Excitatory synaptic transmission and network activity are depressed following mechanical injury in cortical neurons

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In vitro and in vivo traumatic brain injury (TBI) alter the function and expression of glutamate receptors, yet the combined effect of these alterations on cortical excitatory synaptic transmission is unclear. We examined the effect of in vitro mechanical injury on excitatory synaptic function in cultured cortical neurons by assaying synaptically-driven $[\text{Ca}^{2+}]_i$ oscillations in small neuronal networks as well as spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs). We show that injury decreased the incidence and frequency of spontaneous neuronal $[\text{Ca}^{2+}]_i$ oscillations for at least 2 days post-injury. The amplitude of the oscillations was reduced immediately and 2 days post-injury, although a transient rebound at 4 hours post-injury was observed due to increased activity of N-methyl-D-aspartate (NMDARs) and calcium permeable $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (CP-AMPARs). Increased CP-AMPAR function was abolished by the inhibition of protein synthesis. In parallel, mEPSC amplitude decreased immediately, 4 hours and 2 days post-injury, with a transient increase in the contribution of synaptic CP-AMPARs observed at 4 hours post-injury. Decreased mEPSC amplitude was evident after injury, even if NMDARs and CP-AMPARs were blocked pharmacologically, suggesting the decrease reflected alterations in synaptic Glur2-containing, calcium-impermeable AMPARs. Despite the transient increase in CP-AMPAR activity that we observed, the overriding effect of mechanical injury was long-term depression of excitatory neurotransmission that would be expected to contribute to the cognitive deficits of TBI.
INTRODUCTION

Traumatic brain injury (TBI) can produce cognitive, motor, and behavioral dysfunction including deficits in memory, attention, and executive function (Ashman et al. 2006). Deficits following TBI reflect neuronal loss and defective neurotransmission in surviving neurons, including altered connectivity and network circuitry, changes in excitability, unbalanced excitatory and inhibitory input, and abnormal synaptic function and plasticity (Cohen et al. 2007).

Most studies of neuronal electrical activity following experimental TBI have assayed synaptic function and plasticity in the hippocampus (Cohen et al. 2007). These studies reveal region-specific changes in circuit excitability and an inability to induce long-term potentiation (LTP) (D'Ambrosio et al. 1998; Miyazaki et al. 1992; Reeves et al. 1995; Sanders et al. 2000; Schwarzbach et al. 2006), and are attributed to injury-induced cell death (Golarai et al. 2001), aberrant synaptic connectivity (Golarai et al. 2001; Santhakumar et al. 2001), abnormal presynaptic function (Cole et al.; Reeves et al. 2000), and changes in excitatory and inhibitory postsynaptic currents/potentials mediated by glutamate and GABA<sub>A</sub> receptors, respectively (Cohen et al. 2007; Schwarzbach et al. 2006; Witgen et al. 2005). There are fewer studies of cortical electrophysiology following TBI, hindering our understanding of how mechanical injury affects synaptic transmission in cortex.

We therefore examined the effect of in vitro mechanical injury on synaptic and network activity in cortical pyramidal neurons, and focused on alterations of excitatory synaptic transmission mediated by postsynaptic glutamatergic AMPARs and NMDARs. We previously demonstrated alterations in whole-cell NMDAR and AMPAR-mediated currents in cortical pyramidal neurons
subjected to stretch-injury \textit{in vitro} (Goforth et al. 1999; 2004; Zhang et al. 1996). In addition, differences in the expression of the NMDAR subunits NR1, NR2A, and NR2B, and the AMPAR subunits GluR1 and Glur2 are reported in hippocampus and cortex following \textit{in vivo} TBI (Bell et al. 2009; Biegon et al. 2004; Kumar et al. 2002; Osteen et al. 2004; Schumann et al. 2008). Recent studies report a switch in AMPAR composition from Glur2-containing receptors that are mostly calcium impermeable to calcium permeable receptors lacking Glur2 (CP-AMPARs) following mechanical injury of cortical neurons \textit{in vitro}, and that activation of CP-AMPARs post-injury increases neuronal cell death (Bell et al. 2009; Spaethling et al. 2008).

Despite the progress made toward understanding how TBI alters NMDAR and AMPAR expression and function, the effect of these alterations on functional cortical synaptic transmission remains unclear. Previous evidence of increased whole-cell NMDA and AMPA currents and CP-AMPAR upregulation following mechanical trauma of cortical neurons supports the hypothesis that there is enhanced glutamatergic synaptic transmission following injury (Park et al. 2008). In contrast, we demonstrate here that trauma caused prolonged depression of excitatory synaptic transmission and network activity, while only transiently altering the contribution of CP-AMPARs to synaptic transmission following \textit{in vitro} TBI.

\section*{MATERIALS AND METHODS}

\subsection*{Cortical cell culture and injury}

All animal use was in compliance with protocols approved by the University of Michigan Committee on Use and Care of Animals. Primary cultures of neurons and glia were prepared using a modified version of a standard method (Huettner and Baughman 1986). In brief,
neocortices were isolated from 1-2 day old Sprague-Dawley rats (Harlan, Indianapolis, IN), tissue was minced coarsely, and incubated at 34°C for 1.5 hr in a solution containing 10 U/mL papain. Following incubation, the tissue was rinsed with trypsin inhibitors followed by growth media consisting of MEM supplemented with 5% calf serum, 25 mM glucose, 100 U/mL penicillin, 100 µg/L streptomycin, and 500 nM glutamine. The growth medium was first conditioned by overnight incubation in flasks containing confluent astrocytes. After rinsing, tissue was gently tritured in 1-2 mls of growth media using fired-polished Pasteur pipettes of decreasing tip size. The resulting cell suspension was then plated at a density of 5x10^5 cells/25mm well onto a confluent layer of astrocytes grown on deformable Silastic membranes that formed the bottom of 6-well plates (Flexcell International Corp., Hillsborough, NC). Cell cultures were incubated at 37°C in 95/5% air/CO₂ fed twice weekly with conditioned growth media, and were utilized after 14-21 days in vitro (DIV). Mechanical injury was delivered to each well using a Cell Injury Controller II, as described (Ellis et al. 1995). To simulate mild/moderate injury, a 50 ms pulse of compressed air was used to deform the Silastic membranes of the 6-well culture plate (2.5 cm diameter) by 6.5 mm, corresponding to 38% stretch of the membrane and attached cells (Ellis et al. 1995; McKinney et al. 1996). Cells were then washed 3X and growth medium replaced with extracellular recording solution (see below). For studies that were conducted at 4 hours or 2 days post-injury, cells were incubated in media at 37°C following injury. Control cells were treated identically except that no injury was delivered.

