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Original Article

ORIGINAL ARTICLE

Ceftriaxone Treatment Preserves Cortical Inhibitory Interneuron Function via Transient Salvage of GLT-1 in a Rat Traumatic Brain Injury Model

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Abstract

Traumatic brain injury (TBI) results in a decrease in glutamate transporter-1 (GLT-1) expression, the major mechanism for glutamate removal from synapses. Coupled with an increase in glutamate release from dead and dying neurons, this causes an increase in extracellular glutamate. The ensuing glutamate excitotoxicity disproportionately damages vulnerable GABAergic parvalbumin-positive inhibitory interneurons, resulting in a progressively worsening cortical excitatory: inhibitory imbalance due to a loss of GABAergic inhibitory tone, as evidenced by chronic post-traumatic symptoms such as epilepsy, and supported by neuropathologic findings. This loss of intracortical inhibition can be measured and followed noninvasively using long-interval paired-pulse transcranial magnetic stimulation with mechanomyography (LI-ppTMS-MMG). Ceftriaxone, a β-lactam antibiotic, is a potent stimulator of the expression of rodent GLT-1 and would presumably decrease excitotoxic damage to GABAergic interneurons. It may thus be a viable antiepileptogenic intervention. Using a rat fluid percussion injury TBI model, we utilized LI-ppTMS-MMG, quantitative PCR, and immunohistochemistry to test whether ceftriaxone treatment preserves intracortical inhibition and cortical parvalbumin-positive inhibitory interneuron function after TBI in rat motor cortex. We show that neocortical GLT-1 gene and protein expression are significantly reduced 1 week after TBI, and this transient loss is mitigated by ceftriaxone. Importantly, whereas intracortical inhibition declines progressively after TBI, 1 week of post-TBI ceftriaxone treatment attenuates the loss of inhibition compared to saline-treated controls. This finding is accompanied by significantly higher parvalbumin gene and protein expression in

ceftriaxone-treated injured rats. Our results highlight prospects for ceftriaxone as an intervention after TBI to prevent cortical inhibitory interneuron dysfunction, partly by preserving GLT-1 expression.

Key words: ceftriaxone, GLT-1, intracortical inhibition, parvalbumin, traumatic brain injury

Introduction

Traumatic brain injury (TBI) is among the most common causes of morbidity and mortality in the USA across all age groups (Coronado et al. 2011). Beyond the mechanical injury, progressively increasing levels of extracellular glutamate leaking from dead and dying neurons during the acute and subacute post-traumatic period (Luo et al. 2011), coupled with downregulation of glial membrane glutamate transporters in the lesional cortex (Goodrich et al. 2013), lead to continuing excitotoxic neuronal injury (Li et al. 2002; Hardingham and Bading 2003; Goforth et al. 2011; Luo et al. 2011; Choo et al. 2012; Algattas and Huang 2014; Lai et al. 2014; Patel et al. 2014) and to long-term post-traumatic sequelae, such as post-traumatic epilepsy (PTE) and neurocognitive decline including earlier onset of cognitive impairment and dementia (Christensen et al. 2009; Levine et al. 2013; Nguyen et al. 2018). Notably, while some symptoms such as headache and motor deficits may be evident immediately after injury, PTE and other chronic disorders follow injury by latent periods of weeks to months and thus likely reflect slow pathological processes (Miyazaki et al. 1989; Lyeth et al. 1990).

Precise control of extracellular glutamate levels is essential for normal synaptic function and for protection against excitotoxicity (Takahashi et al. 1997; Schousboe and Waagepetersen 2005; David et al. 2009; Ishikawa 2013). Glutamate transporter-1 (GLT-1) encoded by the *SLC1A2* gene, the rodent ortholog of human excitatory amino acid transporter 2 (EAAT2), provides 95% of the total glutamate clearance capacity in the rat brain and is present predominantly on astroglial membranes surrounding synapses, accounting for as much as 1% of total brain protein (Lehre and Danbolt 1998). GLT-1 knockout mice exhibit increased susceptibility to hippocampal neuronal degeneration, lethal spontaneous seizures, and develop greater vasogenic cerebral edema after acute cortical injury (Tanaka et al. 1997). Single-nucleotide polymorphisms within astroglial glutamate transporter genes have also been associated with accelerated post-traumatic epileptogenesis and the development of PTE following TBI in humans (Kumar et al. 2018). Glutamate transport thus constitutes an essential mechanism for preventing glutamate excitotoxicity in the brain, with astrocytic GLT-1 providing the bulk of this protection in the rodent brain (Petr et al. 2015). However, GLT-1 levels are decreased after TBI. Specifically, in a modified rat fluid percussion injury (FPI) TBI model, GLT-1 levels are decreased in the ipsilesional cortex by close to 50% from baseline levels 1 week after injury (Goodrich et al. 2013). Other studies have also demonstrated post-traumatic decreases in GLT-1 mRNA and protein expression, and transporter function (Rao et al. 1998; Dorsett et al. 2017). Possible explanations for these changes range from glutamate-induced clustering of GLT-1 that induces its endocytosis (Nakagawa et al. 2008) and proteolysis of existing GLT-1 by caspase-3 (Boston-Howes et al. 2006), to decreased de novo gene transcription (Rao et al. 2001; Maragakis and Rothstein 2004).

Glutamate release from dead and dying neurons, coupled with decreased synaptic glutamate clearance due to reduced GLT-1 activity, result in increased extracellular glutamate which persists for days after TBI in humans and rodents (Yamamoto et al. 1999; Zhuang et al. 2018). The ensuing glutamate-mediated excitotoxicity is at least partly attributable to increased mitochondrial activity to accommodate the metabolic demands of enhanced neuronal firing, and results in excess generation of toxic reactive oxygen species (ROS) and oxidative stress (Bondy and LeBel 1993; Awasthi et al. 1997; Petronilho et al. 2010; Khatri et al. 2018).

Post-TBI excitotoxicity creates an especially unfavorable milieu for a specific subpopulation of GABAergic interneurons, parvalbumin-positive (PVALB+) cells, which constitute a plurality of the cortical inhibitory interneuronal population (Rudy et al. 2011; Kelsom and Lu 2013) and exert considerable influence on cortical circuits despite their small absolute numbers (Sohal et al. 2009; Cammarota et al. 2013). Their high baseline firing rate and energy demand carry an inherent risk of increasing ROS levels upon excessive exposure to the excitatory neurotransmitter glutamate (Cantu et al. 2015; Hsieh et al. 2017). Additionally, some PVALB+ cells express NMDA-type and calcium-permeable AMPA-type glutamate receptors (Moga et al. 2002), which render them susceptible to toxic calcium overload with resultant death or dysfunction of these units (Peng and Jou 2010). Post-traumatic breakdown of their extracellular matrix, the perineuronal net (PNN), that protects mature PVALB+ cells from oxidative stress (Hsieh et al. 2017) further impairs PVALB+ cell function and resilience to metabolic stress.

We previously reported progressively worsening oxidative stress and a gradual loss of perilesional PVALB+ interneurons after TBI without a significant decrease in overall neuronal count (Hsieh et al. 2017). As GABAergic inhibitory interneurons constitute 10–20% of neurons (Sahara et al. 2012; Kelsom and Lu 2013), and PVALB+ cells make up ~40% of this subpopulation (Rudy et al. 2011), static total neuron counts may imply that excitatory neurons are largely unaffected. Preferential loss of these inhibitory interneurons thus results in a shift of the cortical excitation:inhibition (E:I) balance and may contribute to both epileptogenesis and neurocognitive decline during the latent post-traumatic period and beyond (Hsieh et al. 2017). The development of PTE attests to this imbalance, as cortical ablation with loss of all neurons is not epileptogenic, rather is a treatment for focal epilepsy (Al-Otaibi et al. 2012; LaRiviere and Gross 2016).

