Modulation of Astrocyte Glutamate Transporters Decreases Seizures in a Mouse Model of Tuberous Sclerosis Complex

Ling-Hui Zeng[a], **Adam W. Bero**[a], **Bo Zhang**[a], **David M. Holtzman**[a,b,d], and **Michael Wong**[a,c,d]

[a]Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110

[b]Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110

[c]Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110

[d]Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110

Abstract

Astrocyte dysfunction may contribute to epileptogenesis and other neurological deficits in Tuberous Sclerosis Complex (TSC). In particular, decreased expression and function of astrocyte glutamate transporters have been implicated in causing elevated extracellular glutamate levels, neuronal death, and epilepsy in a mouse model of TSC (*Tsc1*[GFAP]CKO mice), involving inactivation of the *Tsc1* gene primarily in astrocytes. Here, we tested whether pharmacological induction of astrocyte glutamate transporter expression can prevent the neurological phenotype of *Tsc1*[GFAP]CKO mice. Early treatment with ceftriaxone prior to the onset of epilepsy increased expression of astrocyte glutamate transporters, decreased extracellular glutamate levels, neuronal death, and seizure frequency, and improved survival in *Tsc1*[GFAP]CKO mice. In contrast, late treatment with ceftriaxone after onset of epilepsy increased glutamate transporter expression, but had no effect on seizures. These results indicate that astrocyte glutamate transporters contribute to epileptogenesis in *Tsc1*[GFAP]CKO mice and suggest novel therapeutic strategies for epilepsy in TSC directed at astrocytes.

Keywords

epilepsy; seizure; glia; GLT-1; ceftriaxone; epileptogenesis; mice

Introduction

Tuberous Sclerosis Complex (TSC) is an autosomal dominant genetic disorder, resulting from mutation of either the *TSC1* or *TSC2* genes and involving tumor or hamartoma formation in multiple organs (Kwiatkowski, 2003; Crino et al., 2006; Holmes et al., 2007). Neurological involvement, including epilepsy, cognitive deficits, and autism, often constitute the most

Corresponding Author: Michael Wong, MD, PhD, Department of Neurology, Box 8111, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, Phone: 314-362-8713, Fax: 314-362-9462, wong_m@wustl.edu.

disabling symptoms of the disease. TSC is one of the most common genetic causes of epilepsy, and epilepsy in TSC is usually severe and intractable to currently available treatments. Although hamartomas in the brain (tubers) may serve as the foci for seizures in TSC, this is controversial, and the specific cellular and molecular mechanisms of epileptogenesis in TSC are incompletely understood (Wong, 2008). Achieving a better understanding of the cellular and molecular basis of epileptogenesis should lead to more effective, rational therapies for epilepsy in TSC.

Recent studies in both human brain tissue and animal models of TSC suggest that astrocytes may play an important role in epileptogenesis and other neurological deficits in TSC (Uhlmann et al., 2002; Erbayat-Altay et al., 2007; Sosunov et al., 2008). In one putative astrocyte-related mechanism, astrocytes could potentially promote epileptogenesis and neuronal dysfunction through abnormal regulation of extracellular and synaptic glutamate homeostasis. In support of this hypothesis, a knock-out mouse model of TSC ($Tsc1^{\text{GFAP}}$CKO mice) exhibits decreased expression and function of the astrocyte glutamate transporters, GLT-1 and GLAST, and an associated increase in extracellular glutamate levels and excitotoxic neuronal death (Wong et al., 2003; Zeng et al., 2007). Abnormal glutamate homeostasis and neuronal death, in turn, may result in neurological deficits and promote neuronal hyperexcitability and seizures.

Targeting astrocytic mechanisms could represent a novel therapeutic approach for epilepsy and TSC. Beta-lactam antibiotics, such as ceftriaxone, and other pharmacological compounds have recently been reported to increase the expression of astrocyte glutamate transporters and correspondingly protect against glutamate excitotoxicity and neuronal injury (Rothstein et al., 2005; Ganel et al., 2006; Chu et al., 2007; Lipski et al., 2007). Furthermore, modulation of astrocyte glutamate transporter expression improves survival and other neurological deficits in animal models of various neurological disorders, such as amyotrophic lateral sclerosis (Rothstein et al., 2005; Ganel et al., 2006; Lepore et al., 2008). In the present study, we have tested whether a similar therapeutic approach of modulating astrocyte glutamate transporter expression is effective in countering the abnormal glutamate homeostasis, neuronal death, and epilepsy phenotype in a mouse model of TSC.

Methods

Animals and drug protocols

Care and use of animals were conducted according to an animal protocol approved by the Washington University Animal Studies Committee. $Tsc1^{\text{flox/flox}}$-GFAP-Cre knock-out ($Tsc1^{\text{GFAP}}$CKO) mice with conditional inactivation of the $Tsc1$ gene in GFAP-positive cells starting around embryonic day 14.5 were generated as described previously (Bajenaru et al., 2002; Uhlmann et al., 2002). $Tsc1^{\text{flox/+}}$-GFAP-Cre and $Tsc1^{\text{flox/flox}}$ littermates, which have been shown to have normal phenotypes, were used as control animals in these experiments. Two main drug treatment protocols were used, differing only in time of initiation of treatment. In "early treatment" studies, ceftriaxone or saline treatment was initiated at postnatal day 21, which precedes the onset of seizures and other neurological abnormalities in $Tsc1^{\text{GFAP}}$CKO mice. In "late treatment" studies, drug treatment was initiated at six weeks of age, which is typically after the onset of seizures in these mice (Erbayat-Altay et al., 2007). Ceftriaxone (Sigma, St. Louis, MO) was dissolved in 0.9% NaCl. $Tsc1^{\text{GFAP}}$CKO mice and control mice were administered ceftriaxone (200 mg/kg, i.p.) or saline daily until death or the pre-defined endpoint of the experiment. In some studies, mice were monitored daily for survival and weekly for body weight without any other interventions. Other studies involved Western blotting, glutamate microdialysis, histological analysis, or video-EEG monitoring at defined time points, as described below. Both male and female mice were used for all studies, but the male:female ratio was similar for all groups within a study and no differences in seizure frequency or the

effect of ceftriaxone was found between males and females. Littermates were used in an equal distribution between different groups.