**Measurement of intracellular free calcium**

Intracellular free calcium ([Ca^{2+}]_i) was assayed using the calcium indicator fura-2 AM (Invitrogen Corp.). Neuronal cultures were incubated with 5 µM fura-2 AM plus 0.01% pluronic
acid for 1 hr at 37°C, conditions which minimized fura-2 loading of astrocytes. Neuronal fluorescence was imaged using an Olympus BX51W1 (Olympus; Tokyo, Japan) upright microscope equipped with a Hamamatsu ‘Orca’ camera (Hamamatsu, Japan) and Ludl shutter/filter wheel (Ludl, Germany). Intracellular fura-2 was sequentially excited at 340 and 380 nm, the excitation wavelengths of Ca$^{2+}$-bound and Ca$^{2+}$-free fura-2, respectively, and emitted fluorescence was collected at 510 nm. Images were collected using IPLab 3.6.5 software every 2 s and the ratio of fluorescence intensity generated in response to excitation at 340 and 380 nm (F$_{340}$/F$_{380}$) was calculated and plotted versus time. Images were simultaneously collected from clusters of cultured cortical neurons, typically 8-15 neurons per experiment. [Ca$^{2+}$]$_i$ was monitored under basal conditions (no drug treatment) and during the subsequent application of 30 µM bicuculline methiodide (BMI), 20 µM D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV), and 50 µM 1-naphthylacetyl spermine trihydrochloride (Naspm). 30 mM KCl was added at the end of each experiment to activate voltage-gated Ca$^{2+}$ channels to confirm cell viability, and only those cells exhibiting an increase in [Ca$^{2+}$]$_i$ in response to KCl were included in the analysis. Drugs were applied at a flow rate of approximately 1 ml/minute using a peristaltic pump. Oscillation parameters were measured at least 3 minutes following drug application to ensure complete solution exchange and to allow oscillations to reach a steady-state. Neurons were defined as having basal oscillatory activity if at least 3 transient increases in F$_{340}$/F$_{380}$ greater than 0.02 relative ratio units were observed. The presence of basal oscillatory activity was obvious upon visual inspection. Relative basal [Ca$^{2+}$]$_i$ levels were measured as the initial F$_{340}$/F$_{380}$ ratio. F$_{340}$/F$_{380}$ plots were then baseline subtracted and analyzed using a custom program implemented in MatLab software (2007b, The MathWorks, Inc., Natick, MA). Oscillation amplitude was calculated as the change in fura-2 ratio from baseline (ΔF$_{340}$/F$_{380}$).
Recordings were made from control and injured neurons matched from the same culture at each time point post-injury and experiments were repeated using neurons from at least 3 different culture preparations. All experiments were conducted at room temperature.

Measurement of spontaneous and miniature excitatory postsynaptic currents sEPSCs and (mEPSCs)

sEPSCs and mEPSCs were measured from individual cortical pyramidal neurons using the tight-seal whole-cell voltage clamp technique (Hamill et al. 1981) and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Pyramidal neurons were identified by their characteristic morphology. Currents were filtered at 2 kHz and digitized at 5 kHz using a Macintosh G4 computer equipped with an Instrutech ITC-16 computer interface (Instrutech, Great Neck, NY) and Pulse Control (Herrington 1994) and Igor Pro software (Wavemetrics, Lake Oswego, OR).

Patch electrodes were made from borosilicate glass capillaries (WPI, Sarasota, FL) that were pulled to a tip resistance of 3-6 MΩ using a Brown/Flaming P-97 micropipette puller (Sutter Instr. Co., Novato, CA) and filled with a solution containing (in mM): 135 CsAsp, 4 KCl, 2 NaCl, 10 EGTA, 0.2 CaCl₂, 2 MgATP, 0.6 Na₂GTP, 10 HEPES, pH 7.2. The external recording solution contained (in mM): 130 NaCl, 4 KCl, 3 CaCl₂, 2 MgCl₂, 10 HEPES, 11 glucose, 0.01 glycine, 0.0005 TTX, 30 μM bicuculline methiodide, pH 7.3. For low magnesium experiments, extracellular MgCl₂ was reduced to 0.05 mM without substitution of other ions. For sEPSC recordings, TTX was excluded from the extracellular solution and 5 mM QX314 was added to the intracellular solution to block Na⁺-dependent action potentials in the cell from which sEPSC recordings were made. Drugs were applied using a SF-77 ‘Fast-Step’ rapid perfusion system (Warner Ins. Co., Hamden, CT) as previously described (Goforth et al. 1999). Cells were
continuously superfused with bath-applied standard external solution at a rate of approximately 2 ml/min. Neurons selected for analysis had stable series resistances (Rs) < 30 MΩ.

Data analysis and statistics

In our cultures, sEPSCs occur in bursts that can exhibit complex waveforms. For each cell, maximal burst amplitude, charge transfer during the burst, and burst duration were averaged from at least 5 bursts and compared for control and injured neurons. mEPSC events were analyzed using a commercial software package (Synaptosoft; Decatur, GA). Individual events were detected using an amplitude threshold value of 5 pA, and confirmed visually. mEPSC amplitude, 10-90% rise time, half-width, and charge transfer were determined in the presence and absence of drug treatments. Decay time constants (tau) were calculated from single exponential fits of averaged mEPSC traces. To construct cumulative probability distributions, 75 random events were selected from each neuron using Synaptosoft software and events from control and injured cells were respectively pooled for each experimental condition. Statistical comparisons of cumulative distributions were made with MatLab using the non-parametric Kolmogorov-Smirnov test and significance was defined as p<0.001. All other statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) and statistical significance determined using Fisher’s exact test for percentages, student’s t-test for comparison of two groups, or ANOVA and Newman-Keuls multiple comparison post-hoc test or the non-parametric Kruskall Wallis and Dunn’s post test for comparison of multiple groups. Statistical differences were defined as p< 0.05.

RESULTS
Mechanical injury depresses synaptically-driven $[\text{Ca}^{2+}]_i$ oscillations in cortical neurons.

As cortical neurons mature in culture, they develop spontaneous and synchronous $[\text{Ca}^{2+}]_i$ oscillations that have been shown to reflect excitatory synaptic transmission (Murphy et al. 1992; Robinson et al. 1993). These oscillations emerge in parallel with the formation of synaptic connections (Nakanishi and Kukita 1998), and their timing coincides with bursts of action potentials (Robinson et al. 1993) and EPSPs (Murphy et al. 1992) recorded simultaneously in adjacent neurons or in the same neurons, respectively. $[\text{Ca}^{2+}]_i$ oscillations are abolished by blocking glutamate receptors or action potentials, further indicating their mediation by excitatory synaptic transmission. We thus measured simultaneous $[\text{Ca}^{2+}]_i$ oscillations in groups of cortical neurons to assay the effect of mechanical injury on excitatory neurotransmission within small neural networks.

Intracellular free calcium was monitored using fura-2 AM immediately (within 10 minutes), 4 hours, or 2 days following \textit{in vitro} stretch-injury, and the results compared to recordings from uninjured control neurons (Fig. 1). Cortical neurons exhibited spontaneous $[\text{Ca}^{2+}]_i$ oscillations (Fig. 1A) that required action potential firing and excitatory synaptic activity as they were abolished by tetrodotoxin (TTX) or by a combination of the NMDAR and AMPAR antagonists, APV and CNQX (data not shown). Blockade of inhibitory GABA$_A$ receptors with BMI (30 µM) briskly increased the frequency and amplitude of the oscillations (Fig. 1A). The subsequent applications of APV and then Naspm, a selective inhibitor of CP-AMPARs, were used to assay the contribution NMDARs and CP-AMPARs, respectively, to the $[\text{Ca}^{2+}]_i$ oscillations (Fig. 1).
As in other studies, basal $[\text{Ca}^{2+}]_i$ increased soon after stretch-injury, as evidenced by increased baseline $F_{340}/F_{380}$ ratios in recordings that commenced within 10 minutes post-injury (Table 1). Basal $[\text{Ca}^{2+}]_i$ then returned to control levels at 4 hours and remained so for 2 days post-injury (Table 1). Immediately post-injury, 30% (44/149) of injured neurons were incapable of producing $[\text{Ca}^{2+}]_i$ oscillations, even when exposed to BMI. However, these neurons continued to exhibit normal responses to 30 mM KCl, indicating the loss of oscillations we observed was not due to a generalized loss of membrane integrity and/or cell death. The initial increase in basal $[\text{Ca}^{2+}]_i$ was significantly higher in this subset of injured neurons compared to injured neurons that retained the capability to produce oscillations (Table 1). Injured neurons that did not demonstrate $[\text{Ca}^{2+}]_i$ oscillations in response to BMI were excluded from further analysis, as oscillations were the endpoint we chose as a monitor of synaptic function.