Post-TBI loss of intracortical inhibition can be detected non-invasively by transcranial magnetic stimulation (TMS), a method for focal cortical stimulation in which small stimulating electrical currents in the brain are induced by a strong extracranial magnetic field. With stimulation of the motor cortex by a specific TMS protocol termed paired-pulse TMS (ppTMS), a measure of GABA-mediated cortical paired-pulse inhibition can be obtained in vivo from humans and rodents alike after TBI to assess post-traumatic cortical E:I balance (Kobayashi and Pascual-Leone 2003; De Beaumont et al. 2011; Demirtas-Tatlidede et al. 2012; Hsieh et al. 2012; Villamar et al. 2012). Previously, we reported a progressive loss of intracortical inhibition following FPI in rats as measured by ppTMS, reflecting the

growing imbalance between GABA-mediated inhibition and glutamate-mediated excitation, (Hsieh et al. 2017), and which would presumably lead to increased seizure susceptibility.

Among therapeutic strategies aimed at minimizing excitotoxicity and preserve cortical inhibitory tone after TBI is treatment by ceftriaxone, a common β-lactam antibiotic with good blood–brain barrier penetration and a potent stimulator of GLT-1 (EAAT2) expression (Rothstein et al. 2005; Lee et al. 2008; Lewerenz et al. 2009). We have demonstrated that acute post-TBI ceftriaxone treatment in rats restores GLT-1 expression in injured cortex to near-normal levels, reduces post-traumatic astrogliosis, and suppresses post-traumatic seizures (Goodrich et al. 2013). However, the natural history of post-traumatic glutamate transporter gene (*SLC1A2*) and protein (GLT-1) expression and the durability of their ceftriaxone-mediated rescue is unknown. Moreover, while the capacity of ceftriaxone to prophylax against post-traumatic electrographic seizures has been demonstrated (Goodrich et al. 2013), whether ceftriaxone treatment specifically mitigates PVALB+ interneuron loss post-TBI and preserves cortical inhibitory tone is also unknown. In complement to our earlier work we now test, in a modified rat FPI model, whether 1) ppTMS-derived measures of cortical GABAergic inhibition can be used as a biomarker of the therapeutic efficacy of post-TBI ceftriaxone treatment; and whether treatment preserves 2) *SLC1A2* and GLT-1 expression, and 3) parvalbumin gene (*PVALB*) and protein (PVALB) expression.

Materials and Methods

Animals

Twelve-week-old male Sprague Dawley rats (367 ± 36 g) were used. Rats were housed in standard cages in a temperature-controlled facility with a 12-h light/dark cycle and a continuous supply of water and food ad libitum. All procedures were approved by, and in accordance with the guidelines of, the Institutional Animal Care and Use Committee at Boston Children's Hospital and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of rats used in the present experiments.

Cohorts:

Immunohistochemistry: Two cohorts of rats (for subacute and chronic assays) were each divided into 2 experimental groups as follows: 1) Saline-TBI (Sal-TBI), treated with IP saline after TBI surgery; and 2) Ceftriaxone-TBI (Cef-TBI), treated with ceftriaxone (250 mg/kg, IP) after TBI surgery. The first IP injection was 30 min post-TBI and continued once per 24 h for 7 consecutive days thereafter. Brain tissue from 1 cohort each was harvested 1 week (N = 5 per group) or 6 weeks (N = 5–6 per group) after TBI for immunohistochemistry (IHC) to replicate our previous findings of subacute post-traumatic loss and ceftriaxone-mediated salvage of GLT-1.

Quantitative Real-Time Polymerase Chain Reaction: Two cohorts of rats (for subacute and chronic assays) were each divided into 3 experimental groups: 1) Saline-Sham (Sal-Sham), treated with daily intraperitoneal (IP) saline injections after sham TBI surgery; 2) Sal-TBI, treated with IP saline after TBI surgery; and 3) Cef-TBI, treated with ceftriaxone (250 mg/kg, IP) after TBI surgery. The first IP injection was 30 min post-TBI and continued once per 24 h for 7 consecutive days thereafter. Cortical tissue was harvested for quantitative real-time polymerase chain reaction (qPCR) 1 week (N = 7 per group) and 6 weeks after injury (N = 7 per group).

Long-Interval Paired-Pulse Transcranial Magnetic Stimulation with Mechanomyography: One cohort of rats was divided into 2 experimental groups 1) Sal-TBI (N = 6) and 2) Cef-TBI (N = 7), as above. Baseline LI-ppTMS-MMG was obtained from each rat prior to injury, and serial LI-ppTMS-MMG measures were obtained weekly for 6 weeks after injury.

Fluid Percussion Injury

Injury was induced as previously described (Hameed et al. 2014). Briefly, rats were anesthetized using 2–4% isoflurane vapor by nosecone and mounted on a stereotactic frame (Stoelting Co.). During anesthesia, body temperature was maintained with a circulating water heating pad set to 37°C. A 4-mm-diameter craniectomy was made over the left parietal cortex, posterolateral to the bregma without crossing the sutures, with the lateral edge of the craniectomy adjacent to the lateral ridge. The dura was examined to confirm its integrity. A length of plastic tubing was fitted to the male connector of the fluid-percussion device (AmScien Instruments), and the connection was made airtight using polytetrafluoroethylene (PTFE) tape. The free end of the tubing was attached to a 1000-μL pipette tip that had been cut to leave a 4-mm aperture on its free end, again made airtight using PTFE tape. The tubing was then filled with sterile saline at room temperature, and the tip was positioned over the exposed dura using the micromanipulator arm of the frame, such that the edges of the pipette tip fit tightly over the skull. A percussion wave of 2.3 ± 0.1 atm was delivered to induce moderate TBI. During the TBI procedure, each rat's respiratory rate was monitored by visual inspection. Epochs of apnea after injury were recorded, and rats that were apneic >30 s after FPI were excluded from the study. Sham control rats underwent the craniectomy without FPI. After each verum or sham TBI surgery, rats were observed continuously until they were able to right themselves and maintain sternal recumbency and were then placed in their home cages with ad libitum access to food and water. Opioid analgesia (buprenorphine HCl; 0.1 mg/kg) was administered subcutaneously every 8–12 h for 48 h postoperatively. Rats were observed twice daily for the first postoperative week and daily thereafter until their endpoints.

Long-Interval Paired-Pulse Transcranial Magnetic Stimulation with Mechanomyography

For measures of intracortical inhibition in conscious rats, we employed our previously established protocol for coupling LI-ppTMS with MMG, a noninvasive measure of corticospinal activation in unanesthetized rats (Hsieh et al. 2012). Rats were restrained on a platform by straps securing the head, upper and mid-torso as previously described. Hind limb acceleration resultant from each successive TMS pulse was recorded using a miniature 3-axis accelerometer (AGB3V2; Asakusa Giken) secured with adhesive tape to each hind limb. MMG output was quantified as the peak-to-peak sum of the 3 vectors. LI-ppTMS was administered with a figure-of-eight coil (outside lobe diameter = 66 mm, inside diameter = 15 mm; double small coil) connected to a Magstim Rapid stimulator (Magstim). The stimulating coil was positioned by stereotaxic micromanipulator with the center of the coil midline over the dorsal scalp to elicit bilateral hind limb MMG (Fig. 1A).