Western blot analysis

After 1, 3 or 5 weeks of ceftriaxone or saline treatment, Western blotting was performed to assay expression GLT-1 using standard methods as described previously (Wong et al., 2003; Zeng et al., 2008). In brief, neocortex and hippocampus were dissected, sonicated, and centrifuged. Equal amounts of total protein extract were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubating with primary antibodies specific to GLT-1 (1:1000, Alpha Diagnostics, San Antonio, TX) or actin (1:5000, Sigma), the membranes were reacted with a peroxidase-conjugated secondary antibody. Signals were detected by using enzyme chemiluminescence reagent (Pierce, Rockford, IL). Data were quantitatively analyzed with ImageJ software, calculating the intensity of each band using the Gel Analyzer method, dividing the test band intensity (e.g. Glt-1) by a protein-loading control (e.g. actin), and normalizing to the control group (e.g. saline-injected control mice).

In vivo microdialysis and glutamate concentration assay

$Tsc1^{GFAP}$CKO mice receiving ceftriaxone or saline treatment for 3 weeks were implanted with microdialysis probes as previously described (Cirrito et al., 2003; Zeng et al., 2007). Briefly, after guide cannulae (BR-style, Bioanalytical Systems, Indianapolis, IN) and 2-mm microdialysis probes (BR-2, 38 kDa MWCO membrane, Bioanalytical Systems) were inserted into the hippocampus, mice were allowed to recover from anesthesia and were housed in a Raturn Cage system (Bioanalytical Systems), which permitted freedom of movement and ad libitum food and water for the remainder of the experiment. The microdialysis probe was connected to a Univentor syring pump (SciPro) and artificial cerebrospinal fluid [ACSF (in mM): 1.3 $CaCl_2$, 1.2 $MgSO_4$, 3 KCl, 0.4 KH_2PO_4, 25 $NaHCO_3$, and 122 NaCl, pH 7.35] was perfused through the microdialysis probe. To ensure that brain extracellular fluid (ECF) glutamate levels reached a steady-state concentration after probe insertion, six 1-h samples were taken at a constant flow rate during an initial equilibration phase prior to starting the protocol below. After a stable baseline was obtained, an extrapolated zero flow protocol was used to calculate the *in vivo* concentration of glutamate within the brain ECF, by measuring glutamate concentrations from dialysate samples acquired at different flow rates and extrapolating back to zero flow rate, at which point the dialysate should reach equilibrium with and equal the *in vivo* ECF glutamate concentration. Based on a modification of previous methods (Menacherry et al., 1992; Cirrito et al., 2003; Zeng et al., 2007), the extrapolation involved a second order polynomial fit: $y = a*x^2 + b*x + E$, where y = glutamate concentration, x = flow rate, and E = extrapolated *in vivo* ECF concentration at zero flow rate. To assess whether the microdialysis sampling technique and other biological factors were consistent between different conditions (e.g. control versus $Tsc1^{GFAP}$CKO mice), the percentage recovery of glutamate at each flow rate was determined and compared by the following equation: $(C_x/E) * 100$, where C_x is the measured glutamate concentration at a given flow rate and E is the *in vivo* concentration calculated by extrapolation.

All dialysate samples were collected with a refrigerated fraction collector into polypropylene tubes for subsequent measurement of glutamate concentration. Dialysate glutamate concentrations were measured using an Amplex red glutamic acid/glutamate oxidase assay kit (Molecular Probes, Eugene, OR) on the same day microdialysis was performed. For each sample, a total volume of 100 μl per microplate well was obtained by mixing 50 μl of sample with 50 μl of working solution (100 μM Amplex Red, 0.25 U/ml horseradish peroxidase (HRP), 0.08 U/ml L-glutamate oxidase, 0.5 U/ml L-glutamate-pyruvate transaminase, and 200 μM L-alanine). Samples were then incubated at 37 °C for 30 min and analyzed with a FL600

microplate reader (BioTek, Winooski, VT) with 530 nm excitation and 590 nm emission wavelengths. Glutamate concentrations of samples were determined by interpolation from a standard curve derived by measurements of other samples with known, pre-measured concentrations of glutamate. Each point was corrected for background fluorescence by subtracting values derived from glutamate-free control samples.

Histology and immunohistochemistry

After 5 weeks of ceftriaxone or saline treatment, $Tsc1^{GFAP}$CKO and control mice were transcardially perfused with PBS followed by 4% paraformaldehyde. The brains were removed immediately and post-fixed with 4% paraformaldehyde overnight at 4°C. After dehydrating in 30% sucrose for at least 24 hours, the brains were sectioned coronally at a thickness of 50 μm with a vibratome. GFAP staining was performed as described previously (Zeng et al., 2008). Sections were incubated with GFAP antibody (anti-rabbit, 1:500, Sigma, Saint Louis, MO) followed by rhodamine-conjugated anti-rabbit IgG (1:500, Sigma) and then cover-slipped with anti-fade mount solution (Molecular Probes). In separate sections, staining for Fluoro-Jade B (FJB; Histo-Chem Inc., Jefferson, AR) was performed, as described previously (Schumed and Hopkins, 2000; Zeng et al., 2007). In brief, the sections were mounted on gelatin-coated slides and dried at room temperature. After rehydration in 100% ethanol (EtOH; 5 min), 70% EtOH (2 min), and distilled water (dH2O; 2 min), the sections were oxidized in 0.06% potassium permanganate (KMnO4) for 10 min, washed with water, and then immersed in 0.0004% FJB solution for 20 min in the dark. Thereafter, slides were washed in dH2O, air dried, cleared and cover-slipped. TUNEL staining was performed using the kit obtained from Chemicon (Temcula, CA) according to the manufacturer's instructions and as done previously (Zeng et al., 2007).

Images were acquired with a Zeiss LSM PASCAL confocal microscope. GFAP-immunoreactive, FJB- and TUNEL-positive cells in hippocampus were counted in the respective sections by an investigator blinded to the treatment of the mice. The distribution of GFAP-, FJB-, and TUNEL-positive cells, and the effects of ceftriaxone, appeared to be relatively homogeneous throughout multiple regions of hippocampus. For quantitative analysis, images from the anterior, dorsal hippocampus in coronal sections at ~2 mm posterior to bregma and ~1 mm from midline were specifically analyzed. Standardized 200×200 μm^2 regions of interest were identified in hippocampus within striatum radiatum and pyramidale of CA1, and positive cells were quantified in the regions of interest from 3 sections per animal.

