Neurobiology of Aging 71 (2018) 241–254

Contents lists available at ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Altered glutamate clearance in ascorbate deficient mice increases seizure susceptibility and contributes to cognitive impairment in *APP/PSEN1* mice

Deborah J. Mi [a], Shilpy Dixit [a], Timothy A. Warner [b], John A. Kennard [a], Daniel A. Scharf [c], Eric S. Kessler [c], Lisa M. Moore [c], David C. Consoli [d], Corey W. Bown [d], Angeline J. Eugene [c], Jing-Qiong Kang [b], Fiona E. Harrison [a,*]

[a] Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA
[b] Division of Neurology, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA
[c] Undergraduate Program in Neuroscience, Vanderbilt University, Nashville, TN, USA
[d] Interdisciplinary Graduate Program, Vanderbilt University, Nashville, TN, USA

ARTICLE INFO

Article history:
Received 13 November 2017
Received in revised form 31 July 2018
Accepted 2 August 2018
Available online 7 August 2018

Keywords:
Vitamin C
Ascorbate
GLT-1
Glutamate
Seizure
Behavior

ABSTRACT

Ascorbate (vitamin C) is critical as a first line of defense antioxidant within the brain, and specifically within the synapse. Ascorbate is released by astrocytes during glutamate clearance and disruption of this exchange mechanism may be critical in mediating glutamate toxicity within the synapse. This is likely even more critical in neurodegenerative disorders with associated excitotoxicity and seizures, in particular Alzheimer's disease, in which ascorbate levels are often low. Using *Gulo*$^{-/-}$ mice that are dependent on dietary ascorbate, we established that low brain ascorbate increased sensitivity to kainic acid as measured via behavioral observations, electroencephalography (EEG) measurements, and altered regulation of several glutamatergic system genes. Kainic acid–induced immobility was improved in wild-type mice following treatment with ceftriaxone, which upregulates glutamate transporter GLT-1. The same effect was not observed in ascorbate-deficient mice in which sufficient ascorbate is not available for release. A single, mild seizure event was sufficient to disrupt performance in the water maze in low-ascorbate mice and in $APP_{SWE}/PSEN1_{dE9}$ mice. Together, the data support the critical role of brain ascorbate in maintaining protection during glutamatergic hyperexcitation events, including seizures. The study further supports a role for mild, subclinical seizures in cognitive decline in Alzheimer's disease.

1. Introduction

Unprovoked seizures are 5- to 10-fold more likely in Alzheimer's patient populations than in control subjects (Chin and Scharfman, 2013), occurring in cases of familial (early-onset) Alzheimer's disease, and also in more than 50% of sporadic Alzheimer's disease cases (Chin and Scharfman, 2013; Friedman et al., 2012; Larner, 2011; Noebels, 2011). This comorbidity is clinically important because seizures are a significant contributor to cognitive decline (Volicer et al., 1995; Vossel et al., 2013). Nonconvulsive, subclinical, or "silent seizures" (e.g., absence or partial seizures) go under-reported because these events are subtle and difficult to identify, particularly by family caregivers (Pandis and Scarmeas, 2012). Nevertheless, excitotoxicity leading to synaptic degeneration may be a far greater contributor to cognitive decline than has typically been assumed (Lam et al., 2017).

Alterations in glutamate transporters and uptake by glial glutamate transporter 1 (GLT-1), glutamate aspartate transporter (GLAST), and excitatory amino acid carrier 1 (EAAC1) are reported in brains of patients with Alzheimer's disease (Kirvell et al., 2006; Masliah et al., 1996). GLT-1 also decreased with age in the 3×Tg-AD model of Alzheimer's disease, but not in wild-type (WT) controls despite increased GFAP indicating greater astrocyte coverage (Zumkehr et al., 2015). Efficiency of glutamate clearance from the synapse by glutamate transporters is a key regulator of excitatory neurotransmission. The simultaneous release of ascorbate from the astrocyte as glutamate is taken up via GLT-1 (Rebec, 2013; Wilson et al., 2000) helps ensure protection against oxidative stress-related glutamate toxicity within the synapse when glutamate is present. It also ensures that ascorbate, a critical antioxidant in the brain (Harrison and May, 2009), is available for uptake by neurons

* Corresponding author at: Diabetes, Endocrinology and Metabolism, Vanderbilt University Medical Center, 7465 MRB4, 2213 Garland Avenue, Nashville, TN. Tel.: +1 6158755547; fax: +1 6159361667.
E-mail address: Fiona.harrison@vanderbilt.edu (F.E. Harrison).

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https://doi.org/10.1016/j.neurobiolaging.2018.08.002

via SVCT2 (sodium-dependent vitamin C transporters; Fig. 1). Decreased glutamate uptake results in slower or decreased ascorbate efflux from astrocytes when it is needed, as does overall brain ascorbate deficiency. GLT-1 function is highly sensitive to the cellular oxidative state (Trotti et al., 1997, 1998), and oxidative damage to the protein can further decrease the speed or efficiency of glutamate clearance. Plasma ascorbate levels from Alzheimer's patients are typically around half those of controls (Polidori and Mecocci, 2002; Rinaldi et al., 2003; Riviere et al., 1998). Similar declines in Alzheimer's patients are seen in the few studies that have used cerebrospinal fluid measurements as a proxy for brain levels (Glaso et al., 2004; Quinn et al., 2003). Ascorbate deficiency, particularly as a determinant of oxidative stress status, exhibits a strong link with cognitive decline (Gale et al., 1996; Goodwin et al., 1983; Harrison, 2012; Perrig et al., 1997). We propose that disruption of glutamate clearance or ascorbate release via the *glutamate uptake-ascorbic acid release exchange mechanism* in astrocytes contributes to neuronal hyperexcitability, and seizure susceptibility and severity in Alzheimer's disease.