Of neurons capable of producing oscillations in response to BMI, injury decreased the percentage of cells exhibiting basal calcium oscillations prior to BMI addition (Fig. 1 arrow, 2A). Thus immediately post-injury, 52.1% of control neurons exhibited spontaneous basal $[\text{Ca}^{2+}]_i$ oscillations versus 16.2% of the injured neurons. The decrease in the number of neurons exhibiting basal activity persisted at least 2 days, at which time 32.2% of injured neurons displayed basal oscillations versus 66.3% of control neurons. At four hours post-injury, basal $[\text{Ca}^{2+}]_i$ activity was not significantly different from controls (Fig. 2A), most likely due to an increase in the activation calcium permeability of postsynaptic glutamate receptors at this time point (see below).

Injury-induced suppression of basal $[\text{Ca}^{2+}]_i$ oscillations suggests that injury alters the balance of excitatory and inhibitory drive. To further examine the effect of injury on excitatory
neurotransmission only, we compared the frequency and amplitude of the \([Ca^{2+}]_i\) oscillations after blocking inhibitory GABA_A receptors with BMI, which resulted in more regular and larger amplitude \([Ca^{2+}]_i\) oscillations and removed the contribution of fast inhibitory synaptic transmission from network activity. We detected no significant difference in the frequency of \([Ca^{2+}]_i\) oscillations observed in the presence of BMI immediately post-injury; however, a significant reduction in oscillation frequency was apparent at 4 hours and 2 days post injury (Fig. 2B). In addition, the mean amplitude of the \([Ca^{2+}]_i\) oscillations decreased significantly immediately and 2 days post-injury (Fig. 2C). However, at 4 hours post-injury, oscillation amplitude was not significantly different from controls (Fig. 2C). These data demonstrate that there is an overall depression of oscillatory activity mediated by the excitatory synaptic network that persisted for at least 2 days, with a transient mitigation of injury-induced suppression of amplitude apparent at 4 hours post-injury. As discussed below, the rebound of oscillation amplitude occurred during a time when there was a transient upregulation of calcium permeable glutamatergic receptors following injury.

*Injury increases the contribution of NMDA and calcium permeable AMPA receptors to \([Ca^{2+}]_i\) oscillations*

Previous studies reported increased expression of calcium permeable AMPA receptors (CP-AMPAR) (Bell et al. 2009; Spaethling et al. 2008) and enhanced NMDA receptor function (Lea et al. 2003; Zhang et al. 1996) following mechanical injury. Although the upregulation of CP-AMPARs after mechanical injury has been linked to increased cell death (Bell et al. 2009; Spaethling et al. 2008), the consequences of such upregulation for excitatory synaptic function in surviving neurons is unclear. We thus examined the sensitivity of the neuronal \([Ca^{2+}]_i\)
oscillations to the competitive NMDAR antagonist APV, and to Naspm, a selective antagonist for CP-AMPARs that is a synthetic analog of Joro spider toxin (Koike et al. 1997; Takazawa et al. 1996), to determine the relative contribution of these receptors to synaptic and network activity in control vs. injured neurons. BMI, APV and Naspm were thus added sequentially during the experiments. The activity of NMDARs was assessed as the percent inhibition by APV, calculated by comparing $[Ca^{2+}]_i$ oscillation amplitude in BMI versus BMI + APV. Likewise, the contribution of CP-AMPARS was determined by comparing oscillation amplitude in the presence of BMI + APV versus BMI + APV + Naspm. Naspm sensitivity was determined in the presence of APV in order to isolate injury effects on AMPARergic transmission and eliminate the contribution of NMDAR-mediated changes to oscillation amplitude. Oscillations were more sensitive to 20 µM APV immediately and 4 hours post-injury, indicating an early increase in NMDAR activity, with NMDAR activation returning to control levels by 2 days post-injury (Fig. 3A). In addition, we observed an increased contribution of CP-AMPAR activation to oscillation amplitude, evidenced as a larger Naspm-blockable component, at 4 hours post-injury only, with no significant differences noted either immediately or 2 days following injury (Fig. 3B). The combined increase in $Ca^{2+}$ influx mediated by NMDARs and CP-AMPARs at 4 hours post-injury thus appeared to compensate for the suppression we observed immediately and 2 days post-injury, as oscillation amplitude was not significantly different than controls at this time point (Fig. 3C). However, when $Ca^{2+}$ influx though NMDARs and CP-AMPARs was acutely blocked with APV and Naspm, $[Ca^{2+}]_i$ oscillation amplitude was then significantly smaller for injured cells versus control (Fig.3C). These data suggest that the mechanisms responsible for oscillation depression, as observed immediately and 2 days post-injury, are still present at 4 hours but are masked by the transient increase in NMDAR and CP-AMPAR
activation. These findings indicate that while injury alters the function and/or expression of NMDAR and CP-AMPAR receptors, these alterations only transiently affect neuronal function at early time points post-injury, with the more predominant effect of injury being a reduction of the \([Ca^{2+}]_i\) oscillations that persists for at least 2 days post-injury, the latest time point we monitored in this study.

**Injury-induced enhancement of CP-AMPAR function is selectively prevented by inhibiting protein synthesis.**

An upregulation of CP-AMPARs is observed in many neuropathologies, including ischemia (Calderone et al. 2003; Kwak and Weiss 2006; Liu and Zukin 2007), neurodegeneration (Kwak and Weiss 2006), epilepsy (Rogawski and Donevan 1999), inflammatory pain (Jones and Sorkin 2004; Vikman et al. 2008) and neurotrauma (Bell et al. 2007), but also occurs in certain forms of synaptic plasticity (Cull-Candy et al. 2006; Isaac et al. 2007), including homeostatic plasticity, the process by which neurons dynamically scale their synaptic strength to compensate for increases or decreases in overall synaptic activity (Ju et al. 2004; Sutton et al. 2006; Turrigiano 2008). The insertion of synaptic CP-AMPARs during homeostatic plasticity triggered by drug-induced mEPSC blockade requires protein synthesis (Ju et al. 2004; Sutton et al. 2006). We therefore examined whether the injury-induced upregulation of CP-AMPARs we observed at 4 hours post-injury involved protein synthesis. Neuronal cultures were thus incubated with the protein synthesis inhibitor anisomycin (40 µM) starting 30 minutes prior to injury and continuing for 4 hours post-injury. The relative contributions of NMDARs and CP-AMPARs were again assessed by quantifying the sensitivity of \([Ca^{2+}]_i\) oscillation amplitudes to APV and Naspm, respectively. As shown in Fig. 4A, anisomycin prevented the increase in Naspm sensitivity that
was observed at 4 hours post-injury, demonstrating that protein synthesis was required for increased CP-AMPAR activity following injury. We also examined the effect of anisomycin treatment on [Ca\(^{2+}\)]\(_i\) oscillation amplitude, as we hypothesize that increased CP-AMPAR, in conjunction with increased NMDAR activity, compensate for the reduction in [Ca\(^{2+}\)]\(_i\) oscillation amplitude observed immediately and 2 days post-injury. As shown in Figure 4B, in the presence of APV, [Ca\(^{2+}\)]\(_i\) oscillation amplitude measured from untreated injured neurons at 4 hours was not significantly different from control. However, [Ca\(^{2+}\)]\(_i\) oscillation amplitude was significantly decreased in neurons where the potentiation of CP-AMPARs was prevented by anisomycin treatment. These data support the hypothesis that increased CP-AMPAR activity contributes to the rebound of [Ca\(^{2+}\)]\(_i\) oscillation amplitude that we observe 4 hours post-injury. In addition, when the upregulation of CP-AMPARs was prevented by anisomycin treatment, a significantly smaller percentage of treated injured neurons exhibited basal oscillations (Fig. 4C), revealing the same hypo-excitability that we observed immediately and 2 days post-injury (Fig. 2A). Anisomycin also failed to prevent the injury-induced reductions we observed in oscillation frequency, indicating that these alterations must occur independently from enhancement of CP-AMPAR activity (Fig. 4D).

