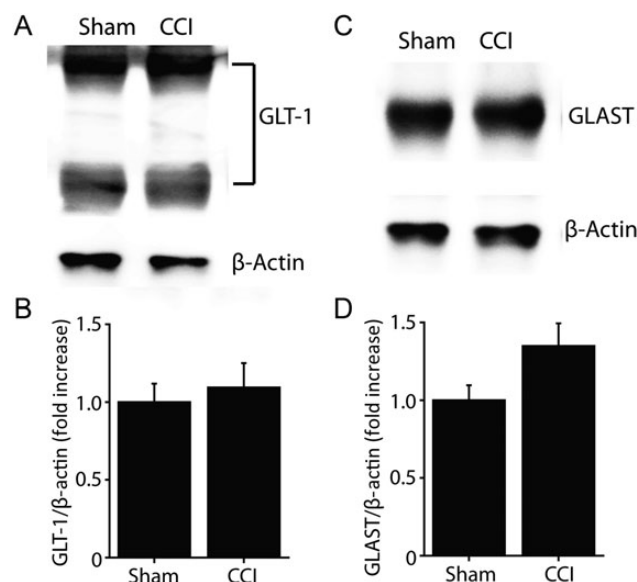


Figure 8. GLT-1 and GLAST protein expression does not change after TBI. Western blot analysis of cortical lysates prepared from sham- and CCI-injured cortex 2–4 weeks postinjury. Samples were probed for the astrocytic glutamate transporters GLT-1 (A) and GLAST (C). Protein expression was quantified by dividing optical density of GLT-1 (B) or GLAST (D) to β -actin, values are expressed as fold increase relative to sham controls, $n = 3$ mice.

(Fig. 9B). Cumulative probability distribution of sEPSC amplitude (Fig. 9C) and average amplitude (Fig. 9D) showed no difference between sham and CCI cortex. However, cumulative probability distribution of sEPSC interevent interval and average frequency demonstrated a significant increase in CCI slices compared with sham (Fig. 9E, $P < 0.001$; Fig. 9F, $P < 0.05$). We next measured sIPSCs from sham (black trace) and CCI (gray trace) cortical slices. sIPSC frequency was decreased in CCI slices compared with sham (Fig. 9G). Similar to our sEPSC recordings, superimposed average sIPSCs showed a similar amplitude between sham and CCI cortex (Fig. 9H). Cumulative probability distribution of sIPSC amplitude (Fig. 9I) and average amplitude (Fig. 9J) did not show a significant difference between sham and CCI cortical slices. On the other hand, cumulative probability distribution of sIPSC interevent interval and average frequency demonstrated a significant decrease in CCI cortex compared with sham (Fig. 9K, $P < 0.001$; Fig. 9L, $P < 0.05$). These data suggest that cortical excitatory input increases and inhibitory control decreases during the latent period following TBI.

Discussion

Four major findings emerge from these studies. First, extracellular glutamate signaling is increased in cortical networks following TBI but before the onset of reported electroconvulsive seizures (Hunt et al. 2009; Statler et al. 2009; Bolkvadze and Pitkanen 2012). Second, increased glutamate signaling during an epileptiform event in the injured cortex follows a layer-specific progression, spreading medial to lateral through the deep cortical layers. Once glutamate signaling initiates in a given deep cortical region, glutamate signaling is detected in the adjacent superficial cortical layers with a ~ 10 ms delay. To our knowledge, this is the first study showing the spatial and temporal properties of cortical network activation following

cortical injury in an in vivo model of TBI. Third, the immunoreactivity of markers for inhibitory interneurons in and around the cortical injury is decreased. Fourth, sIPSC frequency is decreased in pyramidal neurons from cortical layer V after TBI. These results show that inhibitory control in the cortex is lost and suggests that this underlies glutamatergic network dysfunction and increased excitability following TBI. These studies pinpoint PV+ and SST+ interneurons as cell types that are likely compromised following TBI. As normal cortical function relies heavily on feed-forward inhibition, loss of 2 main interneuronal subtypes undoubtedly has multiple effects of how sensory information reaches the cortex and is then processed by both local and long-range circuits.