APP/PSEN1 mice exhibited differences in glutamatergic function despite no difference in hippocampal GLT-1 expression (Minkeviciene et al., 2008). Spontaneous seizures are reported in many *APP* and *PSEN1* mutation–harboring mouse models (Bezzina et al., 2015; Jackson et al., 2015; Minkeviciene et al., 2009; Palop et al., 2007; Steinbach et al., 1998; Warner et al., 2015). We have reported an increased occurrence of home-cage seizures and far higher death rates (3- to 4-fold greater) when *APP/PSEN1* mice also had decreased ascorbate (30%–50% lower in the brain compared with WT), whether by knockout of *SVCT2* or the enzyme gulonolactone oxidase (*Gulo*) (Dixit et al., 2015; Harrison et al., 2010c; Warner et al., 2015). Increased mortality is observed before 6 months when β-amyloid plaque deposition is low in the *APP/PSEN1*$^{+}$ model and was hypothesized to be due to seizures. *APP/PSEN1* mutant mice are more susceptible to kainic acid–induced seizures than their WT litter mates (Steinbach et al., 1998) and show more myoclonic jerks (MJs) and spike discharges following treatment with $GABA_A$ antagonist pentylentetrazol (PTZ) (Warner et al., 2015). Treatment with fluoxetine also increased mortality due to seizures in this model (Sierksma et al., 2016). The greater cognitive deficits we reported in the low-ascorbate *APP/PSEN1* mice (Dixit et al., 2015; Harrison et al., 2010c) may, therefore, have been at least partially due to seizure occurrence. To clarify this relationship, we investigated the role of low brain ascorbate in determining susceptibility to seizures, and whether this could be related to altered glutamate clearance.

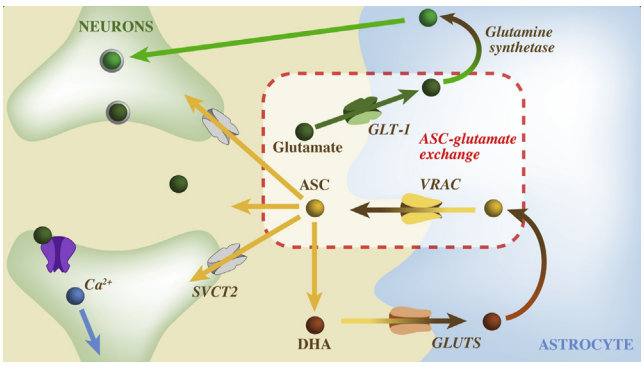

Fig. 1. Glutamate uptake-ascorbate release exchange mechanism in astrocytes. The 2 tethered systems (red box), glutamate uptake via GLT-1 and ascorbate release through volume-regulated anion channels (VRACs), are highlighted. Under normal conditions, as glutamate enters the astrocyte, it causes cellular swelling. This change results in the opening of VRACs, which allow ascorbate (ASC, yellow circles) to efflux from the astrocyte into the synapse. As an antioxidant, ascorbate donates electrons as needed to radical species in the synaptic cleft, eventually becoming oxidized to dehydroascorbic acid within the synapse (DHA, brown circles). Alternatively, some ascorbate is also available for uptake by neurons on the SVCT2. Dehydroascorbic acid is taken up on glucose transporters (GLUTS) where it is reduced back to ascorbate, ready for release owing to its very efficient recycling chemistry (Harrison and May, 2009; Wilson, 1997; Wilson et al., 2000). Glutamate (dark green circles) is converted to glutamine (light green circles), which is released from the astrocyte for reuptake by neurons. Slower glutamate clearance can contribute to hyperstimulation of postsynaptic receptors and contribute to localized oxidative stress, with the potential to further damage GLT-1 function. Abbreviations: GLT-1, glutamate transporter 1; SVCT2, sodium-dependent vitamin C transporter, type 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2. Materials and methods

2.1. Subjects

Homozygous *Gulo*$^{-/-}$ mice were originally obtained from Mutant Mouse Regional Resource Centers (http://www.mmrrc.org, MMRRC:000015-UCD) are bred in-house and maintained on a C57BL/6J background (https://www.jax.org/strain/000664; Jackson Laboratories, Bar Harbor, ME, USA). *Gulo*$^{-/-}$ mice lack a functional copy of the gulonolactone oxidase gene responsible for the final step in ascorbate synthesis and are dependent on dietary intake of ascorbate (Maeda et al., 2000). WT-equivalent levels of ascorbate in tissues are maintained by providing deionized drinking water with 1.0 g/L ascorbate and 20 μL 0.5 μM EDTA per liter, made fresh twice per week, to help maintain stability of ascorbate. Four weeks of low supplementation (0.03 g/L ascorbate) is typically sufficient to establish stable brain ascorbate levels at less than 50% of WT without risking development of scurvy (Harrison et al., 2008, 2010b; Ward et al., 2013).

WT mice were bred in-house from C57BL/6J mice obtained from Jackson Laboratories (stock #000664).

SVCT2$^{+/-}$ mice were originally obtained from Dr Robert Nussbaum and have been backcrossed to the C57Bl/6J strain for more than 10 generations. Heterozygous knockout of the SVCT2 transporter leads to approximately 30% decrease in brain ascorbic acid, with no effect on ascorbate synthesis (Harrison et al., 2010a; Sotiriou et al., 2002).

APP/PSEN1 mice (Borchelt et al., 1997; Savonenko et al., 2005) develop cognitive deficits and accumulate β-amyloid from 5 to 6 months. By 12 months, they exhibit a strong neuropathological profile, including β-amyloid accumulation, neuroinflammatory response, and oxidative stress. *APP/PSEN1* mice were bred in house from founders obtained from Jackson Laboratories (https://www.jax.org/strain/005864). Hemizygous *APP/PSEN1*$^{+}$ were crossed with the *SVCT2*$^{+/-}$ mouse line (Dixit et al., 2015) to yield litters of 4 genotypes: WT, *APP/PSEN1*, *SVCT2*$^{+/-}$, and *SVCT2*$^{+/-}$*APP/PSEN1*.

All mice were 10–14 weeks old at time of experiments unless stated otherwise. All animal experiments were performed in accordance with the local Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1.1. Seizure induction compounds (experiments 1, 3, and 4a, b)

Kainic acid monohydrate (#K0250; 10 mg/kg), pilocarpine (#P6503; 40 mg/kg), scopolamine methyl bromide (#S8502; 1 mg/kg), PTZ (#P6500; 40 mg/kg), and ceftriaxone disodium salt hemi(heptahydrate) (#C5793; 200 mg/kg) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Drugs were either made fresh daily (ceftriaxone, pilocarpine, methyl scopolamine) or reconstituted daily from stock kept at −20° for up to 1 month (Kainic acid). Pilocarpine was given 30 minutes following administration of 1 mg/kg of scopolamine methyl bromide, which was used to limit peripheral cholinergic effects of pilocarpine. All compounds were

made up in physiological saline at an administration volume of 10 mL/kg, and given via intraperitoneal (i.p.) injection. Each mouse was only exposed to a single seizure-inducing drug. Doses for each compound were selected as the lowest of the range reported in literature from comparable studies in mice because our goal was to avoid initiating severe seizure activity (and mortality).

