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Research Paper

Genetic and pharmacological manipulation of glial glutamate transporters does not alter infection-induced seizure activity

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ABSTRACT

The contribution of glial transporters to glutamate movement across the membrane has been identified as a potential target for anti-seizure therapies. Two such glutamate transporters, GLT-1 and system x_c^-, are expressed on glial cells, and modulation of their expression and function have been identified as a means by which seizures, neuronal injury, and gliosis can be reduced in models of brain injury. While GLT-1 is responsible for the majority of glutamate uptake in the brain, system x_c^- releases glutamate in the extracellular cleft in exchange for cystine and represents as such the major source of hippocampal extracellular glutamate. Using the Theiler's Murine Encephalomyelitis Virus (TMEV) model of viral-induced epilepsy, we have taken two well-studied approaches, one pharmacological, one genetic, to investigate the potential role(s) of GLT-1 and system x_c^- in TMEV-induced pathology. Our findings suggest that the methods we utilized to modulate these glial transporters, while effective in other models, are not sufficient to reduce the number or severity of behavioral seizures in TMEV-infected mice. However, genetic knockout of xCT, the specific subunit of system x_c^-, may have cellular effects, as we observed a slight decrease in neuronal injury caused by TMEV and an increase in astrogliosis in the CA1 region of the hippocampus. Furthermore, xCT knockout caused an increase in GLT-1 expression selectively in the cortex. These findings have significant implications for both the characterization of the TMEV model as well as for future efforts to discover novel and effective anti-seizure drugs.

1. Introduction

Epilepsy is a neurological condition associated with recurrent seizures, stigma, psychiatric and cognitive comorbidity, and high economic costs. An estimated one third of epilepsy patients remain resistant to available therapies (Kwan and Brodie, 2000), with a high number of these untreated patients diagnosed with the most frequent epilepsy syndrome, temporal lobe epilepsy (TLE) with hippocampal sclerosis (Kurita et al., 2016). Importantly, tissue from refractory patients with hippocampal sclerosis has been shown to have altered levels of glial glutamate transporters (Proper et al., 2002), and changes in glial transport of glutamate has been implicated in a number of diseases of hyperexcitability (Dossi et al., 2018). Considering also that most to all anti-seizure drugs are targeted to neuronal mechanisms, it is apparent that a greater understanding of the multi-fold physiological mechanisms behind the development of epilepsy is essential to develop novel, more effective therapies (Janigro and Walker, 2014). However, investigating these mechanisms in a model that displays both clinical relevancy as well as pathological sequalae related to changes in glial mechanisms is key for thoroughly investigating glial mechanisms of glutamate homeostasis.

The Theiler murine encephalomyelitis virus (TMEV) mouse model of infection-induced TLE recapitulates clinical observations including hippocampal sclerosis (Libbey et al., 2008; Loewen et al., 2016), and offers a unique opportunity to study the molecular mechanisms underlying epileptogenesis (Libbey et al., 2008; Barker-Haliski et al.,

Abbreviations: CEF, Ceftriaxone; TMEV, Theiler's Murine Encephalomyelitis Virus; TNF-α, Tumor necrosis factor alpha; TSC, Tuberous sclerosis complex

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2015). Intracortical infection of the Daniel's (DA) strain of TMEV into C57BL/6 J mice leads to acute encephalitic seizures from 3 to 8 days post-injection (dpi) (Libbey et al., 2008; Stewart et al., 2010; Patel et al., 2017). In addition, prior publications show that mice then clear the virus by 14 dpi and a proportion of animals which experienced acute seizures go on to develop spontaneous recurrent seizures (~60%) or epileptiform activity after a latent period of 2–3 months (Libbey et al., 2008; Stewart et al., 2010). Tissue from seizing infected mice display hippocampal cell death, microglial activation, the generation of reactive oxygen species, and reductions in glutathione redox status (Libbey et al., 2008; Stewart et al., 2010; Umpierre et al., 2014; Bhuyan et al., 2015; Loewen et al., 2016). Importantly, certain findings from the brains of TMEV infected mice also suggest glial glutamate transporters could specifically be targeted in the model. Seizing animals show evidence of reactive astrogliosis, which has been associated with decreases in glutamate uptake transporter expression and function in sclerotic tissue (Proper et al., 2002; Dossi et al., 2018). Furthermore, the cytokine tumor necrosis factor-alpha (TNF-α), which has been shown to increase glutamate export and decrease uptake through glial glutamate transporters, has been closely associated with TMEV-induced seizures Lewerenz et al., 2009, 2014; Kirkman et al., 2010; Olmos and Lladó, 2014; Patel et al., 2017). However, whether glial mechanisms of glutamate homeostasis do in fact play a role in TMEV pathophysiology and whether modulating these mechanisms can reduce acute seizures and cellular sequalae is unknown.

Glial cells possess a number of ways in which to move glutamate across the membrane and contribute significantly to the control of extracellular glutamate levels. The expression profile and function of two specific glutamate transporters have been implicated in epilepsy. One key transporter, the excitatory amino acid transporter GLT-1 (the rodent homologue of excitatory amino acid transporter 2 (EAAT2)), is expressed in high levels on astrocytes and is responsible for 90% of total glutamate uptake in the brain (Kim et al., 2011). The transporter can also be expressed on microglia, notably in diseased states (Persson and Rönnbäck, 2012; Olmos and Lladó, 2014). GLT-1 knockout results in seizures and mortality (Tanaka et al., 1997), and research has suggested GLT-1 expression and function can be decreased in patient tissue and some models of brain injury, seizures, and infection (Rao et al., 2001; Proper et al., 2002; Wong et al., 2003; Goodrich et al., 2013; David et al., 2016). A promising approach to investigating GLT-1's role in seizures and epilepsy has been the use of the beta lactam antibiotic ceftriaxone (CEF). CEF treatment can increase GLT-1 expression and function (Rothstein et al., 2005) and has been shown to decrease glutamate levels, neurodegeneration, seizure activity, and other hyperexcitability-related pathophysiologies in a number of models (Jelenkovic et al., 2008; Verma et al., 2010; Zeng et al., 2010; Goodrich et al., 2013; Hussein et al., 2016). CEF treatment has further been shown to suppress the expression of TNF-α (Wei et al., 2012), which may play a facilitatory role in glutamate excitotoxicity by inhibiting expression of GLT-1 on astrocytes (Pickering and Cumiskey, 2005; Chu et al., 2007; Patel et al., 2017).

Another key glial glutamate transporter, the cystine/glutamate antiporter system x_c^-, is a plasma membrane antiporter which releases glutamate into the extracellular space while importing cystine for the eventual production of the antioxidant glutathione (Bannai, 1986; Bridges et al., 2012). Recent findings have established that in the forebrain, the system x_c^- specific subunit, xCT, is expressed by a subset of astrocytes (and tanycytes) both in physiological conditions and in two models of neuroinflammation (Ottestad-Hansen et al., 2018; Albertini et al., 2018). System x_c^- expression is induced by both neuronal activity as well as inflammatory stimuli (Lewerenz et al., 2014; Massie et al., 2015). In fact, TNF-α has been shown to increase system x_c^- activity in astrocytes and cells of myeloid origin (Jackman et al., 2010; Figuera-Losada et al., 2014). Accordingly, genetic ablation of xCT in vivo attenuates peripheral and central release of pro-inflammatory cytokines after a single peripheral injection of lipopolysaccharide (Albertini et al., 2018). xCT deletion also leads to a massive decrease of extracellular glutamate in rodent hippocampus and striatum, and has been shown to be anticonvulsant in multiple seizure models (De Bundel et al., 2011; Massie, Schallier et al., 2011; Lewerenz et al., 2014). Thus, given this collective evidence, we sought to investigate whether knockout of system x_c^-, could reduce seizure burden, neurodegeneration and gliosis in the TMEV model.