Video-EEG monitoring

In early treatment studies, saline- and ceftriaxone-treated $Tsc1^{GFAP}$CKO mice underwent weekly video-EEG monitoring starting at 4 weeks of age to assess seizure frequency, as described previously (Erbayat-Altay et al., 2007; Zeng et al., 2008). In late treatment studies, weekly video-EEG monitoring was started at 6 week of age, with the first monitoring session occurring immediately prior to initiation of ceftriaxone (or saline) treatment. Briefly, four epidural screw electrodes were surgically implanted in mice under isoflurane anesthesia. Mice were allowed to recover from surgery for at least 24 hours before recording. Continuous EEG data were saved digitally on personal computers using Grass P-100 AC amplifiers (Astro-Med, West Warwick, RI), Axon Digidata A-D converters, and Axoscope software (Molecular Devices, Sunnyvale, CA). To determine the behavioral correlate of electrographic seizures, simultaneous digital video was recorded using a Sanyo Day-Night camera and a Darim MG-100 MPEG video capture card (Darim Vision Corp., Pleasanton, CA). Forty-eight hour epochs of continuous video-EEG data were obtained once a week from each mouse, until the animal died or the electrodes malfunctioned. The clinical and electrographic characteristics of seizures of $Tsc1^{GFAP}$CKO mice have been reported in detail previously and were analyzed quantitatively by identical methods as described in the previous studies (Erbayat-Altay et al.,

2007; Zeng et al., 2008). Seizure frequency (# seizures/48 hr period, based on analysis of the entire EEG record) were calculated from each 48 hr epoch.

Statistics

Data are expressed as mean values ± SEM. Student's two-tailed *t* test was used for quantitative comparisons between two groups and ANOVA for comparisons for more than two groups, with Tukey multiple comparisons post-tests. Survival of ceftriaxone and saline-treated *Tsc1*GFAPCKO mice was analyzed by a Kaplan-Meier LogRank test. Statistical significance was defined as p<0.05.

Results

Early treatment with ceftriaxone increases GLT-1 expression in both control and Tsc1GFAPCKO mice

Previous studies have shown that daily injections of ceftriaxone at a dose of 200 mg/kg can induce increased expression of the astrocyte glutamate transporter, GLT-1 (Rothstein et al., 2005). In "early treatment" studies, we first initiated daily ceftriaxone (200 mg/kg, i.p., daily) or saline treatment at 3 weeks of age. Consistent with previous studies (Wong et al., 2003), saline-treated *Tsc1*GFAPCKO mice showed decreased GLT1 expression in both hippocampus and neocortex compared to saline-treated control mice. One week of ceftriaxone treatment increased GLT-1 protein expression in control mice by about 1.3 fold. Ceftriaxone increased GLT-1 expression in *Tsc1*GFAPCKO mice by 2-3 fold, correcting the deficient GLT-1 expression seen in these mice (Figure 1A,B). In other experiments, longer ceftriaxone treatment for 3 weeks or 5 weeks maintained this increase in GLT-1 expression in *Tsc1*GFAPCKO mice (Figure 1C,D). Dose-response studies showed that ceftriaxone reached a maximal effectiveness in inducing GLT-1 expression at 200 mg/kg (Supplementary Fig. 1A). In contrast, ceftriaxone did not affect phospho-S6 expression, indicating that any effects of ceftriaxone were not mediated by directly regulating the mTOR pathway (Supplementary Fig. 1B). While *Tsc1*GFAPCKO mice also exhibit decreased expression of the other major astrocyte glutamate transporter, GLAST (Wong et al., 2003), ceftriaxone did not alter the expression of GLAST in *Tsc1*GFAPCKO mice, demonstrating that ceftriaxone's effect was specific for GLT-1 (Supplementary Fig. 1C).

Early treatment with ceftriaxone decreases extracellular glutamate levels and neuronal cell death in Tsc1GFAPCKO mice

As GLT-1, a specific glutamate transporter in astrocytes, mediates synaptic glutamate uptake, a decrease in GLT-1 expression can lead to excessive extracellular glutamate levels and excitotoxic neuronal death. Related to the decreased GLT-1 expression of *Tsc1*GFAPCKO mice, we have previously demonstrated that extracellular glutamate levels are correspondingly elevated in the hippocampus of *Tsc1*GFAPCKO mice *in vivo* (Wong et al., 2003; Zeng et al., 2007). Thus, we performed additional microdialysis experiments to test whether ceftriaxone treatment could decrease extracellular glutamate levels in *Tsc1*GFAPCKO mice. After saline or ceftriaxone treatment for 3 weeks, the average hippocampal ECF glutamate concentration was significantly reduced in ceftriaxone-treated *Tsc1*GFAPCKO mice (Figure 2A,C; 867.6 ± 146.1 nM vs 1762.4 ± 70.1 for saline treated group; p<0.001, n=5 mice per group). By comparison, no difference in percentage recovery of glutamate was observed between *Tsc1*GFAPCKO mice treated with saline and ceftriaxone at each flow rate (Figure 2B), indicating that the microdialysis technique and other potentially confounding biological factors were consistent between the two groups. Although a direct comparison to glutamate levels in control mice was not repeated in the present studies, the extracellular glutamate concentration in ceftriaxone-treated *Tsc1*GFAPCKO mice was comparable to that of control mice in our previous study (Zeng et al., 2007).

We have previously reported that *Tsc1*^GFAP^CKO mice exhibit excitotoxic neuronal death in hippocampus (Zeng et al., 2007). Since decreased extracellular glutamate may protect against neuronal excitotoxicity, we used multiple methods to assay neuronal cell death in control and *Tsc1*^GFAP^CKO mice treated with saline or ceftriaxone for 5 weeks. While control mice showed minimum neuronal death, saline-treated *Tsc1*^GFAP^CKO mice exhibited obvious pyramidal neuronal cell death in hippocampus, particularly in the CA1 region, as assayed by Fluoro-Jade B (Figure 3A,B) and TUNEL staining (not shown), and consistent with our previous studies (Zeng et al., 2007). Ceftriaxone treatment caused a significant decrease in neuronal death in hippocampus of *Tsc1*^GFAP^CKO mice (Figure 3C,G).