2.1.2. Behavioral scoring of seizures (experiments 1, 3, and 4a, b)

Behavioral observations began immediately following administration of kainic acid, PTZ, or pilocarpine and continued for up to 1 hour, after which mice were returned to their home cage or were sacrificed. Cages were not returned to the vivarium until at least 3 hours after drug administration when it was confirmed that no further seizure activities were observed. For experiment 1, mice were scored live at the time of treatment. For experiment 4a in addition to live scoring, treated mice were videotaped for additional coding of activity levels by a third experimenter, who was fully blinded to experimental condition. Mice were rated for immobility time across three continuous 10-minute time bins. Mice were sacrificed, and their brains were removed either immediately after behavioral observations (within 2 hours of seizure induction) or 24 hours later to allow for wash out of drugs. Kainic acid and pilocarpine were scored according to a modified Racine scale (Table 1) in which stage 3 head bobs represents a MJ as reported in the electroencephalography (EEG) experiment. The response in PTZ-treated mice was qualitatively different, and these mice were scored according to full-body tics including tail flicks (Straub's tail phenomenon). These were classed as "small" if tail flick was $<45^{\circ}$ and "large" if the accompanying tail flick was $>45^{\circ}$. Recordings were made in 5-minute time blocks, for 15 minutes. Behavioral response to this compound did not result in the same array of behaviors as are described by the typical Racine scale used for kainic acid–treated and pilocarpine-treated groups. A small cohort of 7 mice was injected with saline and observed for behaviors resembling those scored to establish any investigator bias in recording behaviors. Behaviors were scored by a minimum of 2 experimenters, at least one of whom was blind to the experimental conditions of the mice. Following each session, reports were compared to ensure interrater reliability. Where differences existed between recorded behavior onset times, an average of the 2 latencies was used.

For experiment 4b, immobility time was measured using force plate actimeters (FPAs; Basi, USA). Mice were placed in the box immediately following ceftriaxone (or saline) and kainic acid treatments on the final day, which were given consecutively and in that order. Immobility, or "bouts of low mobility," was defined as lack of movement for 5 seconds. FPAs also provide a measure of distance traveled within the 42 × 42 cm chamber. A trained coder, blinded to mouse treatment groups at the time of analysis, also assessed the activity spectrograph and counted the number of activity spikes that corresponded to head bob behavior. Parameters were established based on visual confirmation of the head bob behavior and corresponded to a single spike of activity greater than 1.0 g above and below the average activity level (example shown in Fig. 4B). Baseline level was adjusted as needed according to changes in activity level.

Table 1
Modified Racine scale and additional behavioral scoring descriptions in response to seizure-inducing compounds

Drug	Classification	Traditional modified Racine scale and additional descriptions for this study
Kainic acid	Stage 1	Immobility/flattening
	Stage 2	Forelimb and/or tail extension, rigid posture
	Stage 3	Repetitive movements, including head bobs/myoclonic jerks
	Stage 4	Rearing and falling
	Stage 5	Continuous rearing and falling; barrel rolling
	Stage 6	Severe tonic-clonic seizures
Pilocarpine	Stage 1–6	Modified Racine scale (as for kainic acid)
	Seizure event	Full-body tremors/shaking, mouse may be stationary or attempting to walk
	Major seizure event	Event lasts more than 5 seconds
Pentylentetrazol	Full-body tic (small)	
	Full-body tic (large)	

2.1.3. Quantitative real-time PCR microarray (experiment 2)

Total RNA was extracted using RNeasy kit (Qiagen), and cDNA was synthesized from 50 ng isolated RNA per reaction. Real-time PCR was conducted using a CFX96 thermocycler. Qiagen profiler array plate "GABA and glutamate" (Qiagen; PAMM-152Z) was used to establish differences in expression of genes between naïve high ascorbate–treated and low ascorbate–treated *Gulo*$^{-/-}$ mice. The plate includes primers for 84 genes for neurotransmitter receptors, signaling downstream of GABA/glutamatergic synapses, transporters and trafficking proteins, and metabolism, plus 5 housekeeping genes. The plates were run according to the manufacturer's instructions.

2.1.4. Headmount affixation surgeries and EEG measurements (experiment 3)

Mice were affixed with a prefabricated headmount (Pinnacle Technology Inc) comprising 3 channels; 2 EEGs to assess the electrical impulses of the brain, and 1 EMG (electromyography) to measure the muscular activity evoked in the nuchal muscles. For surgical procedures, mice were anesthetized by 3%–5% of isoflurane and maintained with 1%–2% of isoflurane, which was lower than the dose (2%–3% of isoflurane) we use for typical EEG surgery to reduce mortality of *Gulo*$^{-/-}$ mice, particularly those on low supplements, as these mice are more sensitive to anesthesia. An incision was made on the scalp to expose the skull. A swab wetted with 3% H_2O_2 was gently applied to the skull surface, which resulted in a clear visualization of bregma and lambda. Four holes were drilled through the skull to dura to place stainless steel electrodes. These holes accommodated a prefabricated mouse headmount that was fastened to the skull with stainless steel screws (Small Parts, Miami Lakes, FL, USA). The headmount was placed between bregma and lambda and centered with the sagittal suture used as a reference point. Two electrodes were placed about 2 mm posterior to the bregma and 2 placed 7.5 mm posterior to the bregma, each being 1.5 mm lateral to the sagittal suture. The headmount was secured to the skull using dental acrylic. Loose skin was sutured around the implant. Only male mice were used because the larger size of male mice at 12 weeks of age makes them better able to tolerate the surgery and headmount procedure. Following a recovery period of up to 7 days, mice were placed individually in cylindrical (diameter, 10 in.) recording chambers and allowed ad libitum access to food and water. Synchronized video-EEG/EMG recordings were conducted to assess baseline brain electrical activity over a 24-hour period followed by a single treatment with kainic acid (10 mg/kg) and a further 1 hour of video-EEG/EMG recordings.