Investigating the roles of non-neuronal mechanisms of glutamate homeostasis in seizures and epilepsy is essential for future anti-seizure drug development. Considering the complex neurological response to TMEV-infection, including neuronal injury, gliosis, and cytokine and ROS production, the model serves as an excellent paradigm in which to investigate the functional role(s) of both system x_c^- and GLT-1 in infection-induced seizures. We accomplished this by investigating on one hand the effect of TMEV infection on expression levels of both transporters and on the other hand the effect of modulating the expression of GLT-1 and xCT - respectively, by the use of the positive modulator of GLT-1 expression, CEF, and by the use of the xCT knockout mouse on TMEV-induced pathology. Although we could not identify a major contribution of either of the glutamate transporters in TMEV-induced seizure development, our findings, as presented here, further contribute to the characterization of the TMEV model.

2. Materials and methods

2.1. Animals

Male C57BL/6 J mice (4–5 weeks old) were purchased from Jackson Laboratories (USA), n = 40 for initial western blot experiments and n = 26 for CEF experiments. Thirty-eight xCT wild-type (xCT+/+, n = 19) and knockout (xCT−/−, n = 19) mice were bred in a heterozygous colony at the University of Utah for system x_c^- experiments. Male and female mice of equal proportion and age were utilized for this specific experiment's groups as obtaining homozygous individuals limited the number of animals available. Genotypes were confirmed pre- and postmortem via PCR with the following primers: 5′-GATGCC CTTCAGCTCGATGCGGTTCACCAG-3′ (GFPR3); 5′-CAGAGCAGCCC TAAGGCACTTTCC-3′ [mxCT5'flankF6]; 5′-CCGATGACGCTGCCGATGA TGATGG-3′ [mxCT(Dr4)R8]. The xCT transgenic mice used in this study were of a C57BL/6 J background and were descendants of the strain previously generated by targeted disruption of the START codon in exon 1 of the Slc7a11 gene (Sato et al., 2005). Animals were 4- to 5-weeks-old at the beginning of the experiments. All animals were group housed in a temperature- and light-controlled (12 h on/12 h off) environment and permitted access to food and water ad libitum throughout the study. Animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Utah Institutional Animal Care and Use Committee. Results are described in accordance to the ARRIVE guidelines.

2.2. TMEV infections and seizure assessment

The TMEV model was performed as previously described (Libbey et al., 2008). The percent of infected mice developing seizures is positively correlated with the titer of the DA strain of TMEV used for infection (Libbey et al., 2011). Briefly, mice were anesthetized in an induction chamber with isoflurane and subsequently intracerebrally injected with 20 μl of DA strain of TMEV stock diluted in 1% PBS (vehicle). Plaque forming units titers equaled 2.6×10^6, unless noted in figure legend. A separate group was injected with 20 μl 1% PBS (vehicle sham). Injections were made at 2.5 mm with a William's collar prepared insulin syringe. Starting from 1-day post-injection (dpi), mice were agitated by briefly shaking their cages and monitored for behavioral seizures twice a day with at least 6 h in between until 7 dpi. Individuals assessing and recording seizure scores were blinded as to experimental

groups. The intensity of the seizure activity was graded using the modified Racine scale (Racine, 1972; Patel et al., 2017). Only generalized seizures (Racine 3 or higher) were scored throughout the experiment. Cumulative seizure burden was determined by summation of seizure intensity over time (3–7 dpi) (Umpierre et al., 2014; Barker-Haliski et al., 2015; Loewen et al., 2016). As previously observed, mice developed acute behavioral seizures starting from 3 dpi and the peak of seizure activity was observed 5–6 dpi, gradually decreasing afterwards.

2.3. Ceftriaxone injection

C57Bl6/J wildtype (4–5 weeks old) purchased for CEF experiments were treated as stated above with one exception. Beginning immediately after TMEV injection, animals were randomly sorted into two treatment groups. Subsequently, groups received only either saline (n = 13) or CEF (400 mg/kg) diluted in saline (n = 13) injected i.p. immediately after each seizure scoring period (2 injections/day) until 7 dpi. This dose was selected based on literature values of a high dose that was well tolerated (Lutsar et al., 1997; Hearing et al., 2016). This schedule was selected so that injections did not interfere with behavioral seizure scoring. Animals were sacrificed at 8 dpi.

2.4. Immunofluorescence

Animals were anesthetized with pentobarbital, and transcardial perfusions were performed with 0.1 M PBS followed by 4% paraformaldehyde solution, after which the brains were removed, post-fixed in paraformaldehyde, and transferred to a 15%/30% sucrose gradient for cryoprotection. Tissue was sectioned on a freezing stage microtome (Leica, Buffalo Grove, IL) at a thickness of 35 μm and stored in 0.1 M PBS at 4 °C. Dorsal hippocampal sections caudal to the septum (3 sections/animal for each stain; 4 animals per group) were batch processed. Sections were washed 3 times with 0.1 M PBS and blocked in a Triton X solution (0.2% Triton, 0.25% bovine serum albumin) for 1 h. Tissue was incubated overnight at 4 °C with primary rabbit antibodies directed to GLT-1 (ab41621, 1:250, Abcam, Cambridge, UK), with some slides being co-incubated with primary goat antibodies against ionized calcium-binding adaptor molecule 1 (Iba1) (NB100–1028, 1:750, Novus Biologicals LLC, Littleton, CO), diluted in the same solution used for the blocking step. Sections were then rinsed in 0.1 M PBS and incubated for 1.5 h in, respectively, anti-rabbit secondary antibody conjugated to Alexa 488 (A-21206, 1:200, Molecular Probes VR, Grand Island, NY), and one of the following: conjugated anti-neuronal nuclear antigen 555 (NeuN-555) (MAB377A5, 1:500, EMD Millipore, Temecula, CA), conjugated anti-glial fibrillary acidic protein Cy3(GFAP-Cy3) (C9205, 1:1000, Sigma Aldrich, St. Louis, MO), or anti-goat secondary antibody conjugated to Alexa546 (A-11056, 1300, Molecular Probes VR) (for slides stained with unconjugated Iba1). All slides were counterstained with 4′,6-diami-dino-2-phenylindole (DAPI) (D9542, 1:100, Sigma Aldrich). Primary and secondary omission controls were batch processed alongside these slides. Slides were then rinsed and coverslipped with Prolong Gold antifade reagent (Molecular Probes VR).

2.5. Imaging and image analysis

Images were captured as reported previously (Vargas et al., 2013; Loewen et al., 2016) with an Olympus FV 1000 confocal microscope using a 20/NA 1.0 air objective and analyzed utilizing ImageJ software (National Institutes of Health). Briefly, regions of interest were identified using a filter set for DAPI to avoid selection bias. Once the region was selected, laser-scanning mode was used to collect images in the z-axis. A minimum of 12 z-stack optical images (1 μm thick) were collected from each region of interest in each tissue section. Optical imaging and colocalization analysis were focused on the contralateral dorsal CA1 region of the hippocampus (including stratum pyramidale and stratum oriens), and the contralateral overlying dorsal cortex. Raw grayscale 16-bit images from the channel of each cell marker (NeuN, Iba1, and GFAP) and GLT-1 were utilized to perform analyses. To remove bias in the analysis, an automated macro created using ImageJ software (National Institutes of Health, Bethesda, MD) was utilized that performed the following functions to each raw image: the image was first converted to 8-bit and a rolling ball background subtraction was performed. Images were then automatically thresholded using the Triangle algorithm. The total field areas stained by each cell marker were measured for each optical section through the stained tissue section and averaged. Primary antibody omission slides were utilized to determine background autofluorescence (mean + 1 SD of control sections).