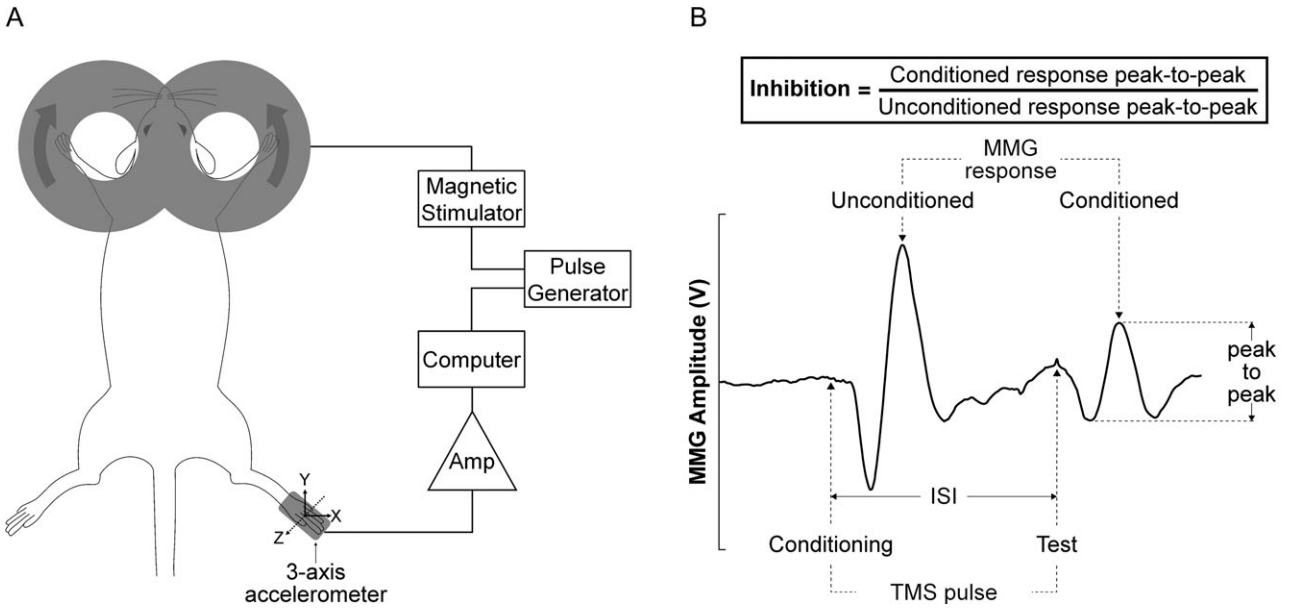

Figure 1. LI-ppTMS-MMG. (*A*) An awake rat is restrained on a platform beneath a figure-of-eight TMS coil that is fixed to the micromanipulator arm of a stereotaxic frame. The coil is centered at the interocular line. Hind limb acceleration resultant from each successive TMS pulse is recorded using a miniature 3-axis accelerometer. (*B*) Intracortical inhibition is calculated as the ratio of the conditioned MMG response to the second, test stimulus and the unconditioned response to the first, conditioning stimulus.

To accommodate the short time period, approximately 3 min, during which an awake, immobilized rat remains relatively motionless beneath the stimulating coil, paired TMS pulses (each individual stimulus of the 2 paired stimuli was at the same intensity) were delivered over a range of progressively increasing stimulus intensities (60%, 70%, 80%, 90%, and 100% maximum machine output). Six stimulus pairs were delivered per stimulation intensity. Each pair of stimuli were 5 s apart. Thirty pairs of stimuli were delivered with an interstimulus interval (ISI) of 100 ms, and 30 pairs of stimuli were delivered with an ISI of 200 ms. Baseline LI-ppTMS-MMG measures were obtained for each rat prior to TBI, and the procedure was repeated weekly after injury till the study endpoint.

Results obtained with 100 ms ISI or 200 ms ISI stimuli were analyzed separately. MMG signals were digitized at 10 kHz, band-pass filtered 1–250 Hz and stored for further offline analysis (PowerLab 8/30; AD Instruments). The paired-pulse inhibition ratio was calculated from each motor response pair where the TMS-evoked MMG following the conditioning stimulus exceeded baseline noise by >3 standard deviations. The calculated ratios (conditioned MMG: unconditioned MMG, Fig. 1*B*) were averaged across stimulus intensities per ISI for each rat at each time point as previously described (Hsieh et al. 2012).

mRNA Isolation from Cortical Tissue

Rats were decapitated using a sharp guillotine. Ipsilesional cortical tissue was rapidly dissected out onto a chilled plate and immersed in RNA later (Life Technologies). Total RNA was extracted using the RNeasy Mini kit (Qiagen) and stored at −80 °C.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was converted to cDNA using the RT^2 First Strand Kit (Qiagen). RT-PCR was carried out to quantify *SLC1A2* and *PVALB* with Ribosomal Protein, Large, P0 (*RPLP0*) as housekeeping gene, using RT^2 SYBR Green ROX FAST Mastermix and RT^2 qPCR Primer Assays (*SLC1A2*: PPR44742A; *PVALB*: PPR42647A; *RPLP0*: PPR42394A. Qiagen) on a Rotor-Gene Q real-time PCR cycler (Qiagen). All reactions were carried out in triplicate. Gene expression was calculated using the ΔΔCt method.

Immunohistochemistry

Rats were processed for immunofluorescence staining as previously described (Hsieh et al. 2017). Briefly, rats were anesthetized and systemically perfused with ice-cold saline followed by 4% paraformaldehyde. Perfused brains were harvested and cryoprotected in 30% sucrose solution before being frozen rapidly in a dry-ice slurry. Brains were stored at −80 °C at least 24 h before sectioning. Floating cryosections of 30 μm were produced using a cryostat and slices were blocked with phosphate-buffered saline (PBS) with 10% goat serum and 0.1% Triton X-100 for 1 h. Brain slices were then incubated with primary antibodies against nGLT-1 (raised in rabbit, 1:100; a gift from Dr Paul Rosenberg) and PVALB (raised in mouse, 1:500; Swant) overnight, washed with PBS, incubated with corresponding secondary antibodies conjugated with Alexa488 and Alexa594, before being mounted with DAPI Fluoromount G medium (SouthernBiotech).

Confocal Microscopy

Perilesional sites in the motor cortex medial to the TBI region (100 μm medial from the edge of lesion) were identified by fluorescence imaging as described previously (Hsieh et al. 2017). Confocal images were taken using an Olympus FV10 confocal microscope (100× (nGLT-1) or 10× (PVALB) objective) with the following parameters: 5% laser output, X1 gain control, 500–600 laser intensity, 10–15% offset for linear signal range. Individual channels were acquired sequentially. At least 6 slices were analyzed per brain.

Data Processing and Statistical Analysis

Data were analyzed using GraphPad Prism 7.0 (GraphPad Software) with the significance level set at $P < 0.05$. All data are

presented as mean ± standard error of the mean (Mean ± SEM). Paired-pulse inhibition was expressed as the percentage of the conditioned evoked MMG to unconditioned evoked MMG, with *higher percentage denoting lower inhibition* (Fig. 1B). Sequential LI-ppTMS-MMG measures after TBI were compared by repeated measures ANOVA with treatment and time after TBI as factors. Post hoc Fisher's LSD tests were used to compare means at individual time points. All gene expression data were expressed as fold change relative to Saline-Sham values. Gene expression and immunofluorescence data comparisons between rats were performed by 2-way ANOVA, with Tukey's post hoc tests.

Results

Acute Ceftriaxone Treatment after TBI Attenuates Progressive Post-Traumatic Intracortical Inhibition Loss

We previously identified that LI-ppTMS-MMG reveals progressive loss of intracortical inhibition for 6 weeks after TBI in rats (Hsieh et al. 2017). At present, we test whether 1 week of ceftriaxone treatment preserves intracortical inhibition. Repeated measures ANOVA on data obtained with a paired-pulse ISI of 200 ms reveals a significant effect of time ($F_{6,66} = 18.12$, $P < 0.001$) and ceftriaxone treatment after injury ($F_{1,11} = 5.51$, $P < 0.05$), and a significant interaction between these factors ($F_{6,66} = 4.45$, $P < 0.001$). Post hoc analysis using the pre-TBI condition as a baseline further reveals that intracortical inhibition is reduced 1 week after injury in both Cef-TBI and Sal-TBI rats. A progressive loss of inhibition then continues in Sal-TBI rats throughout the 6-week testing period. In contrast, intracortical inhibition recovers to baseline values in the Cef-TBI group by the second week, and while depressed relative to baseline after week 4, remains significantly higher than that in Sal-TBI rats starting 3 weeks post-TBI (Fig. 2). In a replicate experiment, essentially identical trends are observed in data collected by LI-ppTMS-MMG with an ISI of 100 ms (Supplementary Fig. S1).