As *Tsc1*^GFAP^CKO mice exhibit increased proliferation of astrocytes (Uhlmann et al., 2002; Zeng et al., 2008) and a difference in astrocyte number may affect extracellular glutamate levels and neuronal death, we tested whether ceftriaxone treatment affected astrocyte number in *Tsc1*^GFAP^CKO mice. Consistent with the previous studies, *Tsc1*^GFAP^CKO mice showed a significant increase in GFAP-positive cells as compared to control mice (Figure 3D,E). However, ceftriaxone treatment did not affect astrocyte number (Figure 3F,H) in the *Tsc1*^GFAP^CKO mice. Correspondingly, *Tsc1*^GFAP^CKO mice exhibit megalencephaly and increased brain weight compared to control mice due to the glial proliferation (Zeng et al., 2008), but ceftriaxone had no effect on brain size or weight (control mice: 0.39 ± 0.03 g *Tsc1*^GFAP^CKO mice + saline: 0.50 ± 0.06 g; *Tsc1*^GFAP^CKO mice + ceftriaxone: 0.51 ± 0.05 g). These results indicate that the effects of ceftriaxone on GLT-1 expression and seizure frequency (below) were independent of changes in astrocyte proliferation.

Early treatment with ceftriaxone decreases seizures and improves survival in presymptomatic Tsc1^GFAP^CKO mice

As elevated glutamate levels and excitotoxic neuronal death may promote epileptogenesis, we next examined the potential neuroprotective effect of ceftriaxone in decreasing or preventing epilepsy. In an "early treatment" paradigm, *Tsc1*^GFAP^CKO mice were treated with daily saline or ceftriaxone injections starting at 3 weeks of age, which precedes the typical age of onset of epilepsy in these mice (Erbayat-Altay et al., 2007), and underwent weekly video-EEG monitoring sessions to detect seizures. Consistent with previous studies (Erbayat-Altay et al., 2007; Zeng et al., 2008), seizures start to develop in saline-treated *Tsc1*^GFAP^CKO mice around 4-5 weeks of age and become progressively more frequent over the ensuing month (Figure 4A,B). By comparison, ceftriaxone-treated *Tsc1*^GFAP^CKO mice exhibited a similar onset and progression of seizures, but had a significantly decreased seizure frequency compared to saline-treated *Tsc1*^GFAP^CKO mice (Figure 4B). There was no significant difference in seizure frequency or the effect of ceftriaxone between male and female *Tsc1*^GFAP^CKO mice, except that the statistically-significant effects of ceftriaxone were observed at an earlier time point in female, compared to male mice (Supplementary Fig. 2A).

Tsc1^GFAP^CKO mice normally develop a wasting syndrome, with poor weight gain, and then die prematurely by 3-4 months of age (Uhlmann et al., 2002; Zeng et al., 2008). Compared to saline-treated *Tsc1*^GFAP^CKO mice, ceftriaxone-treated *Tsc1*^GFAP^CKO mice had improved weight gain and moderately, but significantly, prolonged survival, although all ceftriaxone-treated *Tsc1*^GFAP^CKO mice still died by about six months of age (Figure 4C,D). The beneficial effects of early ceftriaxone treatment on weight gain and survival were observed in both male and female mice (Supplementary Fig. 2C-E). Thus overall, early ceftriaxone treatment had some beneficial effects on seizures and survival of *Tsc1*^GFAP^CKO mice, but did not completely prevent epileptogenesis or neurological progression of these mice.

Late treatment with ceftriaxone fails to decrease seizures or improve survival in already symptomatic Tsc1^{GFAP}CKO mice

As early ceftriaxone treatment had significant effects on the neurological phenotype of *Tsc1*[GFAP]CKO mice, we next tested the effects of later treatment with ceftriaxone, starting at 6 weeks of age, after the typical onset of epilepsy in these mice. Although late ceftriaxone treatment increased GLT-1 expression in both control and *Tsc1*[GFAP]CKO mice (Figure 5A), there was no significant difference in seizure frequency between saline- and late ceftriaxone-treated *Tsc1*[GFAP]CKO mice (Figure 5B). When comparing seizure frequency between mice receiving early versus late treatment, there were trends toward lower seizure frequency in late saline-treated *Tsc1*[GFAP]CKO mice and higher seizure frequency in late ceftriaxone-treated *Tsc1*[GFAP]CKO mice compared to their respective counterparts in the early treatment groups, but these differences were not statistically significant. Similarly, late ceftriaxone treatment had no effect on body weight or survival (Figure 5C,D). Furthermore, the lack of effect of late ceftriaxone treatment on seizures (Supplementary Fig. 2B), body weight (data not shown), and survival (Supplementary Fig. 2F) was similarly observed in both male and female mice. Thus, in order to be effective, ceftriaxone had to be initiated prior to the onset of epilepsy and neurological progression in *Tsc1*[GFAP]CKO mice.

Discussion

As seizures in TSC are often severe and intractable to current treatments, novel therapeutic approaches for epilepsy in TSC are definitely needed. Ideally, to increase the likelihood of effectiveness, new rational treatments for epilepsy would target and correct specific abnormal cellular or molecular mechanisms that mediate epileptogenesis. Although mechanisms of epileptogenesis in TSC are incompletely understood, there is some evidence in animal models and human tissue that abnormal glutamate homeostasis due to impaired astrocyte glutamate transport could be a contributing factor to epilepsy and other neurological deficits in TSC (Wong et al., 2003; Wu et al., 2005; Zeng et al., 2007). In the present study, we have shown that ceftriaxone, a drug that increases astrocyte glutamate transporter expression, restores normal extracellular glutamate levels and, when administered at an early age, correspondingly decreases excitotoxic neuronal death and severity of epilepsy in a mouse model of TSC. These results provide evidence to support the role of deficient astrocyte glutamate transporters in epileptogenesis in the *Tsc1*[GFAP]CKO mice and suggest a potential novel therapeutic option for epilepsy in TSC.