2.6. Western blot

Ipsilateral cortex and hippocampus were collected from 4 to 10 animals per group and homogenized in 300 μL of extraction buffer containing 2% sodium dodecyl sulphate (SDS), 60 mM Tris base, 100 mM dithiothreitol, 1 mM Na2EDTA and 1 mM sodium orthovanadate, pH 6.8, phosphatase and protease inhibitor cocktails (Sigma Aldrich). Samples were incubated for 30 min at 37 °C and centrifuged for 10 min at 9500 × g at 4 °C. Supernatants were collected and stored at −20 °C. Protein concentrations were determined using a fluorometric method (Qubit, Invitrogen). Equal concentrations of protein were loaded on a 4–12% gel (Criterion™ XT Bis-Tris Precast Gels, Bio-Rad) under reducing conditions and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 200 V, 25 W, 200 mA, 45 min; Bio-Rad Laboratories). Next, proteins were transferred to a polyvinylidene fluoride membrane (xCT) or nitrocellulose membrane (GLT-1) using respectively the Criterion® blotter module (25 V, 17 W, 130 mA, 90 min; Bio-Rad Laboratories) or the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories). Nonspecific binding was blocked by incubating the membranes for 1 h in 5% enhanced chemiluminescence (ECL) membrane blocking agent (GE Healthcare). Afterwards, membranes were incubated overnight with rabbit polyclonal anti-xCT (see (Massie et al., 2008) for generation information) (0.13 μg/mL in blocking agent at 4 °C) or anti-GLT-1 (Anti-B12) (Furness et al., 2008)) (0.13 μg/mL in Tris-buffered saline at room temperature). The next day, membranes were incubated for 30 min with horseradish peroxidase-conjugated IgG antiserum (Dako) and immunoreactive signals were visualized using ECL (ECL Plus for GLT-1 or ECL Advance for xCT, GE Healthcare). Densities of immunopositive bands were measured using ImageJ software (National Institutes of Health) and normalized to the density of total amount of proteins loaded, visualized on the same membrane (SERVA Purple, SERVA Electrophoresis GmbH). Prior to the experiment, standard curves were generated to determine the linear range of the optical density to protein concentration relationship and the optimal amount of protein to be loaded on the gel. All samples of a specific time point were analyzed on one membrane and experiments were repeated at least three times. Specificity of the anti-xCT antibody was confirmed using xCT−/− brain samples (Van Liefferinge et al., 2016). A pool for each set of samples was loaded in several dilutions and was set as reference (100%). The relative expression levels are expressed as a percentage of this reference.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.01 software. Data are presented as the mean ± SE, or as mean and 25th to 75th percentiles for immunoreactivity data. Parametric data was analyzed with a Student's *t*-test, while nonparametric data was analyzed with a Mann-Whitney *U* test. When we evaluated more than one variable, we employed a two-way ANOVA followed by Tukey's post hoc tests. The α value was set at 0.05.

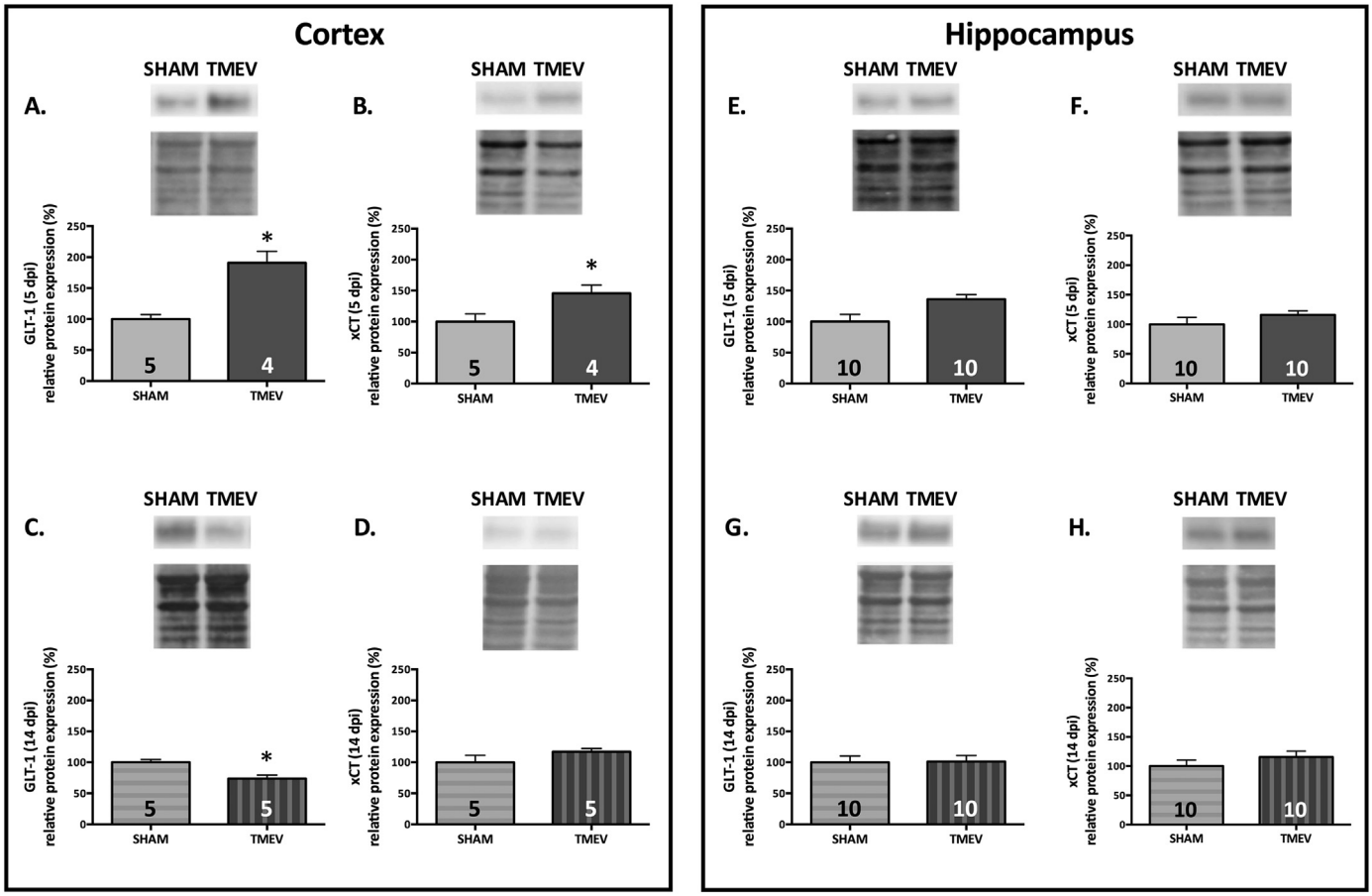

Fig. 1. Relative protein expression levels (%) of GLT-1 in ipsilateral hippocampus and cortex tissue collected at 5 dpi or 14 dpi from naive, wildtype C7Bl6/J mice intracortically injected with either PBS (SHAM) or Theiler's Murine Encephalomyelitis Virus (TMEV) (n = number of animals) TMEV PFU = 3×10^5. % relative protein expression, mean +/− SEM, Mann-Whitney U, *$p < 0.05$, **$p < 0.01$).

3. Results

3.1. Expression of cortical GLT-1 is increased in the acute phase of TMEV-infection

We first sought to investigate whether TMEV infection alone could alter glutamate transporter expression in seizure susceptible brain regions. Thus, we performed western blots for GLT-1 and xCT on hippocampal and cortex tissue collected from wildtype, naive sham (PBS)-injected and TMEV-infected C57Bl/J mice at two time points during the acute and latent periods of TMEV-infection, 5 dpi and 14 dpi respectively. Interestingly, at 5 dpi there was a significant increase in both GLT-1 and xCT in the cortex (Fig. 1, A and B) but not hippocampus (Fig. 1, E and F) of TMEV-infected mice as compared to sham-injected mice. In contrast, at 14 dpi, a timepoint when the virus has largely been cleared, a decrease in GLT-1 expression in the cortex was observed in TMEV-infected animals (Fig. 1C). No such change in expression was observed for xCT (Fig. 1D) at 14 dpi. Western blots of hippocampal tissue showed no significant differences of GLT-1 or xCT expression at either 5 dpi (Fig. 1E and Fig. 1F) or at 14 dpi (Fig. 1G and Fig. 1H). Overall, these results suggest that TMEV infection does result in the alteration of expression of GLT-1 and xCT, though only in the cortex. Furthermore, these alterations are time dependent. Given that the TMEV-infected animals experience seizures despite increases in cortical GLT-1 expression during the acute seizure period, we sought to investigate the effects of further increasing the overall expression of the transporter via pharmacological means.