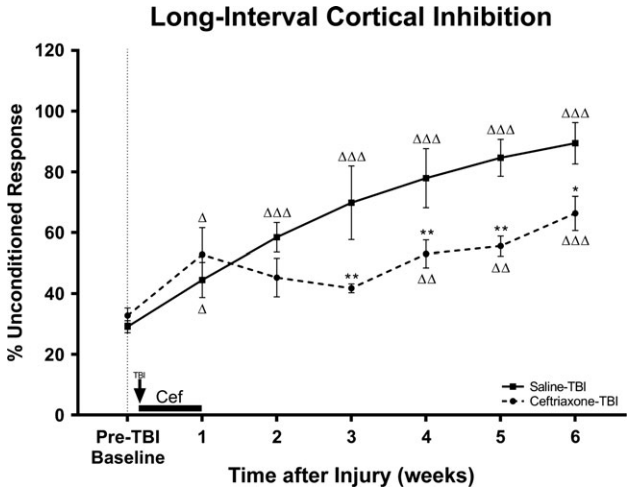

Figure 2. Acute ceftriaxone treatment attenuates progressive post-traumatic loss of GABAergic intracortical inhibition seen with LI-ppTMS-MMG: *Note: Higher y-axis value denotes lower inhibition.* Baseline pre-TBI intracortical inhibition is 29.1 ± 2.0% in Saline-TBI and 32.8 ± 2.5% in Ceftriaxone-TBI rats. Inhibition declines 1 week after injury in both experimental groups. Overall, intracortical inhibition is relatively preserved in Ceftriaxone-TBI rats, where it is significantly greater than in Saline-TBI controls from 3 weeks post-TBI (Cef-TBI: 41.7 ± 1.4%; Sal-TBI: 69.8 ± 12.1%), to 6 weeks post-TBI (Cef-TBI: 66.3 ± 5.6%; Sal-TBI: 89.4 ± 6.8%). Data are shown as mean ± SEM. (*) denotes significance between Saline-TBI and Ceftriaxone-TBI at the same time point ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). ($^{\Delta}$) denotes significance from own group preinjury baseline ($^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$).

Acute Ceftriaxone Treatment Mitigates Subacute post-TBI Loss of GLT-1 Protein and *SLC1A2* Gene Expression in Injured Brains 1 Week after TBI

Next, we sought to identify the cause of this relative preservation of intracortical inhibition. Earlier, we found that ceftriaxone treatment after TBI mitigates the loss of GLT-1 protein caused by injury, as measured by western blot analysis (Goodrich et al. 2013). We aimed to support this finding using IHC and to determine whether this effect is transcriptional or translational in nature.

There is a significant reduction in GLT-1 expression 1 week after injury in Sal-TBI controls compared with Cef-TBI rats. ANOVA showed a significant contribution of both ceftriaxone ($F_{1,4} = 15.58$, $P < 0.05$) and hemisphere ($F_{1,4} = 35.38$, $P < 0.01$) on GLT-1 signal after injury, with a significant interaction between the two ($F_{1,4} = 19$, $P < 0.05$). Post hoc tests reveal a significant reduction of GLT-1 immunoreactivity in ipsilesional Sal-TBI compared with contralesional Sal-TBI (IL Sal-TBI: 604.98 ± 132.98 AU; CL Sal-TBI: 866.91 ± 149.17 AU, $P < 0.05$) as well as the ipsilesional Cef-TBI hemisphere (IL Cef-TBI: 1256.26 ± 51.87 AU, $P < 0.001$). GLT-1 expression is also significantly lower in contralesional Sal-TBI compared with both ipsilesional Cef-TBI ($P < 0.01$) and contralesional Cef-TBI (CL Cef-TBI: 1263.84 ± 42.55, $P < 0.01$). However, no difference is seen in GLT-1 staining between hemispheres in ceftriaxone-treated rats (Fig. 3A,B).

This ceftriaxone-mediated rescue appears to be due at least in part to transcriptional upregulation of the *SLC1A2* gene. qPCR reveals a significant effect of both experimental group ($F_{1,6} = 212.9$; $P < 0.001$) as well as hemisphere ($F_{1,6} = 24.61$; $P < 0.001$), and significant interaction between the 2 factors on *SLC1A2* expression. There is a significant decrease in cortical *SLC1A2* expression in both ipsilesional Sal-TBI and Cef-TBI compared with Sal-Sham tissue (Sal-Sham: 1.01 ± 0.04-fold; IL Sal-TBI: 0.42 ± 0.04-fold, $P < 0.001$; IL Cef-TBI: 0.67 ± 0.06-fold, $P < 0.01$). However, there is no significant difference in *SLC1A2* mRNA levels between Sal-Sham tissue and either contralesional Sal-TBI (0.95 ± 0.06-fold, n.s) or contralesional Cef-TBI (1.1 ± 0.07-fold, n.s) rats. In addition, *SLC1A2* mRNA levels are significantly lower in ipsilesional Sal-TBI tissue compared with ipsilesional Cef-TBI ($P < 0.05$), as well as contralesional Sal-TBI ($P < 0.001$). *SLC1A2* expression in ipsilesional Cef-TBI is also significantly downregulated compared with both contralesional Sal-TBI ($P < 0.05$) and Cef-TBI ($P < 0.001$) (Fig. 3C).

SLC1A2 Gene and GLT-1 Protein Expression Normalize Naturally by 6 Weeks after Injury

GLT-1 and *SLC1A2* expression were then measured 6 weeks after injury, to investigate the long-term effects of 1-week ceftriaxone treatment after TBI. GLT-1 immunofluorescence did not reveal an effect of either ceftriaxone treatment ($F_{1,5} = 2.22$, n.s) or hemisphere ($F_{1,5} = 0.01$, n.s) on GLT-1 protein signal (IL Sal-TBI: 1107.59 ± 34.45 AU; CL Sal-TBI: 1149.79 ± 66.03 AU; IL Cef-TBI: 1194.70 ± 86.54 AU; CL Cef-TBI: 1158.06 ± 53.43 AU; n.s) (Fig. 4A,B). These results are further supported by qPCR data which did not show any significant difference in cortical levels of *SLC1A2* mRNA between Sal-Sham, Sal-TBI, and Cef-TBI rats 6 weeks after injury (Sal-Sham: 1.0 ± 0.06-fold; IL Sal-TBI: 0.94 ± 0.08-fold; CL Sal-TBI: 1.04 ± 0.08-fold; IL Cef-TBI: 0.97 ± 0.05-fold; CL Cef-TBI: 1.01 ± 0.09-fold; n.s) (Fig. 4C). While these results indicate that the effect of ceftriaxone on GLT-1 does not persist beyond the treatment period, they suggest that both *SLC1A2* gene and GLT-1 protein