A deficiency in astrocyte glutamate transporters has been implicated in the pathophysiology of other neurological diseases, as well as other types of epilepsy. For example, expression of the astrocyte glutamate transporter, GLT-1, is dramatically decreased in human ALS and mouse models (Rothstein et al., 1995; Howland et al., 2002). Similarly, abnormalities in glutamate transporter expression have been reported in various animal models of epilepsy (Miller et al., 1997; Akbar et al., 1998; Samuelsson et al., 2000; Ingram et al., 2001; Dutuit et al., 2002; Harrington et al., 2007), as well as in human brain specimens resected from epilepsy patients (Mathern et al., 1999; Tessler et al., 1999; Crino et al., 2002; Proper et al., 2002). Furthermore, genetic or pharmacological manipulations that decrease astrocyte glutamate transporters can induce an epileptic phenotype in rodents (Tanaka et al., 1997; Watanabe et al., 1999; Milh et al., 2007). Conversely, beta-lactam antibiotics, such as ceftriaxone, and other drugs can cause potent upregulation of GLT-1 expression (Rothstein et al., 2005; Ganel et al., 2006), likely by a mechanism involving stimulation of GLT-1 expression through the nuclear factor-kappaB signaling pathway (Lee et al., 2008). Correspondingly, ceftriaxone has been shown to attenuate neurological deficits in animal models of ALS (Rothstein et al., 2005), Huntington's disease (Miller et al., 2008), and stroke (Thone-Reineke et al. 2008). Although it is always difficult to rule out the involvement of complementary or alternative mechanisms

of a drug, the cumulative evidence of similar effects of ceftriaxone on GLT-1 expression and the behavioral phenotype in models of multiple neurological disorders makes GLT-1 regulation the most likely mechanism of action for its neuroprotective effects. Ceftriaxone has recently also been shown to reduce the severity of acute seizures induced in normal rats by the convulsant penetylenetetrazole (Jelenkovic et al, 2008), but, to our knowledge, the present study of $Tsc1^{GFAP}$CKO mice represents the first reported evidence that upregulating astrocyte glutamate transporters can decrease seizures in a chronic epilepsy model.

This study provides some insights into the timing and mechanisms of epileptogenesis in $Tsc1^{GFAP}$CKO mice. Consistent with previous reports (Rothstein et al., 2005), ceftriaxone administration was able to increase astrocyte GLT-1 expression within a week and this was associated with a corresponding decrease in extracellular glutamate levels in $Tsc1^{GFAP}$CKO mice. While both early and late treatments with ceftriaxone were able to restore deficient GLT-1 expression of $Tsc1^{GFAP}$CKO mice back to control levels, the effectiveness of ceftriaxone in subsequently decreasing excitotoxic neuronal death and epilepsy progression was dependent on the age of the mice, only being effective when starting treatment at 3 weeks, but not 6 weeks of age. In the first few weeks of life, $Tsc1^{GFAP}$CKO mice have decreased astrocyte glutamate transporter expression, but otherwise appear normal, with no pathological abnormalities (Uhlmann et al. 2002; Wong et al., 2003). However, by 6 weeks of age, progressive neuropathological and neurological changes develop, including astrocyte proliferation, megalencephaly, and seizures (Uhlmann et al., 2002; Erbayat-Altay et al., 2007). Thus, it is not surprising that modulation of glutamate transporters by ceftriaxone would be more effective when administered at an earlier age before this progressive process occurs, as ceftriaxone did not reverse the pathological changes, as evident by the lack of effect on astrocyte number. Even with the earlier treatment, ceftriaxone was only partially effective in slowing the progression of epilepsy, indicating that modulation of astrocyte glutamate transporter only partially accounts for the neurological phenotype of this mouse model. Other relevant brain abnormalities in $Tsc1^{GFAP}$CKO mice, such as glial proliferation and megalencephaly, were not affected by ceftriaxone and involve upstream signaling mechanisms, such as the mammalian target of rapamycin (mTOR). As the mTOR inhibitor, rapamycin, can completely prevent most of the neurological phenotype of $Tsc1^{GFAP}$CKO mice, including deficient astrocyte glutamate transporters, glial proliferation, and epilepsy (Zeng et al., 2008), it is likely that mTOR is upstream from both astrocyte glutamate transporters and other parallel mechanisms.

The lack of effect of late ceftriaxone treatment could potentially also represent a false-negative result due to technical or experimental issues. The seizure frequency of the late saline-treated $Tsc1^{GFAP}$CKO mice seemed lower than the early saline-treated mice (although the difference was not statistically significant) and our previously-published data on the natural history of seizures in the $Tsc1^{GFAP}$CKO mice (Erbayat-Altay et al., 2007; Zeng et al., 2008), possibly obscuring an effect of late ceftriaxone. Natural variability or experimental factors, such as increased mortality of older $Tsc1^{GFAP}$CKO mice related to EEG surgery or monitoring, could have contributed to an outlier effect in the late treatment groups. The sex of mice can also potentially influence a number of factors examined in this study, but a sub-analysis of the data in male and female mice found minimal influence of sex on the effects (or lack of effects) of ceftriaxone. There appeared to be a slight sex difference in the age at which early ceftriaxone treatment decreased seizure frequency, with female mice showing a beneficial response at an earlier age, relative to male mice. However, this could simply be due to low sample size of the single-sex analysis, as there was a trend toward decreased seizure frequency with early ceftriaxone in both sexes at all time points examined. Additional studies, with larger sample sizes for various subgroups, should improve the likelihood of finding other beneficial effects of late treatment of already symptomatic $Tsc1^{GFAP}$CKO mice, which may have more immediate clinical relevance.