3.2. Ceftriaxone enhances hippocampal GLT-1 protein expression

Cefriaxone (CEF) is a drug that has been shown to increase GLT-1 expression and function (Rothstein et al., 2005) as well as decrease glutamate levels, neurodegeneration, or seizure activity in a number of models (Jelenkovic et al., 2008; Verma et al., 2010; Zeng et al., 2010; Wei et al., 2012; Goodrich et al., 2013; Hussein et al., 2016). First, to confirm that CEF could indeed increase GLT-1 expression following CEF treatment in TMEV-infected mice, animals were sacrificed 24 h after the last i.p. injection and GLT-1 expression was examined in the hippocampus and the overlying cortex using immunofluorescence methods optimized by our laboratory (Vargas et al., 2013; Loewen et al., 2016). In line with previous research, we observed an increase in GLT-1 immunoreactivity in TMEV-infected mice treated with CEF as compared to TMEV-infected mice treated with saline. However, this increase was selectively seen in the CA1 region of the hippocampus (Fig. 2A) No such increase was observed in the overlying cortex, with no significant difference in GLT-1 immunoreactivity levels detected between CEF-treated animals and saline-treated controls (Fig. 2B). Given the dispersed nature of the GLT-1 signal and to confirm that the increase in hippocampal GLT-1 immunoreactivity area detected by our methods was representative of changes in protein expression, we further performed a western blot to assess the effect of CEF on GLT-1 expression in TMEV-infected mice. A western blot for xCT was also performed as CEF, while likely exerting its neuroprotective properties though stimulation of GLT-1 expression (Rothstein et al., 2005), has been shown to alter xCT expression in astrocytes and motor neurons via Nrf2 (Lewerenz et al., 2009; LaCrosse et al., 2017). The western blot results replicated the immunofluorescence findings, showing that GLT-1 expression was

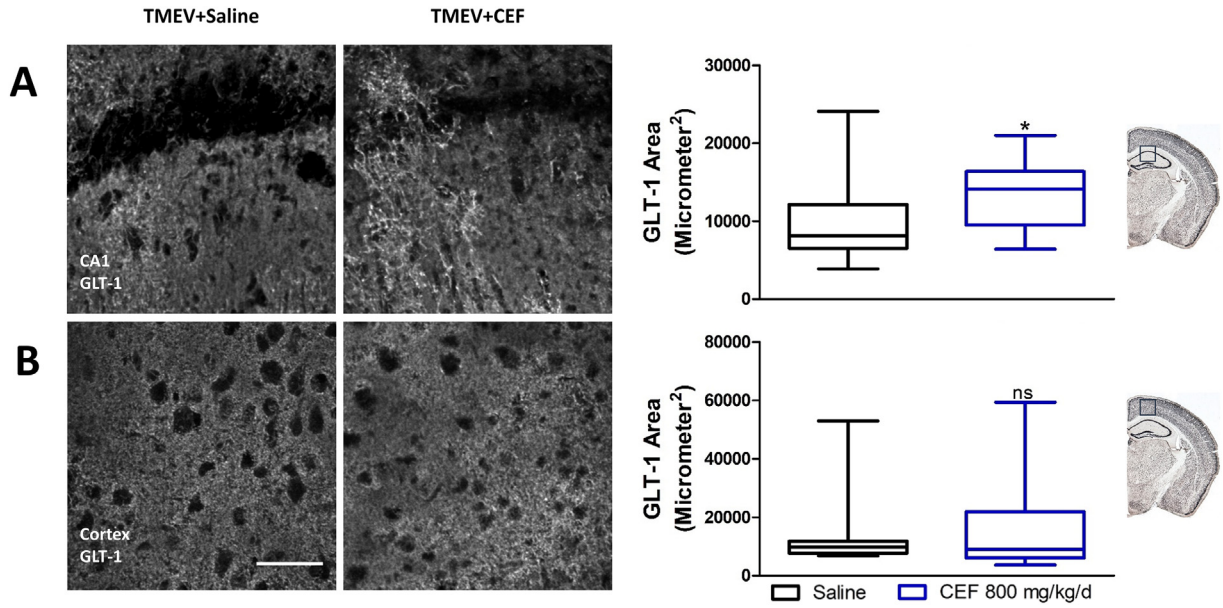

Fig. 2. Ceftriaxone (CEF) treatment increased GLT-1 immunoreactivity area in the CA1 region of the hippocampus of TMEV-infected animals, but not in the overlying parietal cortex. (A) The area of GLT-1 immunoreactivity was significantly increased in CEF-treated TMEV-infected animals. (B) In contrast, such an increase was not seen in the immediately overlying cortex, with GLT-1 immunoreactivity area being similar between the two groups (n = 4 animals per group). Inset of brain sections illustrates region of interest evaluated in each analysis. *p < 0.05, Student's *t*-test. Scale bar = 50 μm.

indeed increased in hippocampus (Fig. 3A) but not cortex (Fig. 3B). However, evaluation of xCT showed that CEF treatment had no effect on xCT expression in either hippocampus (Fig. 3B) or cortex (Fig. 3B), suggesting CEF was only affecting GLT-1 expression in our model.

3.3. CEF treatment does not attenuate acute TMEV-induced seizures

CEF treatment has been reported to reduce seizure activity in several models of brain injury (Goodrich et al., 2013; Hussein et al., 2016). To determine whether the increases in GLT-1 induced by our CEF treatment paradigm could mitigate seizure activity following TMEV infection, an analysis of seizure number and severity was performed on behavioral seizures observed from 0 dpi to 7 dpi in CEF-treated and saline treated groups. Surprisingly, given its antiseizure effects in other animal models (Jelenkovic et al., 2008; Zeng et al., 2010), CEF treatment from 0 to 7 dpi had no effect on acute TMEV-induced behavioral seizures observed over that same time (0–7 dpi). Specifically, no significant differences were detected in total number of seizures (Fig. 4A) or cumulative seizure burden (Fig. 4B) between the TMEV-infected mice treated with either CEF or the saline vehicle (Fig. 4).

3.4. Ceftriaxone does not affect TMEV-induced gliosis or neuronal injury

CEF treatment has also been reported to decrease neurodegeneration and astrogliosis in models of brain injury (Goodrich et al., 2013). However, we discovered that CEF treatment had no significant effect on the degree of neuronal injury or gliosis that occurs in the acute period of TMEV-infection (Loewen et al., 2016). Both TMEV-infected mice treated with vehicle or CEF displayed an equivalent amount of damage in the CA1 region of the hippocampus as represented by a loss of NeuN signal in the stratum pyramidal layer (Fig. 5A). In addition, no difference was seen in the level of GFAP immunoreactivity area (Fig. 5B) between TMEV-infected groups treated with either CEF or vehicle. Furthermore, no differences in NeuN or GFAP immunoreactivity were observed in the overlying cortex (Table 1). Thus, our ceftriaxone treatment paradigm did not affect TMEV-induced neurodegeneration or gliosis in the hippocampus or cortex despite its ability to increase hippocampal GLT-1.

3.5. xCT deletion does not attenuate acute TMEV-induced seizures

The xCT −/− mouse is a reliable model used to attenuate glial glutamate export via system x_c^-, in pathological conditions which enhance xCT expression and/or system x_c^- activity (Sato et al., 2005). Accordingly, xCT −/− animals have been reported to not only have increased seizure thresholds but lowered extracellular glutamate (De Bundel et al., 2011). Thus, we tested whether xCT −/− mice, when infected with TMEV, might demonstrate reduced seizure activity during the acute period. However, no difference in seizure incidence or severity after TMEV-infection was observed between xCT+/+ and xCT −/− animals. Both groups (n = 19) displayed similar total number of seizures (Fig. 6A) as well as similar cumulative seizure burdens (Fig. 6B). This suggests that the function of system x_c^- is not a critical determinant of behavioral seizure activity in this animal model of infection-induced epilepsy.