For measurements of seizure frequency and duration of abnormal EEG discharges, a reviewer blinded to mouse genotype analyzed the EEG recordings offline. For baseline EEG recordings, seizure-related activity (both EEG and corresponding video) was monitored during the final 15 minutes of the 24-hour recording period immediately before kainic acid treatments. For EEG recordings with seizure induction, the first and final 15 minutes of 60-minute recordings following kainic acid administration were scored. Synchronized

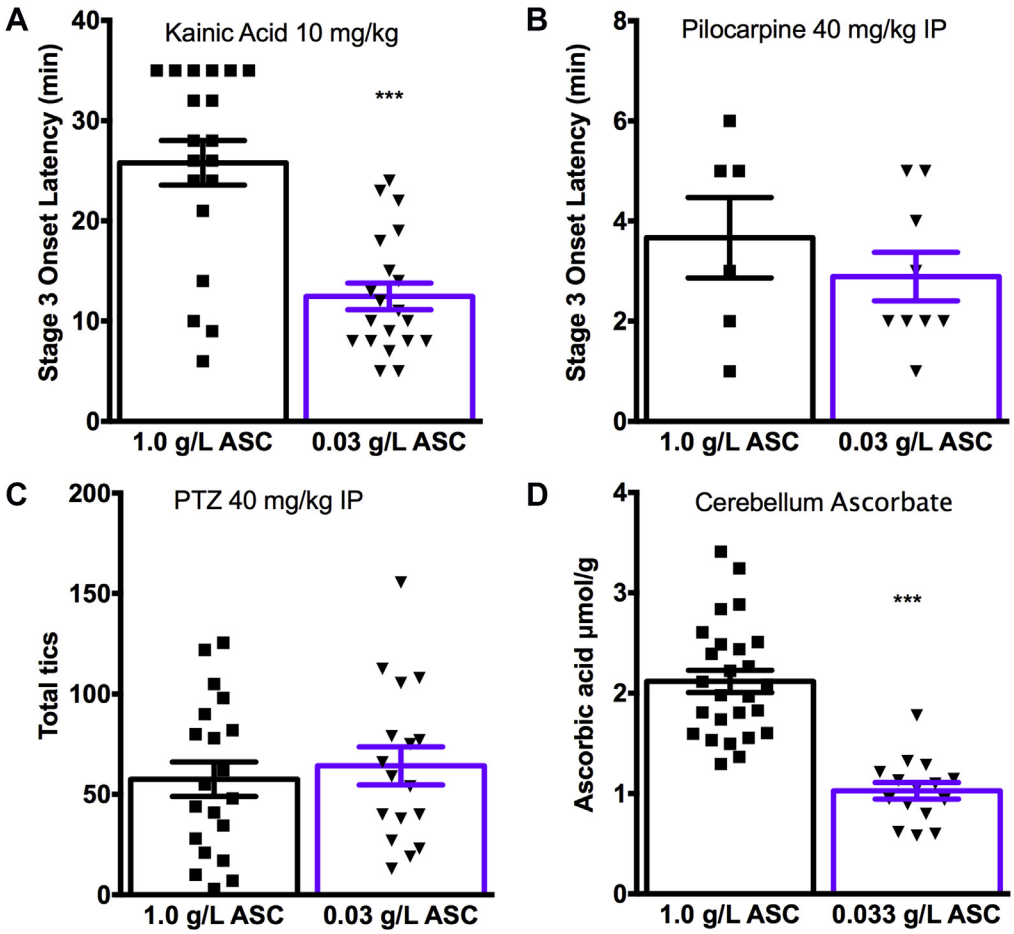

Fig. 2. Increased seizure susceptibility to kainic acid but not pilocarpine or PTZ in low ascorbate-supplemented *Gulo*$^{-/-}$ mice. *Gulo*$^{-/-}$ mice were scored according to severity of behavioral response after treatment with (A) kainic acid (10 mg/kg)—N = 19 high ascorbate (1.0 g/L) and N = 20 low ascorbate (0.03 g/L)—(B) pilocarpine (40 mg/kg)—N = 6 high ascorbate and N = 9 low ascorbate—or (C) PTZ (40 mg/kg)—N = 20 high ascorbate and N = 17 low ascorbate. Primary output measure for kainic acid and pilocarpine was latency to onset of stage 3 of the Racine scale (head bob, and/or other repetitive behavior), as well as noting any overt seizure occurrences corresponding to stages 4–6 of the Racine scale. For PTZ, the numbers of small and large full body tics were scored. (D) Brain ascorbate was measured in the cerebellum—N = 26 high ascorbate and N = 15 low ascorbate. Data analyzed by unpaired *t*-tests with Welch's correction where variances differed significantly between groups. *** $p < 0.001$ different from high ascorbate condition. Abbreviations: ASC, ascorbate; *Gulo*, gulonolactone oxidase; PTZ, pentylentetrazol.

video-EEG recordings were implemented to eliminate EEG artifacts associated with mouse eating, drinking, and mobilization. The same EEG scoring criteria were applied across mouse genotypes. All analyses were made from data collected during the same period of the light cycle (12:00–15:00 hour). We analyzed 15-minute time bins for better comparison of data before and after kainic acid treatment, rather than assessing smaller time periods across the whole 24-hour time block (Arain et al., 2012), because the purpose of the extended untreated period was to ensure all mice were fully habituated to the equipment and environment. A trained observer assessed the spike-and-wave discharges (SWDs) including specific seizure-related events (MJs) following previously determined guidelines (Fig. 4) (Akman et al., 2010; Arain et al., 2012, 2015; Chung et al., 2009). SWDs were correlated with the appropriate behavioral manifestations in the accompanying video of the EEG/EMG recordings. Abnormal discharges (absence seizure–like activity) and spike discharges (MJ-like activity) were quantified separately when observed without a detectable associated behavior with specific seizure-related event (Warner et al., 2015).

2.1.5. Ceftriaxone treatments (experiments 4a, b)

The capacity for ceftriaxone to upregulate GLT-1 expression was first identified through high-throughput screening (Rothstein et al., 2005) and has since been confirmed in a number of disease models including in the hippocampus in 3×Tg-AD mice [200 mg/kg, 2 months (Zumkehr et al., 2015)] and in the cortex and striatum of R6/2 and WT mice [200 mg/kg, 5 days (Sari et al., 2010)].

Experiment 4a utilized WT mice. Mice were treated daily with ceftriaxone (200 mg/kg, N = 13) or saline (N = 15) for 14 days. Of these 2 groups, 4 ceftriaxone- and 4 saline-treated mice were euthanized on the 14th day, 2 hours after the final treatment, and brains were used for protein determination. The remaining animals were treated with 10 mg/kg kainic acid, 1.5 hours after the final ceftriaxone of saline treatment, and observed for seizure behaviors (see Table 1).