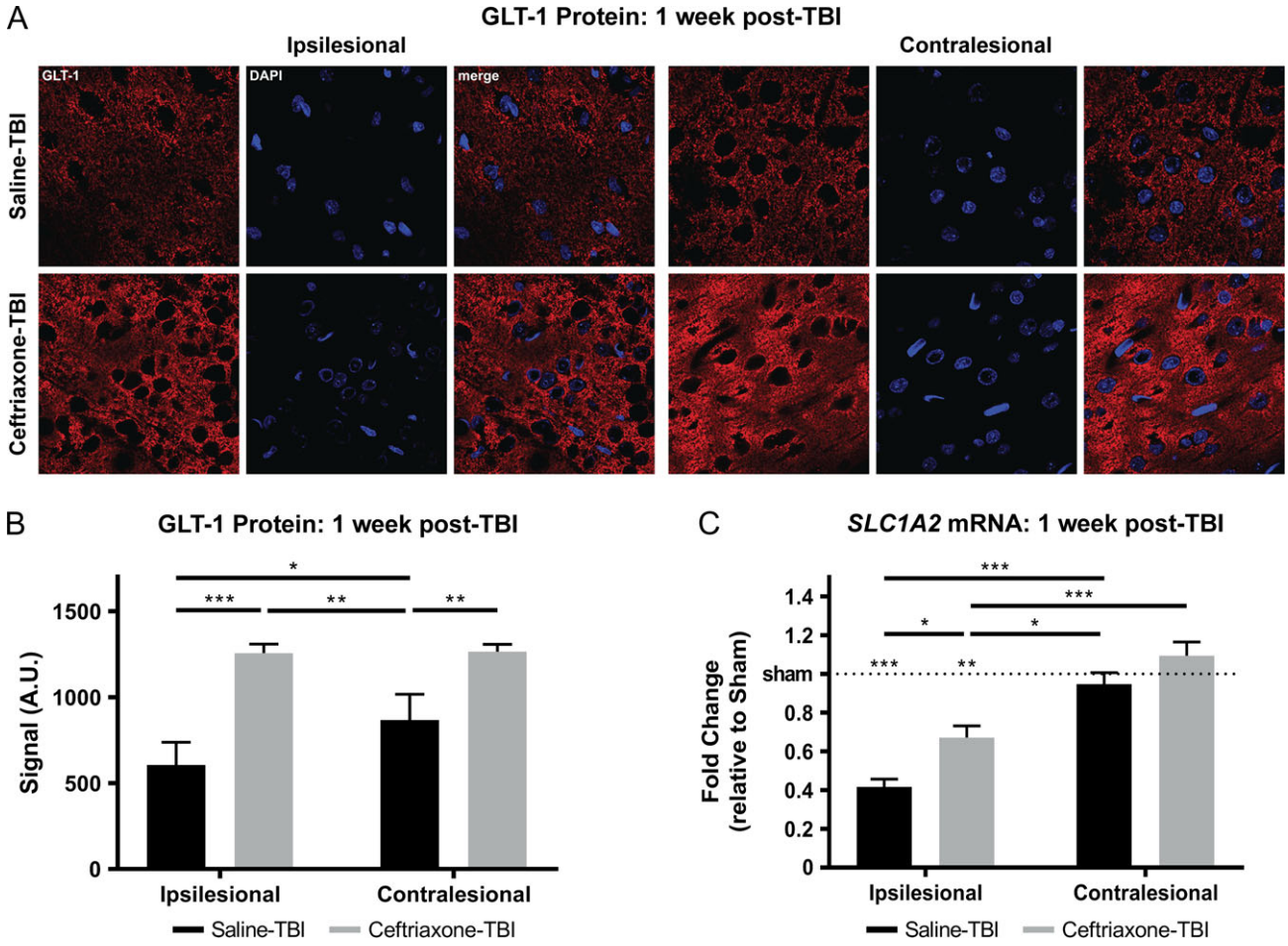

Figure 3. Acute ceftriaxone treatment mitigates subacute loss of GLT-1 protein and *SLC1A2* mRNA after TBI. (*A*, *B*) Reduced GLT-1 signal in ipsilesional Saline-TBI cortex compared with contralesional Saline-TBI as well as ipsilesional Ceftriaxone-TBI hemispheres. GLT-1 expression is also significantly lower in contralesional Saline-TBI compared with both Ceftriaxone-TBI hemispheres. There is no difference in GLT-1 staining between hemispheres in ceftriaxone-treated rats. (*C*) Cortical *SLC1A2* expression decreases in both ipsilesional Saline-TBI and Ceftriaxone-TBI compared with sham cortex. Additionally, mRNA levels are significantly lower in ipsilesional Saline-TBI tissue compared with ipsilesional Ceftriaxone-TBI as well as contralesional Saline-TBI. However, there is no significant difference in *SLC1A2* expression between sham and contralesional Saline-TBI or Ceftriaxone-TBI cortex. Data are shown as mean ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

expression naturally recover over time after TBI and are indistinguishable from control levels by 6 weeks after TBI.

Acute Ceftriaxone Treatment after TBI Preserves Perilesional *PVALB* Gene and PVALB Protein Expression 6 Weeks after TBI

To resolve the apparent discrepancy between ceftriaxone's effects on glutamate transport and intracortical inhibition after TBI, we evaluated the effect of ceftriaxone treatment on one possible mechanism by which intracortical inhibition is lost after TBI by measuring the gene and protein expression of a major inhibitory interneuron marker, parvalbumin, 1 and 6 weeks after TBI.

There is no significant effect of ceftriaxone treatment or hemisphere on *PVALB* mRNA (treatment: $F_{2,12} = 0.15$; hemisphere: $F_{1,6} = 0.00$. Sal-Sham: 1.00 ± 0.08-fold; IL Sal-TBI: 0.96 ± 0.18-fold; CL Sal-TBI: 1.00 ± 0.10-fold; IL Cef-TBI: 1.07 ± 0.17-fold; CL Cef-TBI: 1.05 ± 0.11-fold; n.s) or PVALB+ cell counts (treatment: $F_{1,4} = 0.73$; hemisphere: $F_{1,4} = 0.95$. IL Sal-TBI: 71.67 ± 10.91; CL Sal-TBI: 92.73 ± 10.93; IL Cef-TBI: 89.93 ± 4.61; CL Cef-TBI: 89.47 ± 7.06 cells per mm^2; n.s) 1 week after injury (Fig. 5A–C).

Six weeks after injury, however, qPCR detected a significant decrease in *PVALB* mRNA levels which is partially salvaged with ceftriaxone treatment. ANOVA showed a significant main effect of both ceftriaxone treatment ($F_{2,12} = 7.013$, $P < 0.01$) and hemisphere ($F_{1,6} = 10.45$, $P < 0.05$) on *PVALB* expression. Post hoc tests showed significantly lower *PVALB* mRNA levels in ipsilesional Sal-TBI brains (0.63 ± 0.03-fold) compared with Sal-Sham (1.00 ± 0.05-fold, $P < 0.01$), ipsilesional Cef-TBI (0.90 ± 0.03-fold, $P < 0.05$), as well as contralesional Sal-TBI (0.93 ± 0.06-fold, $P < 0.01$). There is no difference between any of the other experimental groups or hemispheres (Fig. 5D).

We then performed IHC to determine whether this decrease in mRNA after injury and subsequent salvage by ceftriaxone is due to changes in PVALB+ cell counts. ANOVA showed a significant overall effect of hemisphere ($F_{1,5} = 64.52$, $P < 0.001$), and a significant interaction between hemisphere and ceftriaxone treatment ($F_{1,5} = 4.60$, $P < 0.05$) on cortical PVALB+ interneuron counts 6 weeks after injury. There is a significant decrease in PVALB+ cell counts in ipsilesional Sal-TBI tissue (68.33 ± 7.30 cells per mm^2) compared with ipsilesional Cef-TBI (103.23 ± 5.80 cells per mm^2, $P < 0.05$) and contralesional Sal-TBI (124.30 ± 2.93 cells per mm^2, $P < 0.01$) brains (Fig. 5E,F), indicating that ceftriaxone's neuroprotective effect extends beyond the treatment duration independent of its effect on glutamate transport.