3.6. TMEV-induced neuronal loss is slightly attenuated in xCT−/− mice, which coincides with an increase in GFAP immunoreactivity

TMEV is trophic for the limbic structures in the brain and causes not only gliosis but mass neuronal death in the hippocampus (Loewen et al., 2016). While given that no difference was noted between knockouts and wildtype animals' seizures, we examined specific neuropathophysiological characteristics to see whether loss of xCT would impact either neuronal cell death or measures of reactive astrogliosis. While both xCT +/+ and xCT −/− animals displayed disruptions in the CA1 stratum pyramidal layer, as has been reported in mice infected with TMEV (Loewen et al., 2016), tissue from xCT −/− mice showed a slight but significantly greater level of NeuN immunoreactivity in the CA1 region of the hippocampus (Fig. 7A).

This difference suggests that loss of system x_c^- might result in modest neuroprotection. No such difference was seen in the overlying cortex (Table 2), though it should be noted that TMEV infection does not induce cell death in the cortex (Loewen et al., 2016). Paradoxically, it was noted that xCT −/− animals had a small but significant increase in GFAP immunoreactivity in the CA1 region (Fig. 7B).

Given that our group has shown that system xc- is expressed on

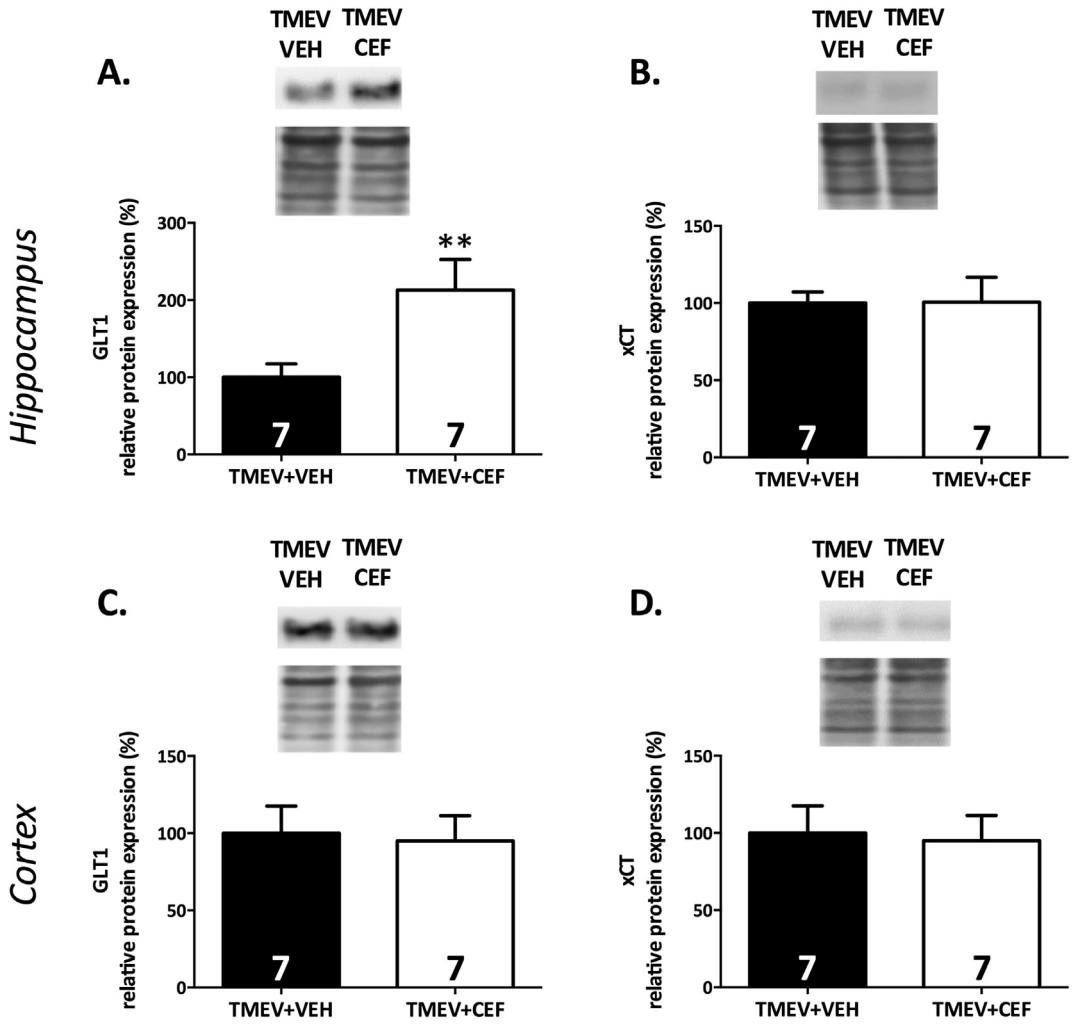

Fig. 3. Western blot confirms ceftriaxone (CEF) treatment increased GLT-1 expression in the CA1 region of the hippocampus of TMEV-infected mice, but did not alter GLT-1 expression in the cortex or xCT expression in either region, as compared to TMEV-infected mice treated with vehicle (VEH). (A) Western blot revealed GLT-1 relative protein expression was significantly increased in the hippocampus of CEF-treated TMEV-infected animals, (B) but not in cortex, paralleling immunoreactivity findings. (C) No difference in hippocampal (D) or cortical xCT expression was found between ceftriaxone or vehicle treated groups. (n = 7 animals per group) Mann-Whitney U, **p < 0.01.

astrocytes and that xCT knockout in astrocytes has an effect on microglial activation, we further pursued an immunohistochemical examination of microglial reactivity in wildtype and knockout animals (Ottestad-Hansen et al., 2018; Albertini et al., 2018). We previously reported that absence of xCT significantly attenuates microglial activation after peripheral lipopolysaccharide administration, despite the lack of xCT expression on microglial cells (Albertini et al., 2018). Thus, we evaluated Iba1 immunoreactivity, as we have previously, in both the

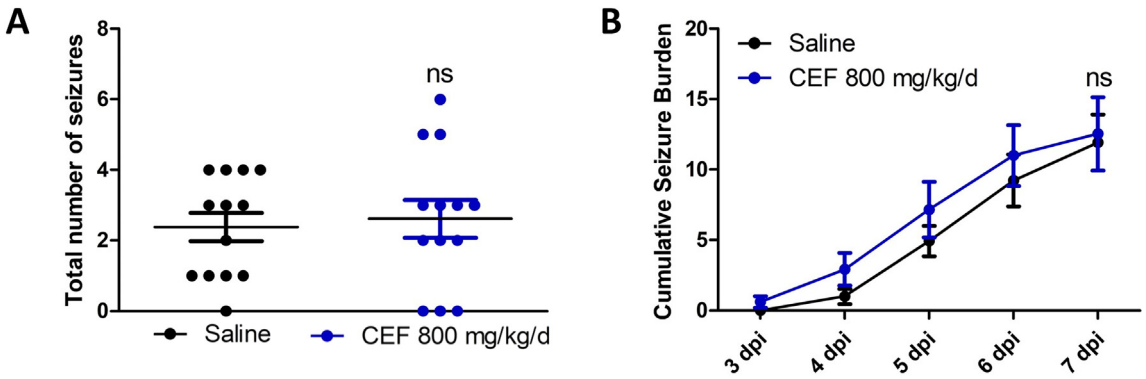

Fig. 4. Ceftriaxone (CEF) had no effect on total number of seizures or cumulative seizure burden in TMEV-infected animals treated with the drug, as compared to TMEV-infected animals treated with saline. (A) Total number of seizures in saline-treated or CEF-treated (800 mg/kg/d) TMEV-infected mice were not significantly different as measured by Student's t-test. (B) Furthermore, using cumulative seizure burden to look at seizure severity, no difference was found between the two groups as measured by ANOVA (n = 13 animals per group). *p < 0.05.