**Mechanical injury diminishes the amplitude of excitatory postsynaptic currents**

Although neuronal [Ca\(^{2+}\)]\(_i\) oscillations are dependent upon synaptic activity and the activation of NMDARs and AMPARs, their generation also involves action potential firing within the neuronal network, membrane depolarization, and the activation of voltage-gated Na\(^+\) and Ca\(^{2+}\) channels (Wang and Gruenstein 1997). Therefore, to confirm that the injury-induced suppression of [Ca\(^{2+}\)]\(_i\) oscillations we observed reflect alterations in underlying excitatory neurotransmission,
we next examined the effect of injury on spontaneous (action-potential dependent) and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs). As shown in Figure 5A, in the presence of bicuculline, sEPSCs occur as bursts of multiple synaptic events. Immediately following injury, maximal sEPSC burst amplitude (Fig. 5B) and charge transfer during the bursts of sEPSCs (Fig. 5C) were significantly reduced compared to control neurons. These reductions in amplitude and charge transfer persisted during the acute application of 20 µM APV, indicating injury primarily decreases AMPAergic synaptic transmission (Fig. 5 B,C). We observed no significant differences in sEPSC burst frequency (Fig. 5D) or sEPSC burst duration (Fig. 5E). We also did not detect any significant differences in the percent inhibition of sEPSC burst peak amplitude or charge transfer by 20 µM APV for injured neurons versus control (data not shown).

sEPSCs result from action potential-dependent activity in the synaptic network and as such, changes in sEPSCs may reflect changes in quantal release at numerous synaptic sites in the network, presynaptic membrane excitability, and the cellular links between action potentials and the release of quanta in multiple neurons. Thus, to more directly assess the function of postsynaptic glutamate receptors following injury, we also examined mEPSCs. Miniature synaptic events occur due to the spontaneous release of a single vesicle or quantum of glutamate onto a postsynaptic neuron, which in turn activates a small ensemble of postsynaptic glutamate receptors. Using whole-cell patch-clamp, mEPSC currents were recorded from individual voltage-clamped pyramidal neurons held at -65 mV in the presence of 0.5 µM TTX to block action potential-dependent synaptic currents and 30 µM BMI to block inhibitory synaptic currents mediated by GABA<sub>A</sub> receptors. The remaining mEPSCs were abolished by the co-
application of APV and CNQX, confirming their mediation by glutamate receptors (data not shown).

Cumulative probability distributions of mEPSC amplitude and kinetics were constructed and compared for control and injured neuronal populations immediately, 4 hours and 2 days post-injury. Paralleling the changes we observed in \([\text{Ca}^{2+}]_i\) oscillation amplitude following injury, mEPSC amplitude was significantly decreased immediately, 4 hours and 2 days post-injury compared to controls (Fig. 6A), as denoted by a leftward shift of the cumulative probability distributions (Fig. 6B). Alterations in mEPSC amplitude also significantly decreased total charge transfer across the membrane during the mEPSC, indicating that injury results in reduced excitatory synaptic strength that persists for at least 2 days (Fig. 6C). There were no significant changes in mEPSC rise time, half-width or mEPSC decay time constant (tau) at any time point following injury (Fig. 6D).

To test whether decreased mEPSC amplitude was due to a reduction in the NMDAR and/or the AMPAR components of the postsynaptic current, 20 µM APV was used to inhibit synaptic NMDARs and pharmacologically isolate the two components. mEPSCs were recorded in 2mM extracellular MgCl\(_2\), to match the conditions of \([\text{Ca}^{2+}]_i\) oscillation recordings, or 0.05 mM extracellular MgCl\(_2\), which reduces Mg\(^{2+}\) -blockade of NMDARs, thus allowing a more thorough examination of the activity of NMDARs at the synapse. As shown in Fig. 7A, mEPSCs contained an APV-sensitive component mediated by NMDARs. To quantify the NMDAergic component of the mEPSC, the average waveform of mEPSCs recorded in the presence of APV was subtracted from the average of mEPSCs in the absence of APV in the same cell, producing an APV-sensitive, NMDAergic difference current (Fig. 7B). The amplitude of APV-sensitive
difference currents measured in either 2 mM or 0.05 mM MgCl₂ did not differ significantly between control and injured neurons at any time point post-injury (Fig. 7C). We also compared the amount of mEPSC charge transfer inhibited by APV for control and injured neurons. As shown in Fig. 7D, although there appeared to be a trend toward a decrease in the amount of mEPSC charge transfer mediated by NMDARs following injury, yet these changes were not statistically significant (Fig 7D).

To confirm that reduced mEPSC amplitude was in fact due to a decrease in synaptic AMPAR activity, we compared mEPSCs recorded in the presence of APV for control and injured neurons. As shown in Fig. 8 (A-D), injury significantly reduced the amplitude of AMPA-mediated mEPSCs immediately, 4 hours and 2 days post-injury. We observed no significant change in the 10-90% rise time or tau of decay of AMPARergic mEPSCs following injury (Fig. 8 E,F). These data taken together therefore indicate that injury-induced suppression of mEPSCs is primarily due to reduced AMPAR but not NMDAR activity.

To determine whether injury increased the contribution of postsynaptic CP-AMPARs, as observed for [Ca²⁺]ᵢ oscillations, we examined the effect of 50 µM Naspm on mEPSC amplitude. We observed a transient increase in the contribution of CP-AMPARs to mEPSC amplitude 4 hours post-injury, as evidenced by changes in the sensitivity of mEPSC amplitude to Naspm (Fig. 9). mEPSCs recorded from uninjured control neurons contained a small CP-AMPAR-mediated component, as the application of Naspm significantly reduced mEPSC amplitude in controls (Fig. 9A,E). Interestingly, immediately post-injury, mEPSC amplitude appeared insensitive to Naspm (Fig 9 B,E), suggesting an early loss of CP-AMPAR activity at the synapse. Naspm sensitivity was also observed for mEPSCs recorded from neurons 4 hours and 2 days
post-injury (Fig. 9 C,D,E). As we observed for $[\text{Ca}^{2+}]_i$ oscillations, the sensitivity of mEPSC amplitude to Naspm was significantly increased at 4 hours post-injury compared to control, indicating enhanced synaptic CP-AMPAR activity at this time point only (Fig. 9F).

When NMDARs and CP-AMPARs were both acutely blocked after injury by the co-application of APV and Naspm, thereby isolating postsynaptic Glur2-containing AMPARs, we observed an overall reduction in mEPSC amplitude at all time points post-injury (Fig. 10). These findings demonstrate that despite the transient modulation of CP-AMPAR synaptic activity we observed with injury, alterations of Glur2-containing AMPARs mediate the persistent depression in the excitatory currents we observed.
Following mechanical injury, cortical neurons exhibited abnormal excitatory synaptic transmission and network activity due, in part, to alterations in postsynaptic AMPARs. Although injury transiently increased synaptic CP-AMPAR activity at 4 hours post-injury, the predominant effect we observed was the depression of mEPSCs and synaptically-driven neuronal \([\text{Ca}^{2+}]_i\) oscillations that occurred immediately post-injury and persisted at least 2 days. This depression most likely reflected decreased function and/or expression of synaptic AMPARs containing Glur2. 