Experiment 4b utilized *Gulo*$^{-/-}$ mice treated with either high or low ascorbate, which were given either ceftriaxone (200 mg/kg, i.p.) or saline for 14 days. On days 10 through 14, all mice received kainic acid (10 mg/kg, i.p.). Behavioral observations in FPA chambers began immediately following kainic acid injections, and mice were sacrificed within 60 minutes of kainic acid treatment. Mice were perfused with 10 mL of cold saline, and 1 hemibrain was immersion-fixed in 4% paraformaldehyde (24 hours), followed by sucrose (30%, 48 hours) as a cryoprotectant, and then kept in PBS at 4° until paraffin embedded and sectioned. The other hemibrain was dissected into hippocampus and cortex and snap-frozen before being kept at −80°.

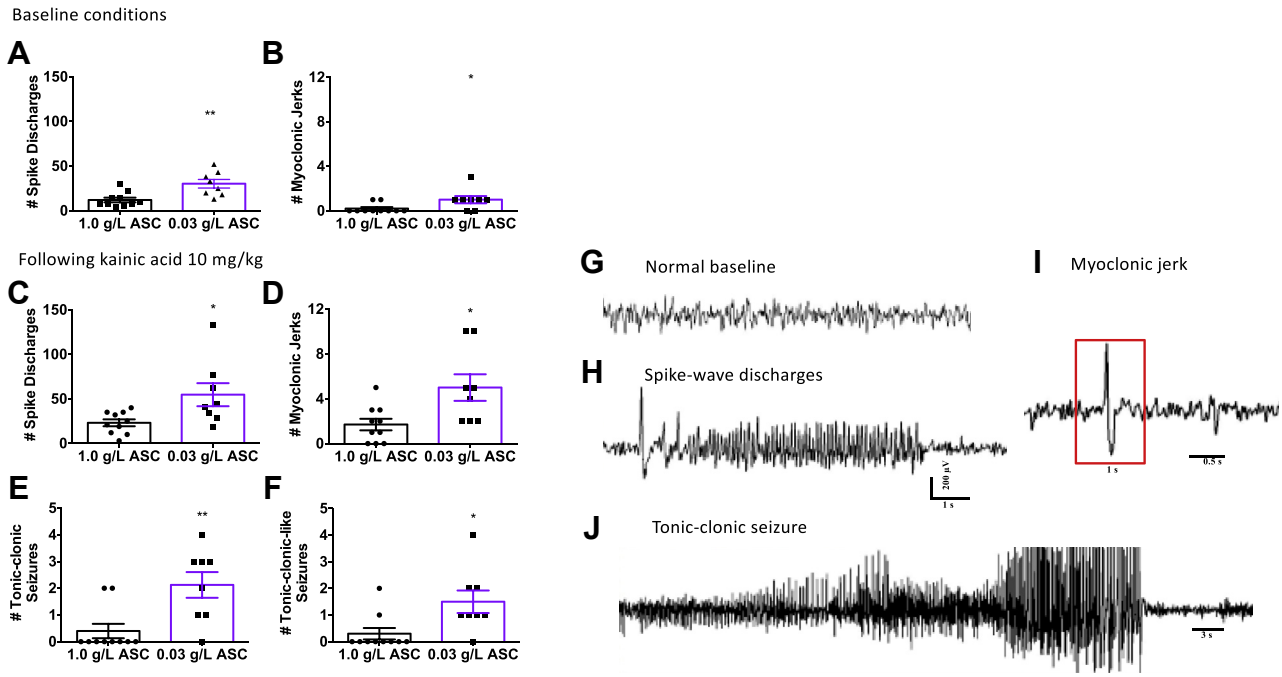

Fig. 3. Greater abnormal EEG and seizures activities in low-ascorbate *Gulo*$^{-/-}$ mice at baseline and following kainic acid. *Gulo*$^{-/-}$ mice on high (1.0 g/L, N = 10) and low (0.03 g/L, N = 8) ascorbate (ASC) supplementation were monitored via skull-mounted EEG devices for 24 hours before and 60 minutes after treatment with 10 mg/kg kainic acid. At baseline, more (A) spike wave discharges and (B) MJs were observed in mice on low-ascorbate treatments. Following kainic acid, low-ascorbate mice experienced more (C) spike discharges, (D) MJs, (E) TCSs, and (F) tonic clonic–like seizures than control and high-ascorbate mice. (G–J) Representative EEG recordings show (G) slow spike-wave discharges (SWDs), (H) spike-wave discharges, (I) MJs, and (J) generalized TCSs from the *Gulo*$^{-/-}$ mice supplemented with low ASC. Unpaired *t*-tests, * $p < 0.05$ and ** $p < 0.01$ from high-ascorbate controls. Abbreviations: EEG, electroencephalography; *Gulo*, gulonolactone oxidase; MJ, myoclonic jerk; TCS, tonic-clonic seizure.

2.1.6. Western blot (experiments 4a and 4b)

Cortical tissue was homogenized using RIPA buffer (Sigma-Aldrich, USA) with protease inhibitors (complete protease inhibitor cocktail, Roche, Switzerland). Gels were loaded with 10 μg of protein, and membranes were prepared using the iBlot system (Life Technologies, USA). Membranes were incubated with GLT-1 (AB1783, 1:4000; Millipore, Bedford, MA, USA) and GFAP (MAB360, 1:2000; Millipore), xCT (NB-300–318; Novus Biologicals, Littleton, CO, USA), and actin (D35E4, 1:5000; Santa Cruz, USA). Appropriate secondary antibodies were selected from anti-goat IgG (A5420, 1:5000), anti-guinea pig IgG (A7289, 1:5000), and anti-rabbit IgG (A0545, 1:5000), all from Sigma-Aldrich (USA).

2.1.7. Immunohistochemistry (experiment 4b)

Slide preparation and staining was completed by the Vanderbilt Translational Pathology Shared Resource Core Facility. Paraffin-embedded hemibrains were sectioned at 5 micrometers. Sections were deparaffinized in xylene, 100% and 70% ethanol, and distilled water. Slides were placed on the Leica Bond Max IHC stainer. Heat-induced antigen retrieval was performed using Epitope Retrieval 2 solution for 15 minutes. Slides were placed in a Protein Block (Ref# x0909; DAKO) for 10 minutes. Slides were incubated with cleaved Caspase-3 (Cat. 9664; Cell Signaling, Danvers, MA, USA; 1:300) or anti-GFAP (Cat.# ab16997; Abcam, Cambridge, MA, USA; 1:500 dilution) for 1 hour. The Bond Polymer Refine detection system was used for visualization. Slides were the dehydrated, cleared, and coverslipped. For Fluoro-Jade C staining, 10-μm sections were cut from the same paraffin blocks and deparaffinized as above. Sections were treated with 0.06% potassium permanganate for 15 minutes to suppress nonspecific fluorescence. Slides were stained with 0.001% Fluoro-Jade performed for 30 minutes in a light-tight box, followed by washing in distilled water. Once dried, sections were coverslipped using Dapi Mounting media.