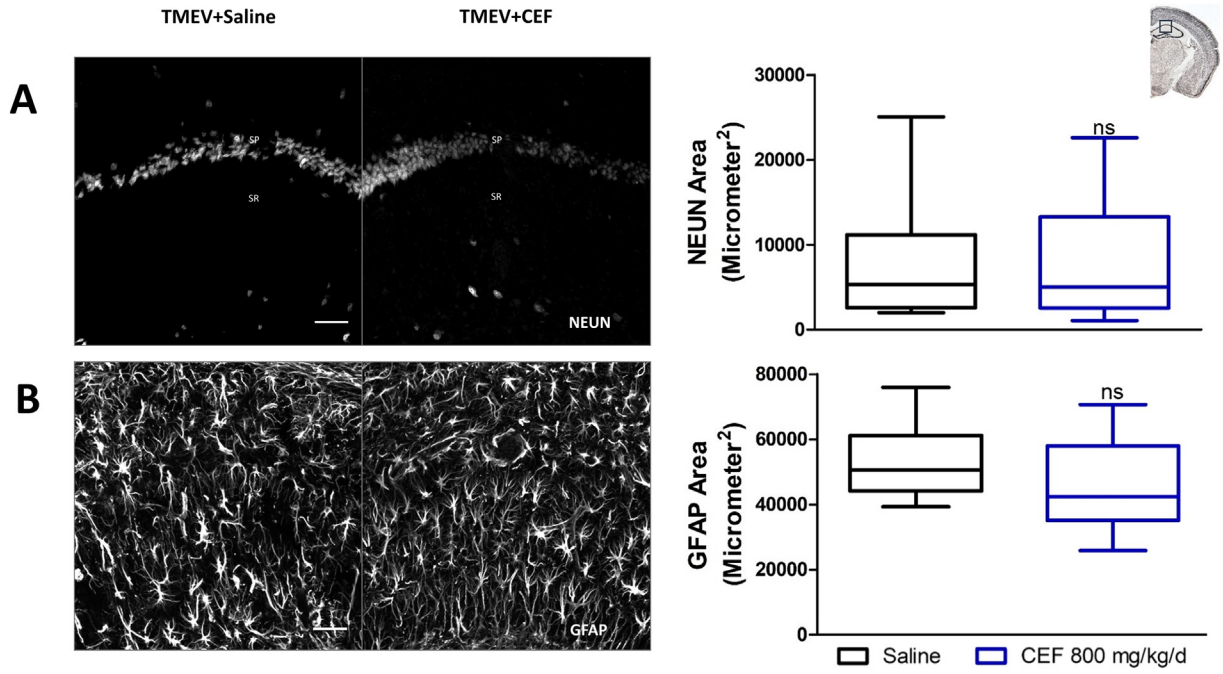

Fig. 5. CEF treatment did not reduce neuronal injury or gliosis present at 8 dpi in the CA1 region of the hippocampus in TMEV-infected mice. (A) Both treatment groups displayed a great degree of neuronal injury in the pyramidal cell layer, further demonstrated by lack of significant differences in NeuN staining. (B) Comparison of GFAP immunoreactivity area revealed no significant difference between saline- and CEF- treated groups. (n = 4 animals per group). *p < 0.05, Student's t-test. Scale bar = 50 μm, SP = Stratum pyramidale, SR = Stratum radiatum.

Table 1
Total field staining area of NEUN, GFAP, and GLT-1 evaluated in tissue collected at 8 dpi from the CA1 region of the hippocampus (CA1) and the overlying cortex (CTX) of TMEV-infected animals treated with 800 mg/kg/d ceftriaxone (CEF) or saline. (Mean +/− SEM, n = 4 animals per group, Student's t-test, *p < 0.05).

			Saline	CEF
NEUN (μm^2)	8 d.p.i.	CA1	7982 ± 2389	8254 ± 2378
		CTX	49,743 ± 2335	55,342 ± 4569
GFAP (μm^2)	8 d.p.i.	CA1	53,140 ± 4203	45,244 ± 4782
		CTX	44,764 ± 3071	45,162 ± 2115
GLT-1 (μm^2)	8 d.p.i.	CA1	9719 ± 1322	13,395 ± 1098*
		CTX	12,797 ± 2833	16,416 ± 4099

hippocampus and cortex of TMEV-infected xCT +/+ and TMEV-infected xCT −/− animals (Loewen et al., 2016). However, there was no significant difference in Iba1 immunoreactivity between the two groups (Table 2), suggesting that there was little to no effect from xCT deletion on microglial activation following TMEV-infection.

3.7. Immunoreactivity of GLT-1 expression was increased in the cortex, but not hippocampus, of xCT deficient mice infected with TMEV

A relationship between xCT and GLT-1 regulatory pathways has been hypothesized, as previous work suggests the transporters might be functionally coregulated, although the mechanism(s) behind such coregulation is/are unknown (LaCrosse et al., 2017). Thus, we further explored whether absence of xCT has an effect on GLT-1 expression in TMEV-infected mice using immunohistochemical methods given our ability to corroborate immunoreactive signal and protein expression levels as shown in Fig. 1 and Fig. 2. Examination of GLT-1 immunoreactive signal in tissue from xCT +/+ and xCT −/− animals infected with TMEV revealed no significant difference in immunoreactive area in the CA1 region of the hippocampus (Fig. 8A). Surprisingly, however, tissue from xCT −/− animals infected with

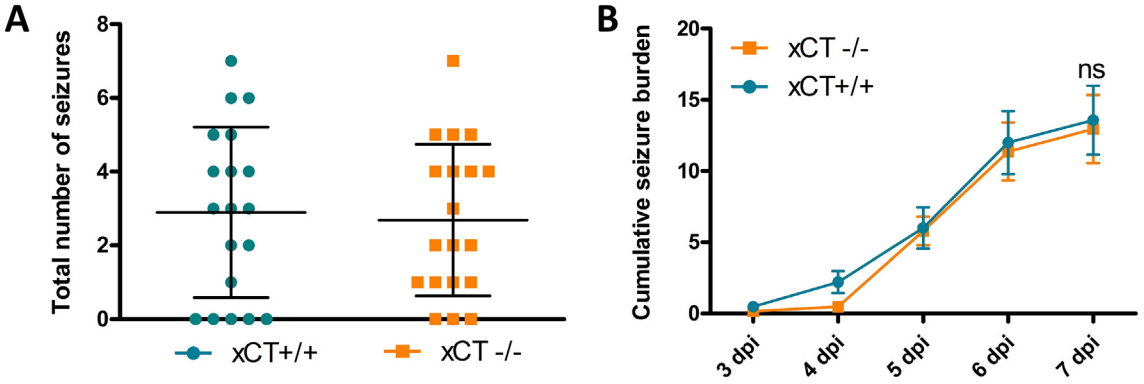

Fig. 6. Neither total number of seizures nor cumulative seizure burden were significantly different between xCT$^{+/+}$ and xCT$^{-/-}$ mice infected with TMEV. A) Total number of seizures in mice with or without xCT were not significantly different (Student's t-test). B) No difference was found between the two groups when comparing cumulative seizure burden (ANOVA; n = 19 animals per group).