\([\text{Ca}^{2+}]_i\) oscillation amplitude decreased immediately post-injury, recovered by 4 hours, but was again decreased at 2 days post-injury. Changes in \([\text{Ca}^{2+}]_i\) oscillation amplitude may reflect calcium influx through glutamate receptors but could also occur via alterations in action potential firing, membrane depolarization, and the activation of voltage-gated calcium channels that indirectly cause a rise in \([\text{Ca}^{2+}]_i\) (Wang and Gruenstein 1997). The rebound of \([\text{Ca}^{2+}]_i\) oscillation amplitude seen at 4 hours was likely due to an increase in NMDAR and CP-AMPAR activity. Although NMDAR activity was also increased immediately post-injury, without the additional potentiation of CP-AMPARs this enhancement was not sufficient to compensate for the decrease in \([\text{Ca}^{2+}]_i\) oscillation amplitude.

The suppression of \([\text{Ca}^{2+}]_i\) oscillation amplitude was paralleled by a reduction in mEPSC amplitude immediately and 2 days post-injury. Decreased mEPSC amplitude persisted when NMDARs and CP-AMPARs were acutely inhibited, indicating that synaptic depression resulted from alterations in synaptic Glur2-containing AMPARs that were insensitive to these agents.
As for $[\text{Ca}^{2+}]_i$, oscillation amplitude, we also observed an increase in CP-AMPAR activation during mEPSCs recorded 4 hours post-injury. Although CP-AMPARs have larger channel conductances compared to Glur2-containing AMPARs (Li et al. 2003; Swanson et al. 1997), in our hands, this did not result in larger mEPSCs. In fact, synaptic charge transfer actually decreased, thereby reducing the strength of the excitatory synapses. Thus, it appears that the contribution of CP-AMPARs to $[\text{Ca}^{2+}]_i$ oscillation amplitude occurs via calcium influx through these channels rather than indirectly through increased membrane depolarization. We also found no significant change in the kinetics of AMPARergic mEPSCs, despite the fact that CP-AMPARs exhibit more rapid kinetics (Grosskreutz et al. 2003; Isaac et al. 2007; Oh and Derkach 2005) and their synaptic incorporation can shorten mEPSC decay time (Guire et al. 2008). This may reflect a more modest change in the composition of synaptic AMPARs in our system following injury. Thus, although the Naspm-sensitivity of mEPSCs is significantly greater 4 hours post-injury versus control, the increased activity of synaptic CP-AMPARs at this time point does not substantially increase mEPSC amplitude (Fig. 6, 8) and the mEPSC waveform may still be governed primarily by GluR-containing AMPARs.

$[\text{Ca}^{2+}]_i$ oscillations exhibited enhanced sensitivity to APV immediately and 4 hours post-injury, indicating an increase in NMDAR contribution to the oscillations at these time points. This is consistent with our previous observation of increased NMDA-elicited calcium influx due to reduced Mg$^{2+}$ blockade of the NMDARs immediately post-injury (Zhang et al. 1996). However, we did not observe a corresponding increase in the NMDAR-mediated component of the mEPSC or sEPSC bursts at any time point post-injury, suggesting that increased NMDAR activity was unlikely to reflect changes in postsynaptic NMDAR function. Thus, enhanced NMDAR activity,
as measured via $[\text{Ca}^{2+}]_i$ oscillations may reflect the activation of extrasynaptic or presynaptic NMDARs that would not be expected to contribute to the mEPSC current, but would be evident when measuring network activity. The effect of injury on different populations of NMDARs clearly requires additional studies.

In addition to depressed oscillation amplitude, we found that fewer injured neurons exhibited spontaneous $[\text{Ca}^{2+}]_i$ oscillations under basal conditions and oscillation frequency was reduced at 4 hours and 2 days post-injury. The lower incidence of basal $[\text{Ca}^{2+}]_i$ oscillations in injured neurons could reflect not only decreased excitatory postsynaptic function, but enhanced inhibitory GABAergic function or a loss of excitatory neurons from the network. Hippocampal IPSCs are known to be altered by fluid percussion injury (Witgen et al. 2005), and GABA-activated currents from cortical neurons are increased by in vitro stretch-injury in our model (Kao et al. 2004). Neuronal death is unlikely here, as the mild injury we used is not associated with a significant degree of neuronal death at these time points (McKinney et al. 1996). Furthermore, alteration of mEPSCs cannot be explained by neuronal death, as these events directly reflect the functioning of individual synapses. Although we observed a trend toward decreased mEPSC frequency post-injury (data not shown), which may reflect decreased presynaptic function after TBI, these changes were not significant. Further studies will thus be necessary to explore the contribution of presynaptic changes to overall changes in excitatory transmission.

Our finding of depressed excitatory postsynaptic function in cortical neurons following mechanical injury qualitatively agrees with reports of reduced field excitatory post-synaptic potentials (fEPSPs) and population spike amplitude in hippocampal CA1 neurons 1 to 48 hours
following FPI (Cohen et al. 2007; Miyazaki et al. 1992; Reeves et al. 2000). Concomitant
decreases in NMDA-mediated postsynaptic potentials and glutamate-activated NMDAR and
AMPAR currents also suggest there are alterations in postsynaptic glutamate receptor function
after FPI injury (Schwarzbach et al. 2006). While our study focuses on mild/moderate injury, it
is possible that differences in injury mechanism and/or severity produce differential synaptic
changes. Thus, models where posttraumatic epilepsy is observed following cortical
deafferation, such as the partially isolated (‘undercut’) cortex model, produce
hyperexcitability and epileptiform activity (Avramescu and Timofeev 2008; Hoffman et al. 1994;
report increased sEPSC and mEPSC frequency and amplitude in layer V pyramidal neurons in
slices from chronically-injured neocortex (Li and Prince 2002). Similarly, deafferentation due to
the removal of superficial cortical layers in an in vitro neocortical slice preparation results in
increased sEPSC amplitude and frequency in layer V pyramidal neurons 2-8 hours after injury
(Yang et al. 2007). Changes in excitatory synaptic function occur in the context of complex
pathophysiology that includes changes in intrinsic membrane properties (Avramescu and
Timofeev 2008; Prince 1993), axonal sprouting (Salin et al. 1995), increased excitatory synaptic
connectivity (Avramescu and Timofeev 2008; Jin et al. 2006), and decreased inhibitory synaptic
transmission (Li and Prince 2002; Yang et al. 2007). Thus, while the findings of these studies
would appear at face value to contradict our study, these other cortical models correspond to
more focal and severe penetrating brain injuries, while our model directly assessed the effect of
mild, non-lethal injury, such as that encountered in closed head concussion. This suggests that
the cortex may respond quite differently depending on type and severity of the brain injury.
Other reports of injury-induced alterations of postsynaptic NMDARs and AMPARs include enhanced NMDAR and AMPA-mediated whole-cell currents immediately following stretch-injury in vitro (Cohen et al. 2007; Goforth et al. 1999; Lea et al. 2002; Zhang et al. 1996); and short-lived hyper-activation of hippocampal NMDARs following in vivo TBI (Biegon et al., 2004). Early enhancement of NMDAR activity is followed by a more sustained depression of activity in the cortex and the hippocampus which lasted hours to days following injury (Biegon et al. 2004). Kumar et al. (2002) report decreased expression of NR1, NR2A, and NR2B subunits in the hippocampus 6 to 12 hours following controlled cortical impact (CCI), and decreased NR2A and NR2B expression is observed in the cortex 1 to 4 days following FPI (Osteen et al. 2004). A biphasic effect of injury on NMDAR function is supported by the evidence for a short (<1 hr) therapeutic time window for applying NMDAR antagonists following trauma, and improvement in neurological deficits observed with more delayed treatment with NMDA or NMDAR agonists following TBI in vivo (Biegon et al. 2004; Yaka et al. 2007). We found there was early transient hyper-activation, but not long-term suppression, of NMDAR activity within the neuronal network. We also observed injury-induced alterations in postsynaptic AMPAR function that contributed to reduced excitatory synaptic strength. Synaptic strength is governed in large part by the trafficking of synaptic AMPARs under both physiological and pathological conditions (Isaac et al. 2007; Malinow and Malenka 2002; Santos et al. 2009). Although TBI activates numerous calcium-dependent kinases (CaMKII, PKC (Atkins et al. 2006; Yang et al. 1993; Zhang et al. 1996)), phosphatases (calcineurin; (Kurz et al.
2005), and proteases (calpain; (Kampfl et al. 1997), which can modify AMPAR trafficking and function, it is unclear how the convergence of these processes affects synaptic function.