To gain a better understanding of these circuits, we used FRET-based glutamate biosensor imaging to create spatial and temporal maps of cortical glutamate network function during epileptiform discharges. Interestingly, CCI slices at threshold stimulation showed similar patterns of glutamate signaling (Fig. 5) when compared with slices from disinhibited sham injured animals. In both control and injured slices, glutamate signaling was highest in deep cortical layers. This is consistent with previous observations showing that cortical layer V propagates epileptiform activity (Telfeian and Connors 1998) likely due to its extensive intralaminar connectivity. Similar to the spatial parameters of glutamate signaling, the temporal properties of CCI-injured cortex at threshold stimulation demonstrated the same local glutamate signal onset signature as disinhibited sham injured slices (Fig. 6). Deep layers proximal to the lesion site were activated first, followed by proximal superficial layers, then distal deep layers, and finally superficial distal layers. This suggests that global loss of inhibitory control

following TBI leads to hyperexcitability. These findings also provide novel insight into the initiation and sequential activation of cortical glutamatergic signaling through the cortex following TBI. The surprisingly similar activation patterns seen in CCI and control cortex suggests that many facets of cortical network connectivity and function in the injured brain may remain relatively unchanged even following severe cortical injuries.

Functional and circuit-level reorganization of both cortex and hippocampus do occur following TBI (Card et al. 2005; Nishibe et al. 2010). How these changes contribute to epileptogenesis and later seizures remains unclear. Our findings showing that sEPSC frequency increases in pyramidal neurons from layer V following TBI (Fig. 9) is consistent with increases in excitatory connectivity seen in multiple models of epilepsy. Mossy fiber sprouting occurs following CCI and correlates with the degree of hippocampal hyperexcitability (Hunt et al. 2009). In both the partial cortical isolation model of neocortical epileptogenesis (Li et al. 2012) and in models of temporal lobe epilepsy (Buckmaster et al. 2002), there is an increase in excitatory synapse number. Lastly, in models of developmental cortical malformations, there is increased excitatory input onto deep layers cortical neurons (Brill and Huguenard 2010).

Compromised inhibitory connectivity is also widely reported both in CCI and in models of epilepsy. Inhibitory interneurons are lost in the hippocampus after TBI in animals models (Lowenstein et al. 1992; Yang et al. 2007; Ding et al. 2011; Pavlov et al. 2011) and PV+ cells are specifically lost following human TBI (Buritica et al. 2009). In models of temporal lobe epilepsy, loss of GABAergic interneurons is commonly reported and thought to be a key epileptogenic event (Obenaus et al. 1993;