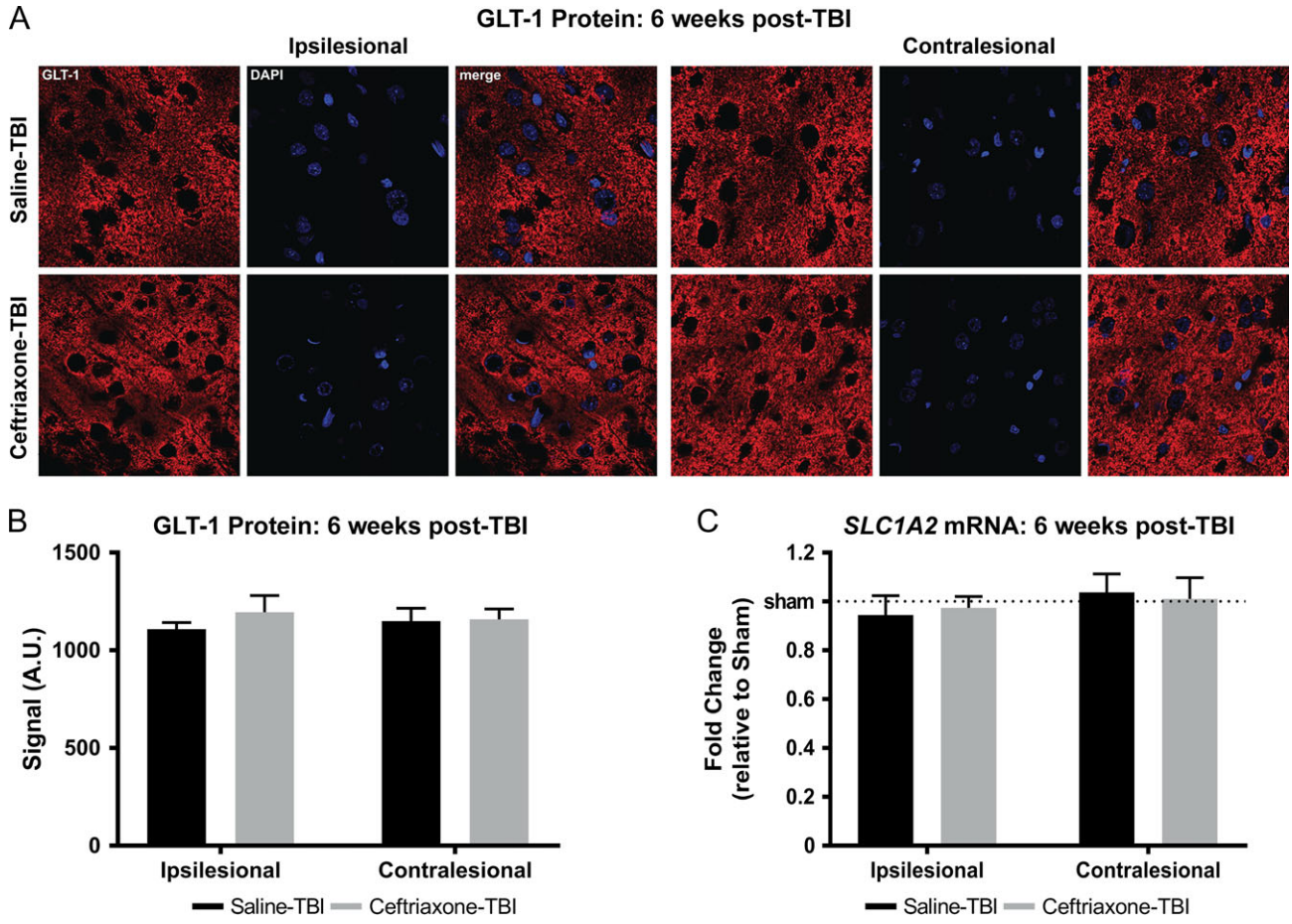

Figure 4. GLT-1 protein and *SLC1A2* gene expression normalize naturally by 6 weeks after TBI. GLT-1 immunoreactivity (A, B) and *SLC1A2* expression (C) do not differ between groups or hemispheres 6 weeks after injury. Data are shown as mean ± SEM.

Discussion

As extensively reported in the literature, TBI effects extend far beyond the mechanical damage, with continuing excitotoxic neuronal injury (Li et al. 2002; Hardingham and Bading 2003; Goforth et al. 2011; Luo et al. 2011; Choo et al. 2012; Algattas and Huang 2014; Lai et al. 2014; Patel et al. 2014) leading to chronic sequelae, such as PTE, insomnia, and neurocognitive dysfunction (Christensen et al. 2009; Wright et al. 2014; Pugh et al. 2015; Azouvi et al. 2017; Wilson et al. 2017; Wood 2017; Botchway et al. 2018; DeGrauw et al. 2018; Elliott et al. 2018; Kalmbach et al. 2018). That these long-term consequences appear after latent periods of weeks to months after the initial trauma suggests the underlying pathological processes are slow, yet pathologic shifts in cortical E:I balance are likely initiated during the critical subacute post-traumatic period and may be amenable to therapeutic interventions (Miyazaki et al. 1989; Lyeth et al. 1990). Our earlier work has also established that although our rat TBI-PTE model reliably results in electrographic seizures 6–12 weeks post-injury (Goodrich et al. 2013), the injury leads to a progressively increasing cortical E:I imbalance measurable as early as the second week after injury and is accompanied by a progressive loss of GABAergic PVALB+ cortical inhibitory interneurons (Hsieh et al. 2017).

The translational relevance of our results may be toward development of antiepileptogenic interventions. One barrier to the successful preclinical development of novel antiepileptogenic therapies has been the lack of a reliable biomarker to both track this progressive imbalance while it remains subclinical and consequently monitor target engagement by experimental antiepileptogenic therapies. Our prior work has highlighted the sensitivity of LI-ppTMS in monitoring the level of GABAergic intracortical inhibition early after TBI, before long-term clinical sequelae such as PTE are evident, indicating the technique's potential to noninvasively track progressive inhibitory deficits after TBI, and its utility in studying the temporal profile of target engagement by experimental therapies that may preserve cortical inhibition.

Ceftriaxone is a promising candidate antiepileptogenic therapy with documented neuroprotective potential (Cui et al. 2014; Jagadapillai et al. 2014; Zumkehr et al. 2015; Krzyzanowska et al. 2017; Tikhonova et al. 2017). It may help minimize secondary glutamate excitotoxity by virtue of being a potent stimulator of promoting GLT-1 (EAAT2) expression at concentrations that are achievable in rodents at approximately 200 mg/kg IP, and in the human central nervous system at doses commonly used to treat bacterial meningitis (100 mg/kg/day IV; Max: 4 g/24 h) (Rothstein et al. 2005; Lee et al. 2008; Lewerenz et al. 2009). We previously reported that ceftriaxone at this dose in rats reliably upregulates cortical GLT-1 protein expression in ipsilesional cortical tissue, attenuates post-traumatic astrogliosis, and partially prophylaxes against chronic post-traumatic seizures (Goodrich et al. 2013).

Using the LI-ppTMS-derived biomarker, we now demonstrate for the first time that daily ceftriaxone treatment started