In contrast to our observation of decreased synaptic AMPAR function, our laboratory previously described an increase in steady-state whole-cell AMPAR-mediated currents due to the slower activation and desensitization of AMPARs in response to exogenous agonist (Goforth et al. 1999; 2004). However, our previous study of whole-cell currents likely included the contribution of both synaptic and extrasynaptic receptors, while the present study included a specific examination of synaptic AMPAR function which may not reveal the altered receptor kinetics such as those observed during prolonged agonist exposure. Our observed changes in synaptic AMPAR function are consistent with a report of decreased GluR1 expression in the cortex 15 minutes after closed head injury (Schumann et al. 2008) as well as reports demonstrating increased CP-AMPAR expression in cortical and cerebellar neurons following in vitro stretch-injury (Bell et al. 2007; Bell et al. 2009; Spaethling et al. 2008) and in CA1 hippocampal neurons following FPI (Bell et al. 2009). As in the present study, Spaethling et al. (2008) found increased \( \text{Ca}^{2+} \) influx and ionic current through CP-AMPARs at 4 hours post injury in cortical neurons. Interestingly, these authors also reported decreased AMPA-evoked \([\text{Ca}^{2+}]\) responses at time points earlier than 4 hours post-injury, consistent with our finding that the contribution of CP-AMPARs to mEPSC amplitude decreased immediately following injury. An increase in postsynaptic CP-AMPAR expression was also reported 1 hour following a combined insult of stretch-injury plus exogenous NMDA in cortical neurons (Bell et al. 2009). In this study, upregulation of CP-AMPARs required the activation of NR2B-containing NMDARs, phosphorylation of Glur2 Ser880 by PKC, and PICK1-mediated endocytosis of Glur2-
containing receptors (Bell et al. 2009). However, in contrast to our findings, Glur2 endocytosis paired with the insertion of CP-AMPARs increased mEPSC amplitude in this model. The differences between the former study and our study are likely due to their use of a milder degree of stretch-injury paired with the coincident application of exogenous NMDA, which may alter the time course of cellular signaling events, and/or activate additional NMDA-dependent signaling pathways targeting postsynaptic glutamate receptor expression and function differently (Cull-Candy et al. 2006; Cull-Candy and Leszkiewicz 2004; Isaac et al. 2007). It is possible that PICK1-dependent endocytosis of Glur2-containing receptors contributes to the decreased excitatory synaptic transmission that we observed following injury, as this mechanism is linked not only to LTP, but long-term depression (LTD) in cerebellar and hippocampal neurons (Chung et al. 2003; Seidenman et al. 2003; Terashima et al. 2008). Both Spaethling et al. (2008) and Bell et al. (2009) provide evidence that the prevention of CP-AMPAR upregulation imparts significant protection against secondary excitotoxic insult and delayed neuronal death 20-24 hours following injury. However, neither study examined CP-AMPAR expression nor synaptic function at time points longer than 6 hours post-injury, as addressed in the present study.

The insertion of synaptic CP-AMPARs plays a role in mediating diverse forms of physiological synaptic plasticity (Cull-Candy et al. 2006; Isaac et al. 2007). In cortical cultures, a 3 hour suppression of neuronal activity and mEPSC blockade results in the insertion of calcium permeable Glur1 homomers in the synapse (Sutton et al. 2006). This process, a form of homeostatic plasticity, depends upon local dendritic protein synthesis and trafficking of Glur1 subunits, and is prevented by protein synthesis inhibitors (Sutton et al. 2006). The upregulation of CP-AMPARs is transient, as the Glur1 homomers are then replaced by Glur2-containing
AMPARs over a 24 hour period (Sutton et al., 2006). Likewise, the increase in CP-AMPAR activity that we observed depended upon protein synthesis and may reflect an attempt by the neurons to restore synaptic strength following an initial depression due to injury. Future studies are needed to confirm whether injury increases the synthesis and trafficking of Glur1 homomers. The short-lived nature of CP-AMPAR enhancement coupled with the persistent depression of mEPSCs following injury, suggests that neurons are unable to sustain the transient increase in AMPARs and may lack the ability to replenish synaptic Glur2-containing receptors, or that extant Glur2-containing synaptic AMPARs are chronically altered by injury.

To our knowledge, this is the first report of decreased AMPAR-mediated excitatory synaptic transmission and neuronal [Ca$^{2+}$], oscillations in cortex following mechanical injury. Because glutamatergic synaptic transmission is essential for proper brain function, it will be a challenge to identify new therapeutic approaches to TBI to mitigate neuronal death and dysfunction while not hindering normal synaptic function and plasticity. Although, pharmacological inhibition of NMDARs and CP-AMPARs appears to be an attractive means for preventing neuronal death after TBI, their blockade may not ameliorate cognitive deficits due to the more persistent suppression of cortical excitatory synaptic transmission we describe here. Elucidation of both mechanisms would thus benefit the development of potential new therapies for TBI.

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FIGURE LEGENDS

**Figure 1.** Mechanical injury depressed [Ca^{2+}]_i oscillations in cortical neurons. A. Recording of [Ca^{2+}]_i from an individual cortical neuron using fura-2. Fura ratios (F_{340}/F_{380}) are plotted versus time. Spontaneous [Ca^{2+}]_i oscillations (arrow) occur prior to the application of BMI, which produced larger amplitude oscillations. APV and Náspm were added sequentially to inhibit NMDARs and CP-AMPARs, respectively. B. Recording of [Ca^{2+}]_i from an individual neuron immediately (within 10 min.) following stretch-injury, C. 4 hours post-injury and D. 2 days post-injury. Spontaneous [Ca^{2+}]_i oscillations are absent from injured neurons prior to BMI application.