2.1.8. Quantification of GFAP-labeled cells

To accurately count astrocyte density, we developed an automated cell counting script with MATLAB. A color threshold was applied to identify GFAP-stained cells and exclude background staining. Optimal threshold settings were set using a random sampling of images and were kept constant throughout coding. Images were converted into binary data (black/white), and a binary large object analysis was utilized to count cells for which the nucleus, cell body, and most processes were visible in the image. Quantification was validated by comparing with a small number of human-counted sections. The same images and thresholds were utilized to provide a measure of total GFAP-positive stained areas.

2.1.9. Brain ascorbate levels (experiments 1 and 4b)

Ascorbate was measured in the cerebellum, which is an accurate reflection of other more critical brain areas including the hippocampus and cortex (Harrison et al., 2010b). Ascorbate was measured by an ion pair HPLC and electrochemical detection as previously described (Harrison et al., 2008).

2.1.10. Behavioral methods

The 4 genotypes included in the present study were: (WT; $n = 17$), *APP/PSEN1* ($n = 8$), *SVCT2*$^{+/-}$ ($n = 9$), and *SVCT2*$^{+/-}$ *APP/PSEN1* ($n = 9$). Average age for each group was between 4 and 5 months at sacrifice. Only female mice were tested. WT mice behaviorally tested included littermates to the mutants bred in-house ($n = 10$) and C57BL/6J mice from Jackson Labs ($n = 7$, stock # 000664). The latter mice were included to control for genetic drift from in-house breeding, and any additional effects of parental genotype on progeny. There were no significant differences in kainic acid response or learning ability between WT mice from Jackson Labs and mice bred in-house. Behavioral testing took place in the facilities of the Vanderbilt Mouse Neurobehavioral Core Facility.