These findings have direct translational applications for developing better treatments for epilepsy in TSC based on rational targeting of specific mechanisms of epileptogenesis. Manipulation of upstream mechanisms most immediately regulated by the *TSC* genes, such as mTOR inhibition by rapamycin, may represent the most effective way of reversing the neurological phenotype of TSC (Zeng et al., 2008; Ehninger et al., 2008; Meikle et al., 2008). However, such an approach may affect numerous downstream pathways and thus also has the most potential for adverse side effects, including disruption of important developmental processes or learning mechanisms (Tang et al., 2002). More selectively targeting a downstream mechanism, such as deficient astrocyte glutamate transporters, may still maintain adequate efficacy for epilepsy, but may avoid other more widespread and unintended consequences. Furthermore, specifically targeting astrocytic mechanisms may also reduce common side effects, such as sedation and cognitive slowing, often seen in drugs that directly decrease neuronal excitability. As more is discovered about the mechanisms of epileptogenesis and other neurological deficits in TSC, the number of rational mechanistic-based treatments should increase, hopefully providing a range of therapeutic options for alleviating the neurological symptoms of TSC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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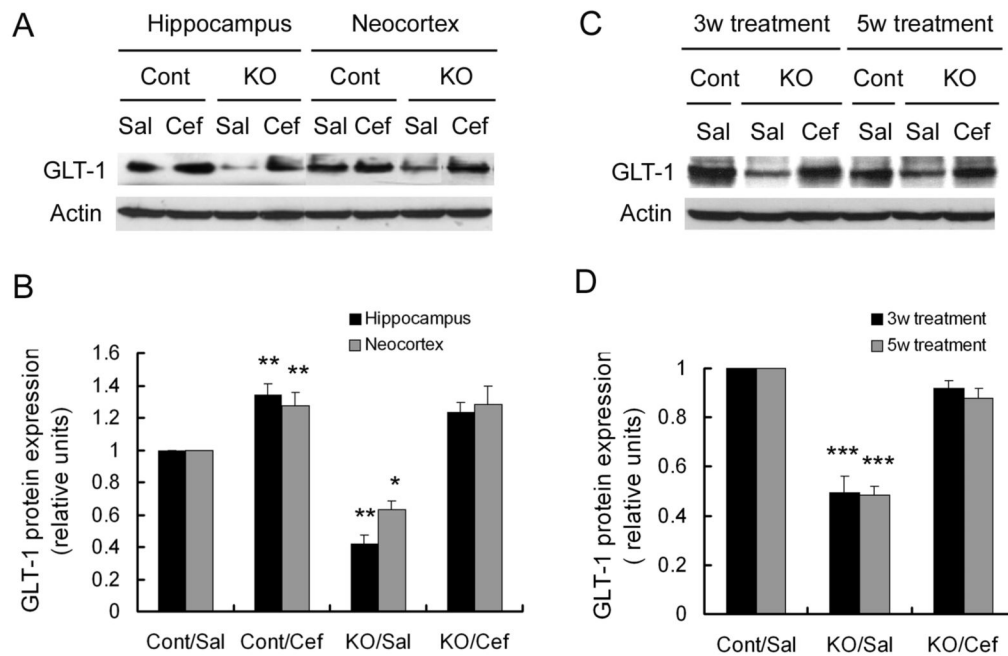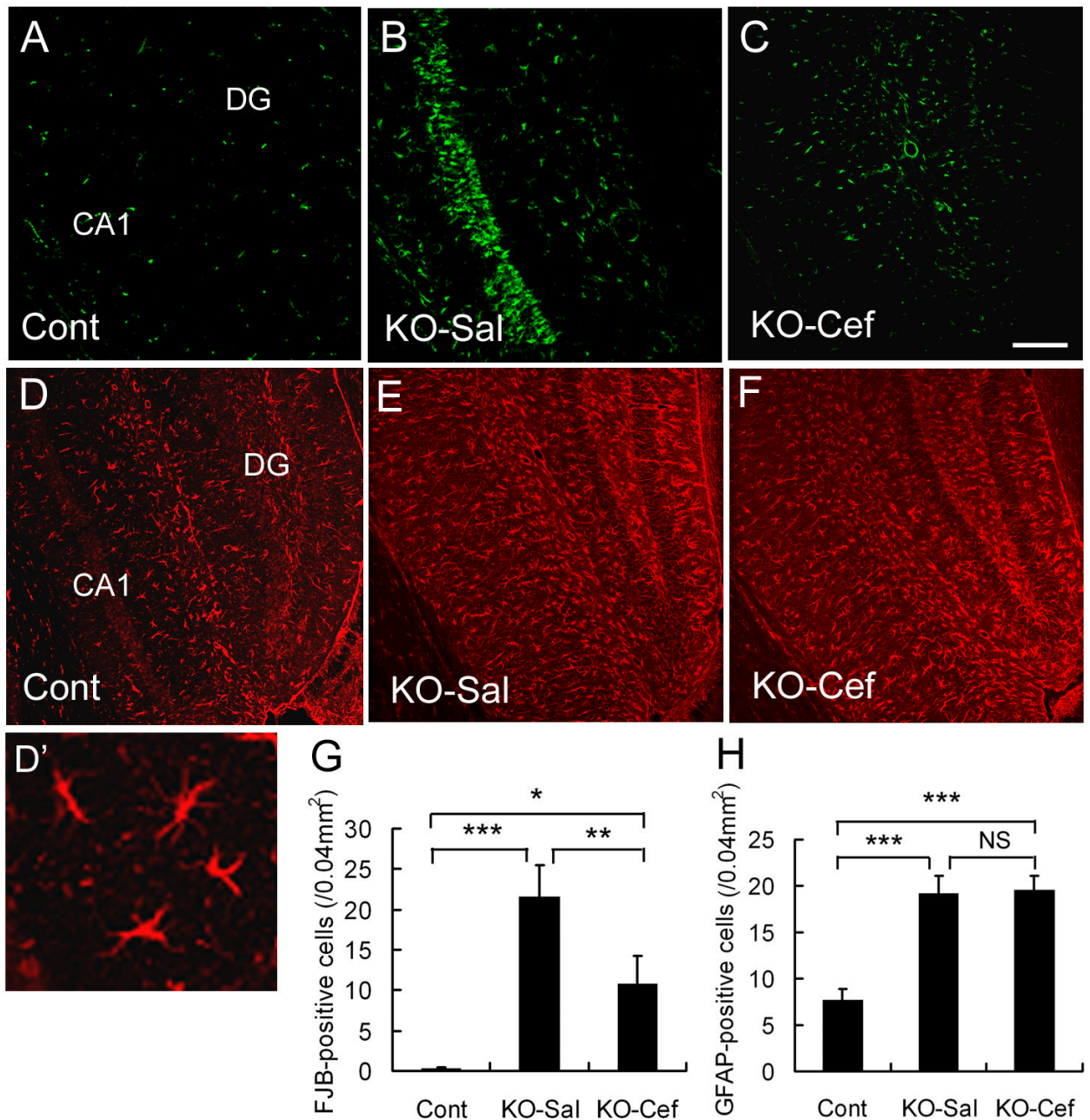
Figure 1.
Ceftriaxone increases astrocyte GLT-1 expression. (A) Three week old $Tsc1^{\text{GFAP}}$CKO (KO) and control (Cont) mice received daily injections of ceftriaxone (Cef) or saline (Sal) for one week. A representative western blot of neocortical and hippocampal extracts shows a decrease in GLT-1 expression in saline-treated $Tsc1^{\text{GFAP}}$CKO compared to control mice. Ceftriaxone