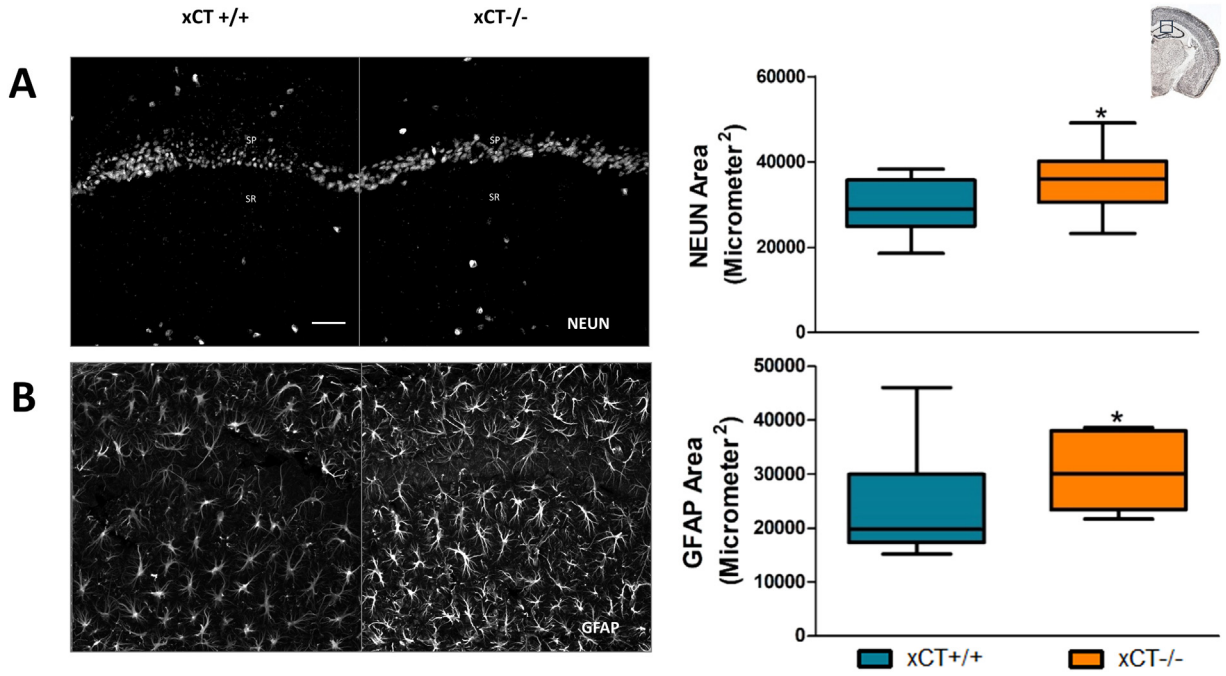

Fig. 7. TMEV-induced increases in GFAP immunoreactivity were enhanced in the hippocampus of $xCT^{-/-}$ mice, which coincided with a slight attenuation of neuronal loss. (A) Both the wildtype and xCT knockout groups displayed a great degree of neuronal injury in the pyramidal cell layer. However, $xCT^{-/-}$ mice displayed a slight reduction in loss of NEUN immunoreactivity caused by TMEV infection. (B) Analysis of GFAP immunoreactivity area also revealed a significant increase in GFAP immunoreactivity in $xCT^{-/-}$ mice (n = 4 animals per group) *$p < 0.05$, Mann-Whitney *U* test. Scale bar = 50 μm, SP = Stratum pyramidale, SR = Stratum radiatum.

Table 2
Total field staining area of NEUN, GFAP, GLT-1, as well as Iba1, evaluated at 8 dpi in the CA1 region of the hippocampus (CA1) and the overlying cortex (CTX) of xCT +/+ and xCT −/− animals infected with TMEV (Mean +/− SEM, n = 4 animals per group, Kruskal-Wallis, *$p < 0.05$).

			$xCT^{+/+}$	$xCT^{-/-}$
NEUN (μm^2)	8 d.p.i.	CA1	29,253 ± 1957	35,913 ± 2000*
		CTX	51,163 ± 3517	51,669 ± 2313
GFAP (μm^2)	8 d.p.i.	CA1	23,305 ± 2800	30,255 ± 1898*
		CTX	24,900 ± 1106	20,498 ± 1808
GLT-1 (μm^2)	8 d.p.i.	CA1	15,665 ± 4152	17,837 ± 3059
		CTX	12,445 ± 2657	37,840 ± 7374*
Iba1 (μm^2)	8 d.p.i.	CA1	80,615 ± 8670	57,765 ± 4245
		CTX	49,811 ± 4874	48,944 ± 2991

TMEV had a significant increase in GLT-1 immunoreactive signal in the cortex as compared to TMEV-infected xCT +/+ mice (Fig. 8B).

4. Discussion

In the present study we employed two approaches to investigate the roles of glial mechanisms of glutamate uptake and export in the TMEV model of viral-induced epilepsy. TMEV-infected mice display acute increases in cortical GLT-1 and xCT expression, and decreases in cortical GLT-1 after seizures have abated. Meanwhile, no difference in hippocampal expression is seen for either transporter when comparing viral infected and sham infected mice. Our results further suggest that increasing or decreasing the expression of two glial glutamate transporters, GLT-1 and xCT respectively, does not have a significant effect on seizure activity induced by TMEV infection. CEF treatment-induced increases in hippocampal GLT-1 did not coincide with a decrease in hippocampal damage or astrocyte hypertrophy. In contrast, deletion of xCT in infected seizing mice modestly reduced neuronal injury and increased astrocyte hypertrophy. Knockout animals infected with TMEV also demonstrated a notable increase in GLT-1 expression selectively in the cortex. These findings contribute to the continuing characterization of the TMEV model, as well as to the investigation of the roles of glial glutamate transporters in epilepsy.

Many published papers have shown GLT-1 levels are altered in disease states and system x_c^- activity has been associated with glial glutamate release and hyperexcitability (Robert et al., 2015; Anderson et al., 2014; Schreiner et al., 2013; Buckingham et al., 2011). However, the literature is inconsistent or, more likely, incomplete concerning which specific glutamate transporter subtypes may change their expression and function profiles and in which regions these changes occur (Coulter and Steinhäuser, 2015). Our lab has previously shown that the TMEV model displays severe and widespread gliosis early on in the acute seizure period, with eventual glial scar formation in the CA1 region (Loewen et al., 2016). However, TMEV-induced neuronal injury is restricted to the hippocampus, with acute decreases in cortical NEUN levels returning to control, sham-infected levels by 14 dpi. Our present findings further show that alongside these cellular changes, molecular expression of cortical GLT-1 expression is altered at both an acute and latent timepoint. Thus, the return of NEUN levels in the cortex might suggest that increases in either astrocyte or microglial GLT-1 occurring in the acute phase may be related to the lack of obvious neuronal injury in this area. However, what cell type could be responsible for this increased GLT-1 expression, and potentially reduced cell injury? While astrocytes express the majority of GLT-1 in the healthy brain, GLT-1 expression on microglia can be increased in inflamed states (Persson and Rönnbäck, 2012). This question of cell specific changes in glutamate transporters is further complicated by the finding that xCT expression is also increased at 5 dpi. TNF-α, which is significantly increased in TMEV-infected mice, has been shown to increase system xc- activity in cells of myeloid origin (Jackman et al., 2010; Figuera-Losada et al., 2014) that are known to infiltrate the brain and contribute to the development of acute TMEV-induced seizures (Cusick et al., 2013). Thus, while recent findings demonstrate the exclusive astrocytic localization of xCT in the forebrain, in both physiological and inflammatory conditions (Ottestad-Hansen et al., 2018; Albertini et al., 2018), we