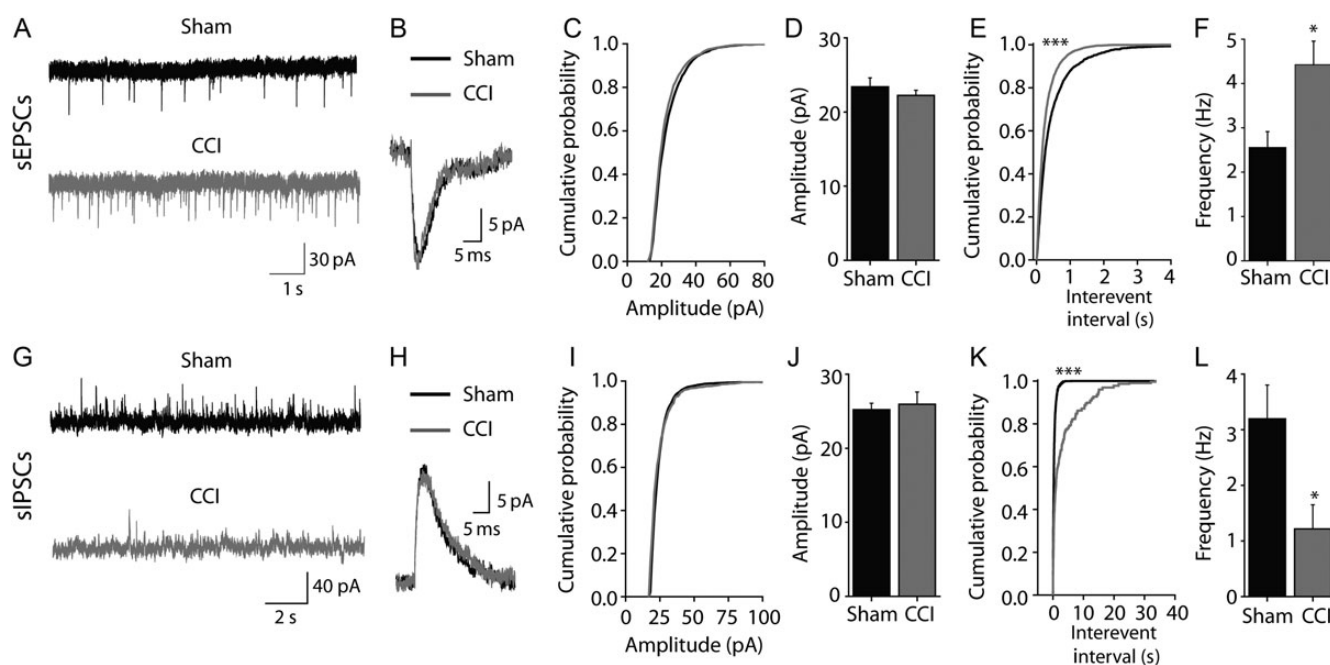


Figure 9. TBI increases the frequency of sEPSCs and decreases the frequency of sIPSCs. (A) Representative traces of sEPSCs collected from cortical layer V after sham (black) or CCI injury (gray). (B) Superimposed representative average sEPSCs show no difference in amplitude between sham and CCI slices. Cumulative probability distribution of sEPSC amplitude (C) and average amplitude (D) show no significant difference between sham- and CCI-injured cortex. Cumulative probability distribution of sEPSC interevent interval (E) and average frequency (F) show a significant increase in CCI-injured cortex compared with sham; *** $P < 0.001$, K-S test; * $P < 0.05$, mean + SEM, two-sample t -test; $n = 9$ sham, 11 CCI cells. (G) Representative traces of sIPSCs from sham (black) and CCI (gray) cortex. (H) Superimposed representative average sIPSCs demonstrate no difference in amplitude between sham and CCI cortex. Cumulative probability distribution of sIPSC amplitude (I) and average amplitude (J) show no significant difference between sham and CCI. Cumulative probability distribution of sEPSC interevent interval (K) and average frequency (L) show a significant decrease in CCI-injured cortex compared with sham; *** $P < 0.001$, K-S test; * $P < 0.05$, mean + SEM, two-sample t -test; $n = 8$ sham, 12 CCI cells.

Houser and Esclapez 1996; Buckmaster and Jongen-Relo 1999). This is consistent with our findings that PV and SST immunoreactivity is globally lost 2–4 weeks post CCI (Fig. 7). However, in the pilocarpine model of temporal lobe epilepsy and the cortical undercut model of post-traumatic epileptogenesis, GABAergic dysfunction rather than cell loss appears to underlie hyperexcitability (Cossart et al. 2001; Ma and Prince 2012). Our findings showing that sIPSC frequency is decreased in CCI cortex (Fig. 9) supports that functional loss of GABAergic control contributes to increased cortical glutamate network activity after TBI.