**Figure 2.** Injury decreased neuronal [Ca^{2+}]_i oscillation frequency and amplitude A. The percentage of neurons displaying spontaneous [Ca^{2+}]_i oscillations prior to disinhibition with BMI was significantly decreased immediately post-injury (n=105 cells, 9 wells) versus control (n=96 cells from 9 culture wells, p<0.001 by Fisher’s exact test) and 2 days post-injury (n=87 cells, 9 wells) versus control (n=92 cells, 8 wells, p<0.01), yet was not statistically different at 4 hours post-injury (n=112 cells, 9 wells) versus control (n=109 cells, 9 wells, p>0.05) B. The frequency of [Ca^{2+}]_i oscillations recorded in the presence of 30 µM BMI did not differ immediately post-injury, 0.042 ± 0.001 Hz for control (n=91) versus 0.042 ± 0.001 Hz for injured neurons (n=105, p>0.05), but was significantly decreased 4 hours post-injury, 0.043 ± 0.002 Hz for control (n=112) versus 0.034 ± 0.001 Hz for injured neurons (n=109, p<0.01), and at 2 days post-injury, 0.033 ± 0.002 Hz for control (n=83) versus 0.022 ± 0.001 Hz for injured neurons (n=78, p<0.001). C. Injury decreased the amplitude of [Ca^{2+}]_i oscillations recorded in the presence of 30 µM BMI immediately post-injury, 0.31 ± 0.01 for control (n=91) versus 0.18 ± 0.01 for injured
neurons (n=105, p<0.001), and 2 days post-injury, 0.32 ± 0.02 for control (n=83) versus 0.24 ± 0.02 for injured neurons (n=78, p<0.01, but was not significantly different at 4 hours post-injury, 0.40 ± 0.02 for control (n=112) versus 0.41 ± 0.02 for injured neurons (n=109, p>0.05).

Measurements are baseline subtracted (F340/F380) relative units.

Figure 3. Injury transiently increased the contribution of NMDARs and CP-AMPARs to neuronal \([\text{Ca}^{2+}]_i\) oscillations. A. The percent inhibition of \([\text{Ca}^{2+}]_i\) oscillation amplitude by the NMDAR antagonist APV (20 µM) was significantly greater immediately post-injury, 44 ± 2.0% for control (n=79 cells from 9 wells) versus 52.4 ± 2.0% for injured neurons (n=87 cells from 8 wells, p<0.05), and 4 hours post-injury, 42.3 ± 1.7% for control (n=110 cells from 9 wells) versus 53.8 ± 1.2% for injured neurons (n=109 cells from 9 wells, p<0.001), and did not differ at 2 days post-injury, 33.6 ± 4.0% for control (n=83 cells from 8 wells) versus 32.6 ± 4.6% for injured neurons (n=66 cells from 8 wells, p>0.05). B. The percent inhibition of \([\text{Ca}^{2+}]_i\) oscillation amplitude by the selective CP-AMPAR antagonist Naspm (50 µM) was significantly greater 4 hours post-injury, 15.5 ± 3.4% for control (n=95 cells from 8 wells) versus 33.8 ± 1.7% for injured neurons (n=92 cells from 8 wells, p<0.001), yet did not differ from controls immediately post-injury, 12.9 ± 1.9% for control (n=58 cells from 5 wells) versus 6.3 ± 3.6% for injured neurons (n=33 cells from 3 wells, p>0.05) or at 2 days post-injury, 18.3 ± 2.8% for control (n=44 cells from 4 wells) versus 20.5 ± 3.5% for injured neurons (n=33 cells from 4 wells, p>0.05). All measurements were made in the presence of 30 µM BMI. C. Calcium oscillation amplitude did not differ at 4 hours when measured in the presence 30 µM BMI only; yet was significantly decreased when measured in the presence of 30 µM BMI + 20 µM APV, 0.22 ± 0.01 for control (n=111 cells from 9 wells) versus 0.19 ± 0.01 for injured (n=109 cells from 9 wells; p< 0.01).
Oscillation amplitude was also significantly reduced when recorded in the presence of BMI + APV + 50 µM Naspm, 0.18 ± 0.01 for control (n=96) versus 0.14 ± 0.01 for injured (n=92), p < 0.001.

**Figure 4. Injury-induced upregulation of CP-AMPARs was dependent on protein synthesis**

**A.** Incubation with the protein synthesis inhibitor anisomycin (40 µM) prevented upregulation of CP-AMPARs at 4 hours post-injury. The percent inhibition of \([\text{Ca}^{2+}]_i\) oscillation amplitude by Naspm (50 µM) was significantly greater at 4 hours post-injury for untreated injured neurons (47.8 ± 6.4 %, n=57 cells from 5 wells) compared to untreated control neurons (7.1 ± 5.6%, n=50 cells from 5 wells, p<0.001, **), anisomycin-treated injured neurons (8.5 ± 4.1%, n=55 cells from 5 wells, p<0.001,###), or anisomycin-treated controls (10.3 ± 6.3%, n=30 cells from 3 wells, p<0.001,+++). % inhibition by Naspm did not differ between anisomycin-treated injured neurons, untreated control neurons or anisomycin-treated control neurons, p>0.05.) **B.** \([\text{Ca}^{2+}]_i\) oscillation amplitude was significantly decreased for anisomycin-treated injured neurons 4 hours post-injury (0.18 ± 0.02) compared to control (0.25 ± 0.02, **p < 0.01) or untreated injured neurons (0.25 ± 0.01, ## p < 0.01). Recordings were made in the presence of BMI and APV. **C.** The percentage of anisomycin-treated injured neurons displaying basal \([\text{Ca}^{2+}]_i\) oscillations (23.6%) was significantly smaller than untreated control neurons (50.9%, **p<0.01, ), anisomycin-treated control neurons (47.4%, ## p<0.01, ) and untreated injured neurons (62.8%, n=43, ++ p<0.001,). **D.** \([\text{Ca}^{2+}]_i\) oscillation frequency was significantly decreased for untreated injured neurons (0.038 ± 0.002 Hz,) and anisomycin-treated injured neurons (0.037 ± 0.002 Hz,) compared to untreated control (0.056 ± 0.004 Hz, ***p<0.001,) and anisomycin treated control neurons (0.062 ± 0.006 Hz, ###p<0.001,).
**Figure 5.** Injury reduces sEPSC amplitude and charge transfer A. Examples of bursts of sEPSCs in a control and injured neuron immediately post injury. Scale bars - 200pA, 0.5 s. B. Injury reduced the maximal amplitude of sEPSC bursts immediately post-injury (732.4 ± 107.7 pA, n=12) versus control (1228.0 ± 196.8 pA, n=14 *p<0.05). The maximal amplitude of AMPAR-mediated sEPSC bursts, recorded in the presence of APV, was also reduced (701.6 ± 81.6 pA for injured neurons versus 1302.1 ± 148.5 pA for control, p<0.05). C. Injury reduced the charge transfer during sEPSC bursts immediately post-injury in the absence of APV (109.3 ± 23.9 pC for injured versus 217.4 ± 47.1 pC for control, p<0.05) and in the presence of APV (61.4 ± 9.8 pC for injured versus 114.1 ± 24.1 pC, p <0.05). D. sEPSC burst frequency recorded from neurons immediately post-injury in either the presence or absence of APV did not differ from control neurons, p >0.05). E. sEPSC burst duration recorded from neurons immediately post-injury in either the presence or absence of APV did not differ from control neurons, p >0.05).