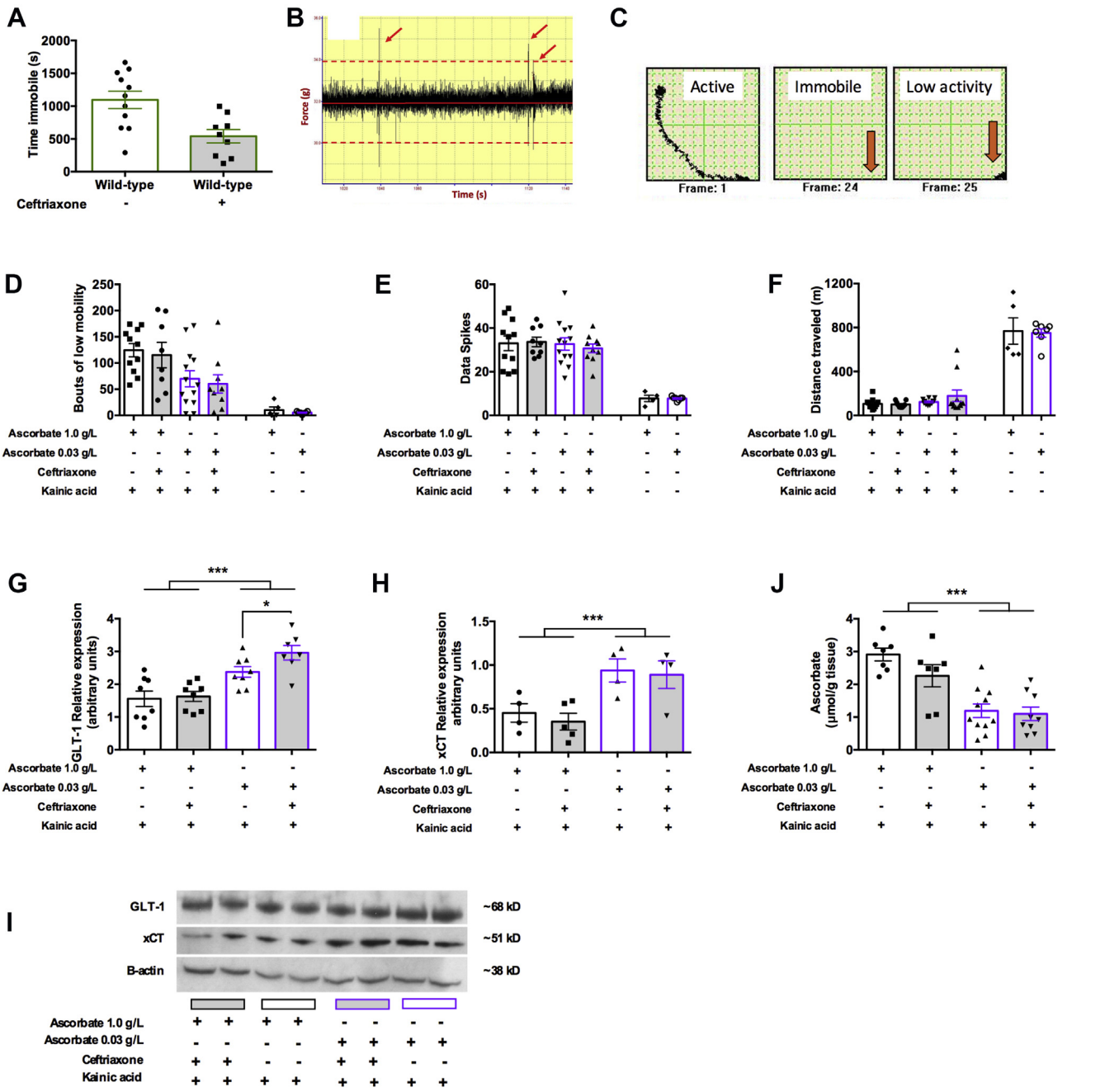

Fig. 4. Ceftriaxone upregulates GLT-1 but does not protect against kainic acid–induced behavioral changes. (A) WT mice pretreated with 200 mg/kg ceftriaxone daily for 14 days spent less time immobile in the 30 minutes following treatment with 10 mg/kg kainic acid. Saline N = 11 and ceftriaxone N = 9. (B–C) Illustration of activity data output from FPA chambers showing (B) spikes representative of MJs (red arrows) and (C) movement in the chambers in the initial few minutes of testing following kainic acid administration. (D–F) High ascorbate– (1.0 g/L) and low (0.03 g/L) ascorbate–treated *Gulo*$^{-/-}$ mice pretreated with 14 days ceftriaxone (200 mg/kg) did not differ in 3 automated or semiautomated measures of activity in the force plate actimeters, (D) bouts of low mobility, (E) spikes reflecting possible MJs, and (F) distance traveled. (high saline N = 9, high ceftriaxone N = 11, low saline N = 11, and low ceftriaxone N = 13). (G and I) GLT-1 expression was significantly increased in low-ascorbate *Gulo*$^{-/-}$ mice with a further increase in low-ascorbate mice according to ceftriaxone treatment (N = 7–9 per group, from 4 separate blots). (H and I) xCT expression was increased in low ascorbate *Gulo*$^{-/-}$ mice but did not change in response to ceftriaxone (N = 4–5 per group). (J) Brain ascorbate levels reflected dietary supplementation regimens. $^{*}p < 0.05$ and $^{***}p < 0.001$ differences between groups as marked. Abbreviations: GLT-1, glutamate transporter 1; *Gulo*, gulonolactone oxidase; MJ, myoclonic jerk; WT, wild-type. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.1.11. Locomotor activity

Locomotor activity was assessed to examine genotype-dependent baseline activity differences that could affect water maze performance. Mice were placed in the activity chamber (ENV-510; MED Associates, Georgia, VT; 27 × 27 × 20.3 cm [L × W × H]) and activity was automatically recorded by the breaking of infrared beams as the mouse explored the chamber. Mice were given a 30 minutes session 24 hours prior to water maze training.

2.1.12. Rotarod

Motor learning and coordination were tested using an accelerating rotarod (Ugo Basile model 7650; Stoelting Co, Wood Dale, IL, USA). The rotarod accelerates from 6 to 50 RPM during the 300 seconds trial. The latency to fall from the rotarod and to the first rotation (mouse clings to the rod and rotates along with it) was recorded. Two sessions were conducted on consecutive days, with 3 trials per session.

2.1.13. Morris water maze

Spatial memory was tested in a Morris water maze using a 120 cm diameter pool with clearly visible cues fixed around the perimeter of the testing room. (1) Mice were first trained to locate the 10 cm circular acrylic platform that was marked and visible above the surface of the water. Mice were given 4 trials (maximum 60 seconds) per day for 3 days. The visible platform phase accounts for sensorimotor differences that may affect spatial learning (i.e., visual acuity, swim speed). (2) Hidden platform testing was conducted with the platform submerged 1 cm below the surface of the water. Mice received 4 acquisition trials per day for 5 days. To assess retention of the platform location, a 60 seconds probe trial was conducted with the platform removed. The first probe trial was given 24 hours following the final acquisition session. In addition, mice received another acquisition trial immediately following the first probe trial to minimize extinction of the learned location before the second probe trial. (3) Mice were treated with kainic acid 1–2 hours after the first probe trial. Probe 2 was given 24 hours following the first probe trial. (4) Mice were then trained to locate a new hidden platform location with 4 trials per day for 3 days. Sessions were captured by an overhead camera and analyzed in real time using ANY-maze software (Stoelting, Wood Dale, IL, USA) on a PC. Latency to escape and path length (distance) were used to index search accuracy during acquisition and relearning. Retention of the platform position during the probe test was assessed using time spent swimming within the target quadrant versus non-target quadrant, as well as the more sensitive measures of time spent swimming with 20 cm of the platform, and average distance from the platform (termed "Search Error") (Gallagher et al., 1993).

2.1.14. Experimental design and statistical analysis

Animal numbers for all experiments are provided in Table 2. Differences between 2 groups (e.g., high vs. low ascorbate in *Gulo*$^{-/-}$ mice, ceftriaxone vs. saline in WT mice) were analyzed in GraphPad Prism 5 for Mac OS X. Single dependent variables were analyzed using unpaired *t*-test (2-tailed). If group variances were significantly different, Welch's correction for unequal variances was applied. For tests between 2 groups requiring non-parametric testing Mann-Whitney U or Kruskal-Wallis tests were used. Where there were 2 independent variables (e.g., ascorbate level in *Gulo*$^{-/-}$ mice and ceftriaxone treatment, or *SVCT2* and *APP/PSEN1* genotype), data were analyzed using IBM SPSS Statistics, version 24, for Mac. Univariate data were analyzed using 2 × 2 analysis of variance (ANOVA). Tests that occurred across multiple days of testing (e.g., water maze acquisition) were analyzed using repeated-measures ANOVA tests with test day as the repeated measure. Following a significant omnibus, ANOVA pairwise comparisons were conducted, with Bonferroni corrections for multiple comparisons, to assess the simple effects of either *APP/PSEN1* or *SVCT2* genotype at each level of the other genotype, for example, the effect of *APP/PSEN1* genotype within either *SVCT2*$^{+/+}$ or *SVCT2*$^{+/-}$ groups. (These comparisons do not allow for direct comparison between WT and *SVCT2*$^{+/-}$ *APP/PSEN1* groups).

For gene array data, genes that had at least 2 samples with Ct values greater than 32 were excluded from analyses, leaving a total of 71 genes. These were analyzed by separate independent *t*-tests, which were corrected using the Benjamini-Hochberg procedure for a subset of genes, to protect against false discovery rates.

3. Results

3.1. Low ascorbate increased susceptibility to seizures induced by kainic acid, but not by pilocarpine or PTZ

The goal of this research was to investigate changes that occur during and following mild seizures that do not present with traditional or easily-observable behaviors. A low dose of kainic acid (10 mg/kg) was chosen at which most WT mice reach stage 3 on the Racine scale (repetitive movements or head bobbing), but few mice progress past stage 3 into more severe seizures. Similarly, doses of pilocarpine (40 mg/kg) and PTZ (40 mg/kg) were also selected to elicit only a moderate behavioral response.