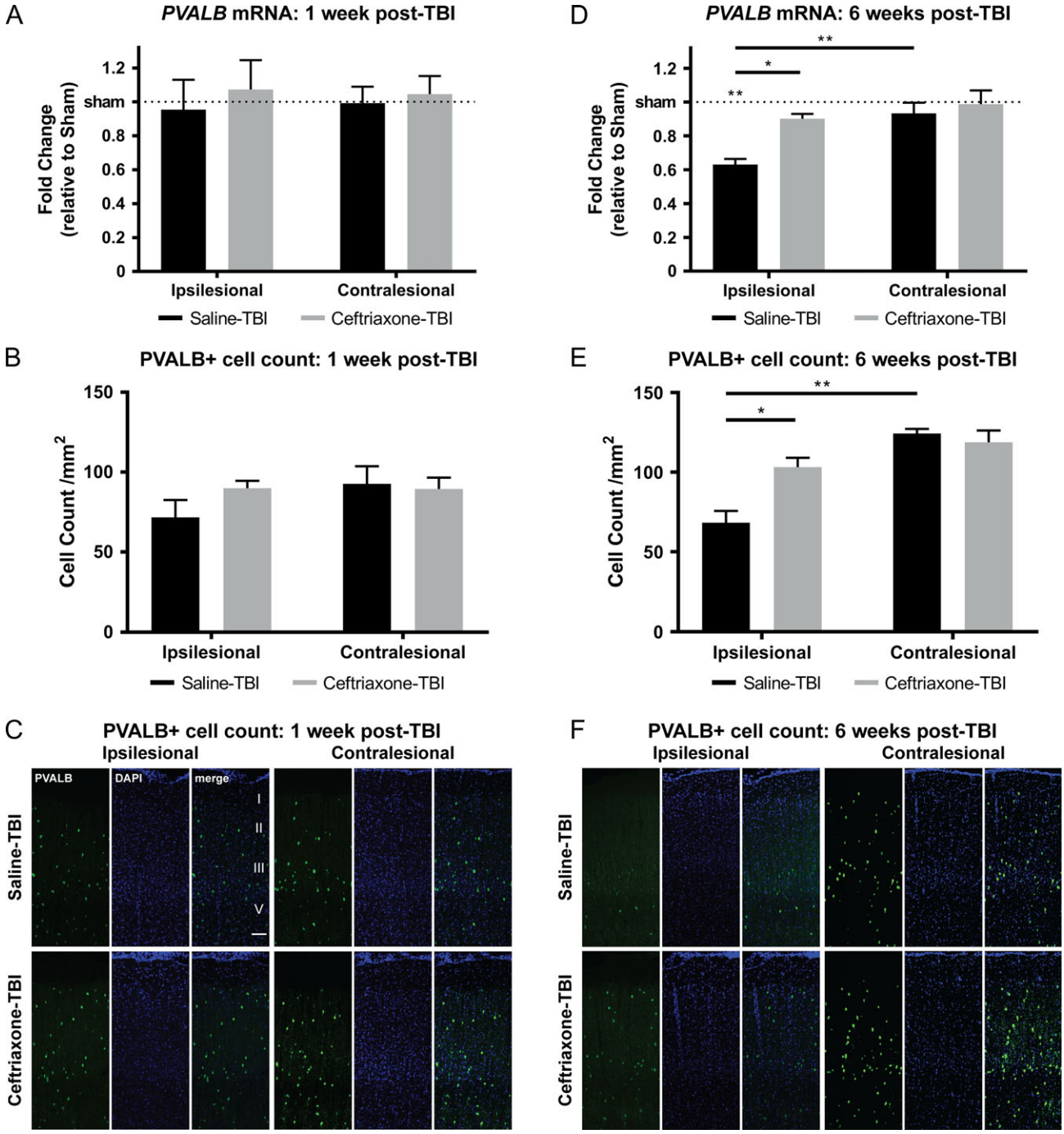

Figure 5. Acute ceftriaxone treatment after TBI preserves *PVALB* gene and PVALB+ interneuron counts 6 weeks after injury: (*A–C*) No difference in either *PVALB* gene expression or PVALB+ cell counts 1 week after TBI. (*D*) Six weeks after injury, *PVALB* expression in ipsilesional Saline-TBI cortex, but not in Ceftriaxone-TBI tissue, is lower than in sham. *PVALB* gene expression is also significantly lower in the ipsilesional hemispheres of Saline-TBI rats compared with contralesional cortex in the same rats, and to ipsilesional expression in Ceftriaxone-TBI rats. (*E*, *F*) PVALB+ cell counts are also lower in ipsilesional Saline-TBI cortex compared with contralesional cortex in the same rats, and to ipsilesional hemispheres in Ceftriaxone-TBI rats, 6 weeks after TBI. However, there is no difference in cell numbers between ipsilesional and contralesional cortex in Ceftriaxone-TBI rats. Data are shown as mean ± SEM (*$P < 0.05$, **$P < 0.01$).

immediately after TBI and continued for a week thereafter successfully attenuates post-TBI inhibitory dysfunction. Our results also confirm our prior findings that the well-tolerated LI-ppTMS metric is a sensitive noninvasive method to track and document post-traumatic changes in intracortical inhibition (Hsieh et al. 2017). Available in both rats (Hsieh et al. 2012) and humans (Hameed et al. 2017), we show here that it may be used as a biomarker to both track the progressive loss of normal cortical inhibitory tone which follows TBI, as well as confirm and monitor therapeutic target engagement in the development of novel post-traumatic antiepileptogenic interventions aimed at preserving cortical inhibitory tone post-TBI.

We did not functionally monitor rats in the current study for the long-term consequences of injury or efficacy of the

treatment such as impact on seizure duration and frequency or perform other post-TBI behavioral testing and instead focused on direct measures of intracortical inhibition. However, we use a well-characterized rat TBI-PTE model with universal findings, (Hicks et al. 1993, 1996; McIntosh et al. 1996; Conti et al. 1998; Bolkvadze and Pitkanen 2012), particularly seizures that reliably develop after an initial latent period (D'Ambrosio et al. 2004, 2005; Curia et al. 2011; Goodrich et al. 2013; Smith et al. 2018), that have been extensively reported by multiple laboratories, including ours. We have also previously reported that ceftriaxone significantly suppresses both the duration and frequency of post-traumatic electrographic seizures weeks after injury in this model (Goodrich et al. 2013). Here, our aim was to identify whether preservation of inhibition was a possible mechanism by which ceftriaxone's observed mitigation of PTE may be achieved. Follow-up studies should further characterize how the loss of intracortical inhibition as measured by LI-ppTMS and its rescue by ceftriaxone correlates with the range of clinical consequences that follow TBI.

Acute reductions in both *SLC1A2* mRNA and GLT-1 protein, 24 h after injury, have been reported after TBI in rats (Rao et al. 2001; Yi et al. 2005; Yi and Hazell 2006). At a slightly longer timescale, we also previously demonstrated that ipsilesional cortical GLT-1 protein levels are decreased by ~50% when measured 7 days after TBI (Goodrich et al. 2013). Our present results support our earlier findings of GLT-1 downregulation in the immediate post-traumatic period and its salvage by ceftriaxone treatment. Novel to this study, we complement our earlier western blot findings with IHC and qPCR analyses. We demonstrate that the injury primarily stays localized to the ipsilesional hemisphere, as reported in our and others' earlier studies (Hicks et al. 1996; Conti et al. 1998; Xiong et al. 2013; Hameed et al. 2014), and that the post-traumatic loss of GLT-1 protein in the ipsilesional cortex is at least partially transcriptional, with an accompanying reduction in *SLC1A2* gene expression post-TBI. Conversely, the ceftriaxone-mediated increase in GLT-1 levels after injury is at least in part achieved by increasing the expression of *SLC1A2*. Using microscopy to quantify region-specific protein levels also teases out significant differences between ceftriaxone-treated injured rats and saline-treated controls in GLT-1 expression in contralesional hemispheres that may have been "washed out" in earlier western blot analyses due to the larger volumes of tissue required, supporting earlier reports that the drug can also increase GLT-1 protein levels in healthy brain (Rothstein et al. 2005).

SLC1A2 mRNA and GLT-1 protein dynamics have not been measured to date at longer timescales of weeks to months after injury. Our data show that both *SLC1A2* gene and GLT-1 protein expression return to near-normal levels in the cortex by 6 weeks post-TBI in both ceftriaxone-treated rats and saline-treated controls. While our findings support the conclusion that ceftriaxone treatment after injury mitigates both *SLC1A2* and GLT-1 loss for the duration of treatment, the natural history of the durability of this effect and of the recovery of glutamate transporter expression in untreated brains will require further study. Accurate therapeutic windows thus constructed will be instrumental in the design of novel therapies aimed at minimizing the short- and long-term effects of TBI.