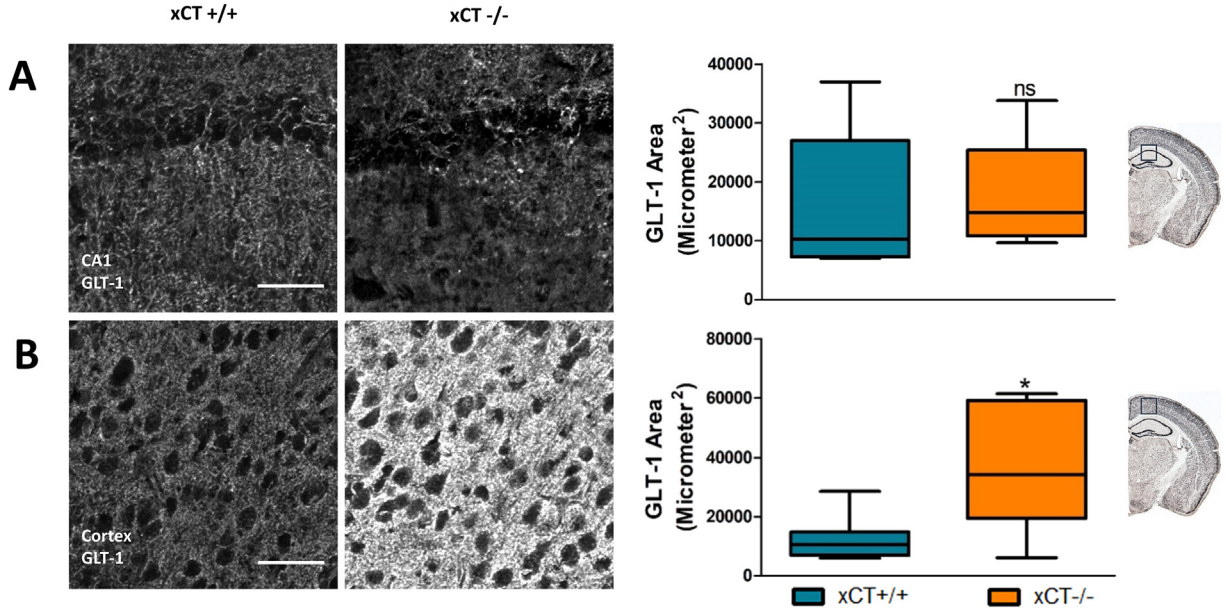

Fig. 8. GLT-1 immunoreactivity area was not altered in the CA1 region of the hippocampus, but was increased in overlying cortex of TMEV-infected xCT$^{-/-}$ mice at 8 d.p.i. GLT-1 immunostaining appears as a diffuse, widespread signal due to the ubiquitous expression of the transporter on astrocytes. (A) The area of hippocampal GLT-1 immunoreactivity was unchanged between the wildtype and xCT knockout groups following TMEV infection. (B) In contrast, a significant increase was seen in the immediately overlying cortex, with GLT-1 immunoreactivity area being increased significantly in xCT$^{-/-}$ mice. (n = 4 animals per group). *p < 0.05, Mann-Whitney U test. Scale bar = 50 μm.

cannot exclude that other cell types, such as infiltrating macrophages or resident microglia, contribute to seizure development in this model. In sum, it remains to be investigated as to which cell type(s) alter their expression of GLT-1 and xCT over the course of TMEV infection, as well as where and when these changes occur. Furthermore, the mechanisms behind these changes have implications for future testing of antiseizure drugs in the TMEV model.

Our findings from modulating GLT-1 provides an additional dimension to our understanding of these two transporters in TMEV infection. While we did demonstrate that CEF treatment was able to increase hippocampal GLT-1, the drug had no effect on either acute TMEV-induced seizures or the reported cellular pathophysiology found in TMEV-infected mice (Loewen et al., 2016). One theory might be that while CEF treatment is able to increase GLT-1 expression, these transporters may not be functional or properly localized to mediate reductions in excitotoxic levels of glutamate (Capuani et al., 2016). Of more interest is the regional specificity of the increase. First, it must be noted that there is data to suggest blood brain barrier (BBB) breakdown occurs in the acute phase of TMEV-infection (Libbey et al., 2008). It has been shown that higher antibiotic penetration correlates with the extent of inflammatory response (Nau et al., 2010; Prásil et al., 2010). Thus, should this disruption of the BBB be more severe in the CA1 region, it is possible the availability of CEF is increased in this area as compared to the relatively unaffected overlying cortex. Deeper investigation into the extent and variability of BBB breakdown across the entire TMEV-infected brain and its relevance to anti-seizure drug activity in the model is warranted. Nonetheless, we must also consider the potential differential transcriptional regulation of the GLT-1 transporter. CEF treatment alone has been shown to induce the expression of GLT-1 mRNA in the frontal cortex and striatum, but not GLT-1 protein (Krzyzanowska et al., 2016). However, in neonatal rats, it is has been shown that CEF treatment induced GLT-1 protein in the cortex but not hippocampus or striatum (Lai et al., 2011). Others still have shown that no change in hippocampal, frontal cortex, and striatal GLT-1 occurs at all after CEF treatment (Thöne-Reineke et al., 2008; Krzyzanowska et al., 2016). Regardless, our finding that seizures persist despite CEF treatment increasing the already heightened level of GLT-1 induced by TMEV infection leads to two conclusions: (1) pretreating with CEF (as utilized in Zeng et al., 2010, for example) or examining outcomes at the point of spontaneous recurrent seizure development (2–3 months) after prophylactic treatment would likely not provide the field with beneficial data concerning the efficacy of CEF in the TMEV model, however, (2) the TMEV model does provide a novel paradigm in which to investigate innate, and induced, regional and temporal mechanisms of GLT-1 transcriptional regulation.

Interestingly, we discovered that while seizures were also not altered in xCT knockout animals infected with TMEV as compared to wildtype littermates, deletion of xCT modestly reduced neuronal injury and increased hippocampal astrogliosis and significantly increased cortical GLT-1 expression in seizing animals. The finding that neuronal injury might be decreased in knockout animals is consistent with previous reports showing xCT knockout reduces extracellular glutamate levels in hippocampus (De Bundel et al., 2011). Furthermore, inhibition of system xc- might modulate microglial functioning in the CNS toward a protective state (Domercq et al., 2016; Mesci et al., 2015). So, in this case, absence of system x_c^- may decrease the ability of astrocytes (and/or microglia) to export glutamate, thus potentially decreasing extracellular levels of the neurotransmitter and reducing TMEV-induced neuronal injury. However, the corresponding increase in GFAP immunoreactivity should also be considered, as these findings may not be coincidental. Astrogliosis is a heterogenous reaction to injury (Sofroniew, 2015). Findings from our lab have suggested reactive astrocytes in the hippocampus of a model of epilepsy can in fact take up glutamate faster (Takahashi et al., 2010), thereby providing potential neuroprotection. Whether this is the case in TMEV-infected xCT −/− mice is yet to be elucidated. Further research is needed to investigate whether the modest neuroprotection observed in xCT −/− mice is sufficient to attenuate the development of spontaneous recurrent seizures once the virus has been cleared.

Surprisingly, we also observed a significant increase in GLT-1 immunoreactivity in the cortex of xCT knockout animals following TMEV infection. Naïve xCT−/− mice do not show alterations in hippocampal GLT-1 expression (De Bundel et al., 2011). Thus, an interaction between the genetic knockout of xCT and TMEV infection might result in an

increase in GLT-1 expression. We also demonstrate here that TMEV-infection alone increases GLT-1 and xCT expression in the cortex in the acute phase of the infection, but that GLT-1 expression is then selectively decreased in the cortex of TMEV-infected animals at 14 dpi. No difference between hippocampal GLT-1 was noted at this latent time point. Thus, if xCT and GLT-1 are coregulated and xCT is removed from cortex, these yet unknown mechanisms of coregulation by TMEV may mediate a compensatory increase in GLT-1 in this area (LaCrosse et al., 2017). Nonetheless, even though GLT-1 expression was increased in knockout animals following TMEV infection, seizures were still able to spread to cortical regions as evidenced by behavioral, secondarily generalized seizures. Thus, as was the case with our findings from TMEV animals treated with CEF, it is unclear if the GLT-1 transporters in this region are functionally able to reduce hyperexcitability. Regardless, our results provide a foundation upon which more research can be done into system x_c^- involvement, and treatment potential, in epilepsy and viral induced seizures.

In conclusion, we have shown that our modulations of glutamate homeostasis by either increasing glial mechanisms to take up extracellular glutamate or decreasing glial mechanisms by which to release glutamate had no effect on acute TMEV-induced seizures. Our results illuminate the fact that the differences in regional, temporal, and co-regulation may further compound the issue of where, when, and if glutamate transporters might be valid anti-seizure drug targets. We feel that our present findings contribute to the current understanding of this intricacy behind glutamate transporters' role in disease, as well as show that the TMEV model could be utilized to investigate viral-induced regional and temporal changes in glutamate transporter regulation, expression, and function.

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Disclosure of conflicts of interest

None of the authors have any conflict of interest to disclose.

Ethical publication statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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