increases GLT-1 expression in both control and $Tsc1^{\text{GFAP}}$CKO mice. (B) Quantitative summary of all experiments confirms that ceftriaxone causes a significant increase in GLT-1 expression in both control and $Tsc1^{\text{GFAP}}$CKO mice. The ratio of GLT-1/actin was normalized to the saline-treated control group. *p < 0.05, **p < 0.01 for both neocortex and hippocampus compared to the saline-treated control group by ANOVA (n = 6 mice per group). (C,D) Treatment of ceftriaxone for three and five weeks causes a sustained increased in GLT-1 expression in hippocampus of $Tsc1^{\text{GFAP}}$CKO mice. ***p < 0.001 compared to the saline-treated control group by ANOVA (n = 6 mice per group).
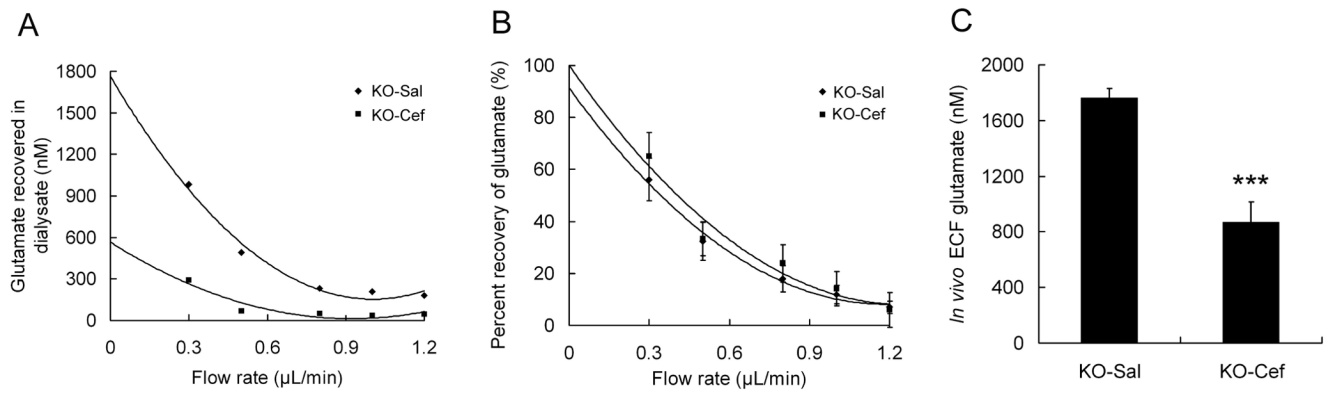
Figure 2.
Ceftriaxone decreases extracellular glutamate levels in hippocampus in *Tsc1*^{GFAP}CKO mice.
(A) Extracellular glutamate concentrations were measured from saline- (KO-Sal) and
ceftriaxone-treated (KO-Cef) *Tsc1*^{GFAP}CKO mice with microdialysis at different flow rates.
Representative examples from two mice are shown. A fitted polynomial curve shows the
dependence of the measured dialysate glutamate concentration on flow rate for each mouse
and allows extrapolation of the ECF glutamate concentration by the extrapolated zero flow
method (See Methods). (B) Recovery percentage of glutamate at various flow rates was not
significantly different between control and KO mice, indicating that the microdialysis
technique and other biological factors were consistent between the two groups. (C) Average
in vivo ECF glutamate concentration of saline- and ceftriaxone-treated *Tsc1*^{GFAP}CKO mice
was determined based on the extrapolated ECF glutamate concentration calculated individually
for each mouse. Ceftriaxone-treated *Tsc1*^{GFAP}CKO mice had significantly decreased ECF
glutamate concentrations compared to saline-treated mice ***$p<0.001$ by t-test (n = 5 mice
per group).