The transient loss of GLT-1 after TBI and the resultant depressed glutamate clearance may be enough to trigger a measurable amount of delayed excitotoxic damage, particularly to the vulnerable inhibitory interneuron system. Such an eventuality is indicated by the progressive post-traumatic loss of intracortical inhibition we found on LI-ppTMS-MMG, with ceftriaxone's stabilization of GLT-1 levels in the acute and subacute post-traumatic period potentially mitigating this loss. To investigate, we studied PVALB+ inhibitory interneurons in injured rats 1 and 6 weeks after injury. We focused on PVALB+ interneurons for 3 reasons. First, they constitute a plurality of the cortical inhibitory neuronal population (Rudy et al. 2011; Kelsom and Lu 2013). Prior research has shown that PVALB+ interneurons (also called fast-spiking inhibitory interneurons) are primarily responsible for the GABAergic inhibitory tone opposing the propagation of focal seizures across the cortex (Cammarota et al. 2013). Second, PVALB+ cells have a high baseline energy demand due to unique electrophysiological and structural characteristics. Oxidative phosphorylation in mitochondria that supports their fast firing rate carries an inherent risk of generating ROS, and therefore PVALB+ cells generate higher ROS concentrations than other neurons even under physiological conditions (Kann 2016). This renders them susceptible to increased oxidative stress due to enhanced mitochondrial activity and ROS accumulation caused by exposure to excess glutamate after TBI (Bondy and LeBel 1993; Awasthi et al. 1997; Lewen et al. 2001; Petronilho et al. 2010; Cantu et al. 2015; Khatri et al. 2018). Calcium overload due to the activation of ionotropic glutamatergic calcium-permeable receptors (Moga et al. 2002) may cause additional mitochondrial dysfunction and exacerbate this free radical accumulation (Peng and Jou 2010), and the breakdown of the protective extracellular matrix surrounding PVALB+ cells may similarly render these cells even more vulnerable (Hsieh et al. 2017). Third, PVALB+ cell numbers correlate strongly with the loss of intracortical inhibition measured by LI-ppTMS-MMG post-TBI, and their impairment has been shown to contribute to the collapse of local GABAergic inhibition, allowing focal epileptic events to propagate to distant neurons (Schwaller et al. 2004; Cammarota et al. 2013; Hsieh et al. 2017).

Our results revealed no effect of injury or subsequent ceftriaxone treatment on either *PVALB* gene expression or PVALB+ interneuron count 1 week after injury. Yet, LI-ppTMS-MMG detected a demonstrable loss of intracortical inhibition from baseline in both Sal-TBI and Cef-TBI rats as early as 1 week after injury. This discrepancy may indicate a multipronged pathophysiology, with mechanisms that differ depending on time after injury. Recent studies have shown, for instance, that mild TBI causes diffuse axonal injury, resulting in the structural and functional disconnection of PVALB+ interneurons in the first 24 h after injury (Vascak et al. 2018). This may also explain why ceftriaxone-mediated attenuation of the loss of intracortical inhibition becomes apparent only after the completion of ceftriaxone treatment, reaching significance weeks after injury once significant PVALB loss occurs (Hsieh et al. 2017).

Ceftriaxone's protective effect on cortical *PVALB* gene and PVALB+ cell counts appears to last for up to 6 weeks after injury, with both measures exhibiting a significant decrease in saline-treated controls as compared with ceftriaxone-treated rats. However, LI-ppTMS measures indicate that treatment may delay, rather than arrest, the loss of intracortical inhibition, with the loss of cortical inhibitory tone resuming after the third week post-injury. Perhaps GLT-1 salvage and other ceftriaxone-mediated neuroprotective pathways "drop off" and return to pretreatment levels following the cessation of treatment, allowing excitotoxicity to nevertheless proceed to some degree, with histological and molecular changes lagging neurophysiological measures by a few weeks. It is also possible that *PVALB* gene and protein loss are only partly responsible for the post-traumatic cortical E:I imbalance detected by LI-ppTMS, and that the surviving PVALB+ cells

are dysfunctional due to loss of trophic support (Croll et al. 1994; Marty et al. 1997; Rutherford et al. 1997; Turrigiano 1999; Baldelli et al. 2005) derived from corticospinal neurons (Kohara et al. 2007). This is in accordance with studies in models of injury-induced neocortical epileptogenesis showing that surviving PVALB+ interneurons make fewer synapses onto pyramidal neurons, and that their axon terminals are structurally abnormal and show significant reductions in vesicular GABA transporter, GAD-65, and GAD-67 (Gu et al. 2017).

Targeting the entirety of pathophysiological pathways activated after injury (Veenith et al. 2009; Frati et al. 2017; Hsieh et al. 2017; Anthonymuthu et al. 2018; Hall et al. 2018; Zhang and Wang 2018) to achieve optimal neuroprotection may require a multipronged approach combining multiple concurrent therapies. However, our results highlight the significance of immediate post-traumatic preservation of glutamate clearance as one plausible prophylactic intervention in combination with other interventions, for post-traumatic syndromes related to loss of cortical inhibitory tone. Further experiments will be necessary to understand the cellular mechanisms via which ceftriaxone protects the structure and function of PVALB+ cells after TBI. Putative mechanisms include an increased GLT-1 expression which alleviates excitotoxicity and oxidative stress in the cortex (Goodrich et al. 2013; Hsieh et al. 2017), and direct ceftriaxone-mediated activation of the Nrf2 antioxidant response element signaling pathway and the system x_c^- cystine/glutamate antiporter which plays key roles in antioxidation via glutathione synthesis (Lewerenz et al. 2009).

Limitations

We note limitations to the present study that warrant further investigation. Although our data show that transient *SLC1A2* mRNA and GLT-1 protein upregulation by ceftriaxone is associated with an attenuated post-traumatic loss of intracortical inhibition, additional experiments are required to conclusively establish a causal relationship between the two, and study the effect of ceftriaxone on other pathways including cellular antioxidant defenses such as system x_c^- (Lewerenz et al. 2006, 2009) after injury.

Further studies are required to investigate whether the attenuated loss of *PVALB* expression and PVALB+ cell counts with ceftriaxone treatment is due to increased PVALB+ interneuron survival, or merely the prevention of PVALB loss from these cells, where appropriate cell death markers are employed to adequately assess for survival. However, delayed cell death due to secondary injury following FPI has been demonstrated to be spatially and temporally variable, especially after the initial wave of cell death 1 week after injury and may not be exclusively apoptotic or necrotic. Apoptotic cell death, for example, is undetectable in several studies 2 months after injury (Conti et al. 1998; Luo et al. 2002), yet progressive cortical atrophy continues for months to years after TBI (Bramlett and Dietrich 2002) and the epileptogenic latent period can extend to a year or more (Kharatishvili et al. 2006). Such studies will therefore likely require a higher temporal resolution and greater animal numbers than used in the present study and were therefore beyond the scope of this proof-of-concept report aimed at testing whether perilesional cortical inhibitory tone is preserved by transient ceftriaxone treatment.

For the present manuscript, we focused on perilesional tissue, which, by definition, is without profound neuronal loss as confirmed by NeuN stain in a prior study (Hsieh et al. 2017). However, beyond the scope of this project focused on a specific form of intracortical inhibition, we anticipate that the analysis of lesion volume and a plurality of neuron types may identify a broader ceftriaxone neuroprotective effect.

Another confound of our experiment is the lack of data beyond 6 weeks after injury. While we infer that the loss of intracortical inhibition, *PVALB* expression, and PVALB+ cells observed up to 6 weeks will continue and lead to the development of post-traumatic sequelae observed in our model and others,(Goodrich et al. 2013) we recognize that further characterization of this phenomenon will be critical in establishing the validity and efficacy of serial measurements of intracortical inhibition as a biomarker.

We also did not test the effects of varying injury-to-treatment time windows, doses, and durations of ceftriaxone administration on the post-traumatic loss of intracortical inhibition, *SLC1A2* and *PVALB* gene and GLT-1 protein expression, or PVALB+ cell counts, which warrant additional study to pinpoint the optimal therapeutic regimen. Such systematic variation of treatment parameters will be necessary before translation of the present work to clinical trials.

Finally, isoflurane has previously been described as having an antiepileptogenic effect in a rat model of chemical-induced temporal lobe epilepsy (Bar-Klein et al. 2016). While the authors are unaware of published literature to support a similar effect in animal models of TBI, the possibility is nevertheless controlled for as a variable in our experiments.

Conclusion

We cautiously conclude that our data from a rat TBI model provide evidence that ceftriaxone therapy shortly after injury offers lasting neuroprotective benefits by mitigating an immediate loss of *SLC1A2* mRNA and GLT-1 protein and attenuating the progressive loss of intracortical inhibition and PVALB expressing inhibitory interneurons in the subacute and chronic post-traumatic period.

Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

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