Our studies do not strictly differentiate between changes in inhibitory and excitatory systems in the cortex following CCI and, in fact, suggest mixed changes. The lack of difference between glutamate network signaling in the disinhibited CCI and sham cortex (Fig. 4), the large global loss of PV+ and SST+ interneurons (Fig. 7), and the decreased sIPSC frequency in CCI cortex (Fig. 9) suggest that changes in inhibitory, rather than excitatory systems may be more significant following CCI. On the other hand, the increased rate of activation of glutamate signaling (Fig. 6), the decreased threshold stimulation intensity in CCI cortex, the ability to block epileptiform activity using an NMDA receptor antagonist (Fig. 2), and the increased sEPSC frequency in CCI cortex (Fig. 9) suggest that glutamatergic signaling drives epileptiform activity following TBI. Considering the traumatic nature of CCI, it is not surprising that multiple cell types and neurotransmitter systems are affected. The similarities in the localization of interneuron loss and enhanced glutamate signaling is perhaps the most interesting component of these studies and underscores the potential for synergistic dysfunction of excitatory and inhibitory systems. Furthermore, local dysfunction of the remaining inhibitory systems in cortical areas proximal to the site of the lesion may explain why glutamate signaling is activated in a layer-specific manner following TBI. Our studies do not differentiate between loss of inhibitory interneurons versus decreased function and expression of PV and SST. It is important to note that while almost all CCI animals had a significant loss of PV+ and SST+ cells and increased glutamatergic signaling, only a small fraction will go on to develop epilepsy (Hunt et al. 2009; Statler et al. 2009; Bolkvadze and Pitkanen 2012). What causes seizures to occur or not occur in the injured brain? This is an unanswered question of much debate. There are likely a multitude of both proconvulsive and anticonvulsive factors at play including stress, sleep disruptions, hormonal modulation, fluctuation in neuromodulators, and many more. These specific questions are of great interest but remain unanswered.

Evidence supports that many molecular, biochemical, and physiological alterations may lead to network reorganization and increased glutamate signaling in the brain following injury. For example, extracellular glutamate levels increase (Nilsson et al. 1994; Folkersma et al. 2011) while glutamate transport decreases (Sullivan et al. 1998) in rat cortex within the first hour following TBI. Although neuronal glutamate transporters are capable of removing extracellular glutamate, the majority of synaptic glutamate is removed by astrocytic glutamate transporters (Anderson and Swanson 2000). Our studies do not directly address the possibility that neuronal glutamate transport is altered following TBI which could significantly affect synaptic glutamate signaling (Scimemi et al. 2009). The ability of astrocytes to effectively remove synaptic glutamate may contribute to increased glutamate signaling

immediately following injury. However, expression of astrocytic glutamate transporters return to normal levels during the latent period, suggesting that alterations in glutamate transport are not responsible for cortical hyperexcitability in our study. This is supported by observations showing that protein expression of GLT-1 and GLAST return to normal levels 1 week postinjury (Rao et al. 1998; but also see Zou et al. 2013) and remain unchanged between sham and CCI cortex 2–4 weeks postinjury (Fig. 8). The initial burst of extracellular glutamate coupled with decreased glutamate transport within the first hours following TBI may begin to drive synaptogenesis and changes in neuronal connectivity. This could facilitate feed-forward excitation; leading to further glutamate increases, pathological changes in cortical connectivity, and eventual development of spontaneous seizures. This is supported by previous studies showing that glutamate can drive synapse formation during development (Cline and Haas 2008; Kwon and Sabatini 2011).

Undoubtedly, many other mechanisms also contribute to TBI pathology including increased inflammation (Fan et al. 1995; Frugier et al. 2010; Helmy et al. 2011), caspase activation (Sullivan et al. 2002; Larner et al. 2005; Krajewska et al. 2011), oxidative stress (Paolin et al. 2002; Pratico et al. 2002), and changes in mitochondrial metabolism (Verweij et al. 1997; Xiong et al. 1998). How these cellular and metabolic changes contribute to, or are driven by, synaptic and network level dysfunction following TBI remains to be seen. Perhaps, using agents such as 2-deoxyglucose (Stafstrom et al. 2008) or cyclosporine A (Yokobori et al. 2013) to alter cellular metabolic function or focal cooling (D'Ambrosio et al. 2013) immediately following TBI can reduce the effects of increased epileptogenesis. In conclusion, this work demonstrates that extracellular glutamatergic signaling is increased and GABAergic inhibitory control is decreased 2–4 weeks following CCI. These changes undoubtedly mediate cortical network dysfunction during the latent period, and likely contribute to spontaneous seizures. Our findings provide novel insight into how cortical networks function in the injured brain and suggest potential circuit-level mechanisms which may contribute to post-traumatic epileptogenesis.

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