Figure 3.
Ceftriaxone reduces neuronal death in $Tsc1$[GFAP]CKO mice, but has no effect on astrocyte number. (A-C) Representative examples of Fluoro-Jade B positive cells in CA1 region of hippocampus in a control mouse (Cont) and $Tsc1$[GFAP]CKO mice treated with saline (KO-Sal) or ceftriaxone (KO-Cef). Saline-treated $Tsc1$[GFAP]CKO mice exhibit an increase in Fluoro-Jade B (FJB) positive cells compared to control mice, and ceftriaxone treatment significantly reduces the amount of neuronal death in the CA1 region of $Tsc1$[GFAP]CKO mice (G). (D-F) Representative examples of GFAP staining in hippocampus of a control mouse and $Tsc1$[GFAP]CKO mice treated with saline or ceftriaxone (High power image showing astrocyte morphology in D'). Both saline- and ceftriaxone-treated $Tsc1$[GFAP]CKO mice exhibit an

increase in GFAP-positive astrocytes compared to control mice, but ceftriaxone treatment has no effect on GFAP expression in *Tsc1*^{GFAP}CKO mice (H). Calibration bars = 100 μm for all panels. *p < 0.05, **p < 0.01, ***p < 0.001 by ANOVA (n = 6 mice per group).
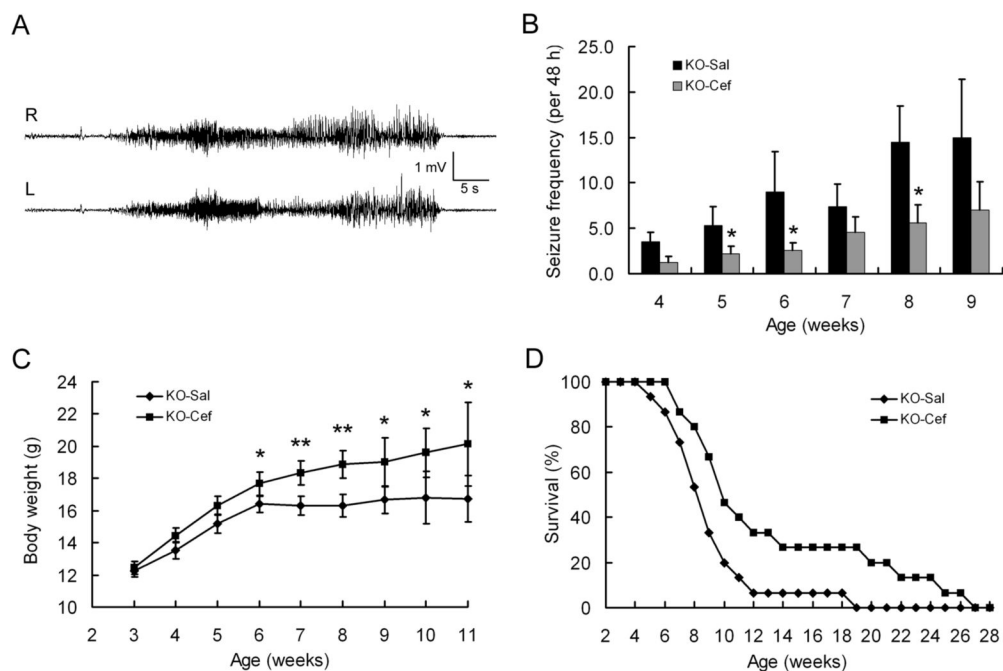
Figure 4.
Early treatment with ceftriaxone decreases seizures and improves survival in presymptomatic
*Tsc1*GFAPCKO mice. (A) Representative EEG recording of a typical seizure in a
*Tsc1*GFAPCKO mouse, recorded with right and left frontal epidural electrodes. Behaviorally,
mice typically display rearing and repetitive forelimb clonus. (B) Saline-treated *Tsc1*GFAPCKO
mice (KO-Sal) exhibit a progressive increase in seizures starting at 4 weeks of age.
*Tsc1*GFAPCKO mice treated with daily ceftriaxone starting at 3 weeks of age (KO-Cef) exhibit
a similar time course of epilepsy, but have a significant decrease in seizure-frequency. *p <
0.05 by ANOVA (n = 15 mice per group). (C) Ceftriaxone-treated *Tsc1*GFAPCKO mice have
improved weight gain compared to saline-treated *Tsc1*GFAPCKO mice, which exhibit minimal
weight gain after 6 weeks of age. *p < 0.05, **p < 0.01 by ANOVA (n = 15 mice per group).
(D) Survival analysis shows that ~50% of vehicle-treated *Tsc1*GFAPCKO mice died by 8 weeks
of age, with all dead by about 4 months. In contrast, ceftriaxone causes a small but significant
increase in survival of *Tsc1*GFAPCKO mice. p<0.05 by Kaplan-Meier LogRank test (n = 15
mice per group).
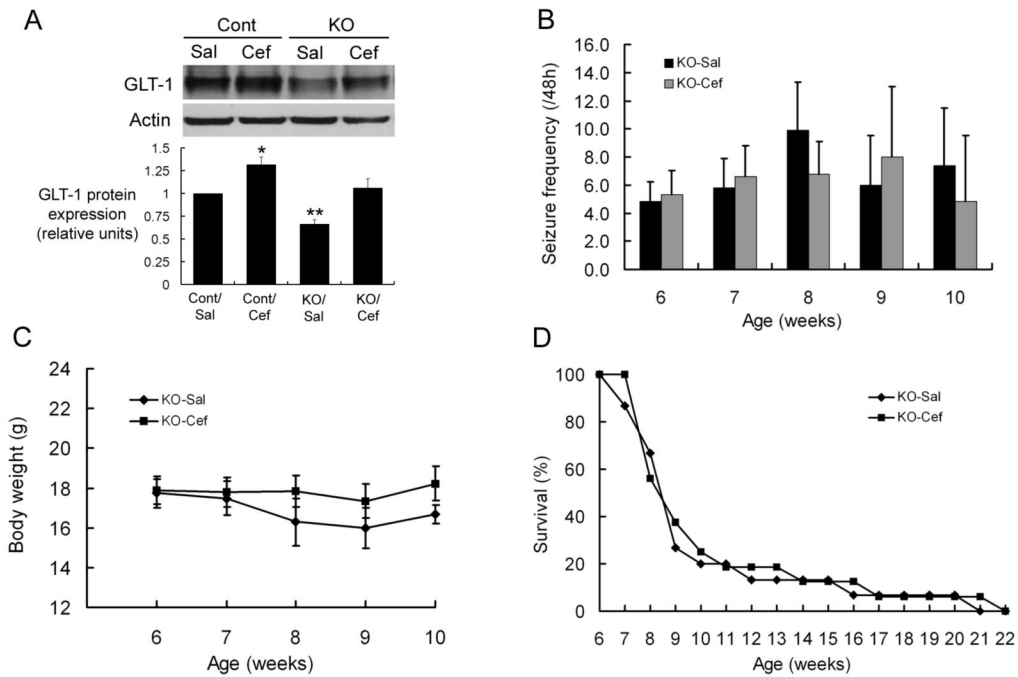
Figure 5.
Late treatment with ceftriaxone fails to decrease seizures or improve survival in already symptomatic $Tsc1^{GFAP}$CKO mice. (A) Initiation of ceftriaxone at six weeks of age (after the typical onset of epilepsy in $Tsc1^{GFAP}$CKO mice) for one week causes a significant increase in GLT-1 expression in hippocampus of both control and $Tsc1^{GFAP}$CKO mice. *p < 0.05, **p < 0.01 compared to the saline-treated control group by ANOVA (n = 6 mice per group). (B-D) Daily late ceftriaxone treatment has no effect on seizure frequency, body weight, or survival of $Tsc1^{GFAP}$CKO mice p > 0.05 by ANOVA (n = 15 mice per group).