**Figure 6.** Injury alters mEPSC amplitude A. Voltage-clamp recordings of mEPSCs from individual cortical pyramidal neurons. B. Cumulative probability distributions of mEPSC amplitude for control neurons (1800 events, n=24 cells) and injured populations immediately (900 events, n=12 cells), 4 hours (1050 events, n=14 cells) and 2 days post-injury (1050 events, n=14 cells). Control distributions are indicated by a solid black line, injured distributions by the dashed grey line. Injury decreased mEPSC amplitude, as indicated by a significant leftward shift of the cumulative probability distribution immediately, 4 hours and 2 days post-injury (***, p<0.001). Inset: average mEPSC traces for cells in A. (scale=5pA, 5 ms) control=black trace, injured=grey. Mean mEPSC amplitude averaged from individual cells was 17.2 ± 1.0 pA (n=24) for control neurons, 13.1 ± 1.1 pA (n=12) immediately, 14.4 ± 0.8 pA (n=14) 4 hours and 13.0 ±
1.2 pA (n=14) 2 days post injury (*=p<0.05 versus control). C. Injury significantly decreased mEPSC charge transfer, as indicated by significant leftward shifts of the cumulative distributions immediately, 4 hours, and 2 days post-injury (***, p<0.001). Mean mEPSC charge transfer averaged from individual cells was 75.0 ± 7.9 fC for control neurons, 55.4 ± 9.1 fC immediately post-injury, 53.3 ± 8.0 fC 4 hours post-injury and 43.5 ± 4.7 fC 2 days post injury. *=p<0.05 versus control. D. Injury did not alter mEPSC kinetics. Mean mEPSC 10-90% rise time was 1.32 ± 0.08 ms for control neurons, 1.41 ± 0.11 ms immediately, 1.29 ± 0.08 ms at 4 hours and 1.19 ± 0.11 ms at 2 days post-injury, p>0.05. Mean mEPSC half-width duration was 3.9 ± 0.3 ms for control neurons, 3.7 ± 0.3 ms immediately, 3.7 ± 0.4 ms at 4 hours and 3.0 ± 0.2 ms at 2 days post injury (p>0.05). Mean tau decay was 4.6 ± 0.3 ms for control neurons, 5.0 ± 0.5 ms immediately, 4.8 ± 0.4 ms at 4 hours and 4.1 ± 0.2 ms at 2 days post injury (p>0.05).

**Figure 7.** Injury does not alter synaptic NMDAR function. A. Average mEPSC waveform recorded from an individual control neuron, in 0.05 MgCl₂, in the presence (black trace) and absence (grey trace) of APV. B. APV-sensitive difference current, calculated as the average mEPSC-no APV minus average mEPSC + APV. C. Mean APV-sensitive mEPSC currents did not differ for control and injured neurons either in 2 mM MgCl₂ (control n=22, immediate n=10, 4 hour n=11, 2 days n=10, p > 0.05) or 0.05 mM MgCl₂ (control n=15, immediate n=8, 4 hour n=7, 2 days n=6). D. APV-sensitive difference in mEPSC charge transfer did not differ significantly for injured neurons versus control in either 2 mM MgCl₂ or 0.05 mM MgCl₂.

**Figure 8.** Injury reduces the AMPAergic component of mEPSCs. A-C. Cumulative probability distributions of mEPSC amplitude recorded in the presence of 20 µM APV for control neurons (n= 25 cells) and injured populations immediately (n=11 cells), 4 hours (n=19...
cells) and 2 days post-injury (n=22 cells). Control distributions are indicated by a solid black line, injured distributions by the dashed grey line. Injury decreased mEPSC amplitude, as indicated by a significant leftward shift of the cumulative probability distribution immediately, 4 hours and 2 days post-injury (***, p<0.001). D. Mean mEPSC amplitude averaged from individual cells was 16.5 ± 0.9 pA for control neurons, 11.5 ± 0.9 pA immediately, 14.1 ± 0.7 pA 4 hours and 12.8 ± 0.8 pA 2 days post-injury (*=p<0.05, **p<0.01 versus control). E. Injury did not alter the kinetics of the AMPAergic component of the mEPSC. Mean mEPSC 10-90% rise time in the presence of APV was 0.83 ± 0.03 ms for control, 0.81 ± 0.04 ms immediately, 0.84 ± 0.06 ms at 4 hours and 0.78 ± 0.04 ms at 2 days post-injury, p>0.05. F. Mean tau decay of mEPSCs recorded in the presence of APV was 3.9 ± 0.2 ms for control neurons, 4.4 ± 0.4 ms immediately, 4.1 ± 0.4 ms at 4 hours and 3.7 ± 0.2 ms at 2 days post injury (p>0.05).

**Figure 9. Injury increases postsynaptic CP-AMPAR function.** A-D. Cumulative probability distributions of mEPSC amplitude recorded in the presence (dashed grey line) and absence (solid black line) of 50 µM Naspm (+BMI and APV). Insets: average mEPSC in the presence (black) and absence (grey) of Naspm from individual neurons. Naspm significantly decreased mEPSC amplitude (noted as a leftward shift of the cumulative distribution) for control neurons, 4 hours post-injury and 2-days post injury (***, p<0.001). E. Naspm decreased mean mEPSC amplitude in control neurons (n=21, p< 0.05), 4 hours post-injury (n=12, p<0.01) and 2 days post-injury (n=19, p< 0.05); but not immediately post-injury (n=10, p>0.05). F. The percent inhibition of mEPSC amplitude by Naspm was significantly increased at 4 hours post-injury when compared to control (22.1 ± 3.7 % vs. 8.9 ± 4.1%, p<0.05); but not immediately (0.01 ± 4.5 %, p>0.05) or 2 days post-injury (6.0 ± 3.5 %, p >0.05).
Figure 10  Injury alters Glur2-containing AMPAR function. A.  Cumulative probability distributions of mEPSC amplitude recorded during blockade of NMDAR and CP-AMPAR with 20 µM APV + 50 µM Naspm show a significant decrease in mEPSC amplitude at all time points post-injury (noted by a leftward shift of the cumulative distribution, ***, p<0.001). Control distributions are indicated by the solid black line, injured distributions by the dashed grey line B. Mean mEPSC amplitude recorded in APV+ Naspm averaged from individual neurons *=p<0.05 versus control.
Table 1

<table>
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<th>Initial F$<em>{340}$/F$</em>{380}$ ratio</th>
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<tr>
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<tr>
<td>immediate (+) oscillations  (n=105)</td>
<td>0.65 ± 0.03 ***</td>
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*** p < 0.001 vs. control
### p < 0.001 vs. injured (+) oscillations
Figure 1

A. control

B. immediate

C. 4 hours

D. 2 days

Time (min)

F340/F380

KCl 30 μM bicuculline 20 μM APV 50 μm naspm

Time (min)

F340/F380

KCl 30 μM bicuculline 20 μM APV 50 μm naspm

Control immediate 4 hours 2 days

A. D.C.
Figure 2

A. Basal Activity

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<td>2 days</td>
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B. [Ca^{2+}]_i oscillation frequency

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<td>4 hours</td>
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<td>2 days</td>
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C. [Ca^{2+}]_i oscillation amplitude

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<th>Control</th>
<th>Injured</th>
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<tr>
<td>2 days</td>
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</table>
Figure 3

A. % inhibition by APV

B. % inhibition by Naspm

C. 4 hours post-injury
Figure 5

A. control vs. injured

B. EPS amplitude (pA)

C. EPSC burst charge transfer (pC)

D. EPSC burst frequency (Hz)

E. EPSC burst duration (ms)
Figure 6

A. control immediate 4 hours 2 days

B. immediate 4 hours 2 days

C. immediate 4 hours 2 days

D.
Figure 7

A. APV-sensitive mEPSC current control immediate 4 hours 2 days control immediate 4 hours 2 days

B. APV-sensitive charge transfer control immediate 4 hours 2 days control immediate 4 hours 2 days

C. APV-sensitive mEPSC current

D. APV-sensitive charge transfer

2 mM MgCl$_2$ 0.05 mM MgCl$_2$ 2 mM MgCl$_2$ 0.05 mM MgCl$_2$
Figure 10

A. immediate

B. 4 hours

2 days

B. Amplitude (pA)

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>immediate</th>
<th>4 hours</th>
<th>2 days</th>
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<tbody>
<tr>
<td>Amplitude (pA)</td>
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