Mice were observed for 1 hour following administration of kainic acid, but all mice that were observed to have head bobs (stage 3) had done so by 32 minutes. Mice for which a head bob was not observed (N = 6, high ascorbate) were given a latency score of

Table 2
Animal numbers for all data included in statistical analyses

Experiment	Mouse model	Groups	N, sex
1) Seizure induction	*Gulo*$^{-/-}$	Kainic acid, high ascorbate	7M, 12F
		Kainic acid, low ascorbate	9M, 11F
		PTZ, High ascorbate	12M, 8F
		PTZ, Low ascorbate	10M, 8F
		Pilocarpine, high ascorbate	5M
		Pilocarpine, low ascorbate	9M
2) Gene expression	*Gulo*$^{-/-}$	High ascorbate	3M, 3F
		Low ascorbate	3M, 3F
3) EEG [a]	*Gulo*$^{-/-}$	High ascorbate	10M
		Low ascorbate	8M
4) Ceftriaxone studies [b]	Wild-type	Saline	4M, 5F
		Ceftriaxone	5M, 6F
	Gulo$^{-/-}$	High ascorbate, Saline	9M, 2F (11)
		High ascorbate, Ceftriaxone	6M, 3F (8)
		Low ascorbate. Saline	6M, 7F (14)
		Low ascorbate, Ceftriaxone	3M, 6F (9)
5) Behavioral studies [c]	*SVCT2*$^{+/-}$ × *APP/PSEN1*$^{+/-}$	*Wild-type*	17F
		APP/PSEN1	8F
		SVCT2$^{+/-}$	9F
		SVCT2$^{+/-}$ *APP/PSEN1*	9F

Key: EEG, electroencephalography; *Gulo*, gulonolactone oxidase; M, male; F, female.
No mice were excluded from analyses, except for health concerns as listed below. These mice are not included in group numbers given above:
[a] Death after or during surgery because *Gulo*$^{-/-}$ mice are more sensitive to the effects of isoflurane anesthesia (N = 2 low ascorbate).
[b] Mice were excluded if they exhibited illness due to repeated injections, for example, weight loss or swelling, internal bleeding at sacrifice (N = 3 high ascorbate). This experiment was repeated in 2 separate cohorts of animals.
[c] One mouse escaped from the locomotor activity apparatus and so was excluded from analyses.

35 minutes (not 60 minutes to avoid skewing the results, although this may slightly underestimate the magnitude of difference between the groups). Following 10 mg/kg kainic acid, stage 3 head bobs were observed in mice under low ascorbate supplementation (0.03 g/L; N = 20) with a significantly shorter latency than in high ascorbate-supplemented mice (1.0 g/L; N = 19) (Mann-Whitney $U = 52$, $p < 0.0001$; Fig. 2A). Following pilocarpine administration, stage 3 head bobs were observed very quickly, and all but 1 mouse (high ascorbate) also suffered more severe seizure events encompassing shakes or tremors, which did not include rearing/falling, barrel rolls, or tonic-clonic seizures (TCSs). There was no difference in latency to stage 3 [Unpaired *t*-test, $t(13) = 0.88$, $p = 0.39$; Fig. 2B], or latency to the first severe seizure event [high 14.4 minutes ± 1.63, low 10.89 minutes ± 1.84, $t(12) = 1.27$, $p = 0.23$, *not shown*]. Three of 6 (50%) high-ascorbate mice and 5 of 9 low-ascorbate mice (55.6%) mice suffered a major seizure event lasting greater than 5 seconds. Following PTZ administration, mice were classified according to the number of full-body tics. There were no differences between groups on total body tics (small and large, across the 15 minutes, scoring period; $t(35) = 0.52$, $p = 0.61$; Fig. 2C). No head bobs, tail extensions, or seizure behaviors at stage 4 or above were observed, although mice were observed to have periods of immobility, without rigid or abnormal postures. For saline-treated mice (N = 7, *data not shown*), 4 or fewer tics were observed per mouse within the 15-minute scoring time frame. We confirmed that ascorbate levels in the brain (cerebellum) were decreased by at least 50% in the low-ascorbate mice (N = 15) compared with high-ascorbate mice (N = 26) (Welch-corrected $t(39) = 7.86$, $p < 0.0001$; Fig. 2D).

These data confirmed that low ascorbate rendered mice more susceptible to seizures induced through direct activation of the glutamatergic system (kainic acid; kainate receptor agonist), but not when the GABA (PTZ, $GABA_A$ receptor antagonist) or cholinergic (pilocarpine, muscarinic receptor agonist) systems were challenged directly.

3.2. Low ascorbate alters expression patterns of glutamatergic but not GABAergic transport genes

To further investigate the sensitivity of the glutamate system to low ascorbate, we determined the expression profile of a number of genes in the hippocampus in both glutamatergic and GABAergic systems using premade microarray plates (GABA and Glutamate PCR array; Qiagen). Expression patterns were only considered for genes with a Ct value of <32, and data were normalized to expression of Hsp90ab, which was determined by the software to provide the most stable expression via a non-normalized calculation of the 5 possible housekeeping genes (Actb, B2m, Gapdh, Gusb, and Hsp90ab1). We observed moderate changes in gene expression according to ascorbate treatment in 5 genes from the glutamatergic array, including the 3 major glutamate transporters, and only one gene from the GABA array [comparison made by uncorrected independent *t*-tests: Slc1a2/GLT1 ($p = 0.0046$; Table 3), Slc1a1/EAAC1 ($p = 0.0037$), Slc1a3/GLAST ($p = 0.016$), Grm8 ($p = 0.037$), Snca ($p = 0.012$)]. Because our hypothesis was that changes would be observed in "transport and trafficking genes," we subjected the 18 genes in that subset, as defined by the manufacturer, to the Benjamini-Hochberg procedure for multiple comparisons. Only EAAC1 and GLT-1 were found to be significantly altered by ascorbate level in $Gulo^{-/-}$ brains (p's = 0.0409, Table 3). The results from this small study combined with the pharmacological data already presented prompted us to continue to explore our hypotheses in a more targeted way.

Together these data support the hypothesis that low ascorbate in the brain can alter expression of a number of genes linked to glutamate signaling and may therefore be critical in situations of increased excitotoxicity, such as during seizures.

3.3. Low ascorbate triggers aberrant EEG activity under baseline conditions and following kainic acid injections

Given the differences in gene expression in the glutamatergic system in mice with low ascorbate supplementation, we next investigated whether there were also functional differences in baseline electrical activity measured by EEG, or whether differences arose only under conditions of increased stress (such as following kainic acid treatment). No baseline differences were reported in EEG from $SVCT2^{+/-}$ mice, although some minor differences were observed when $SVCT2^{+/-}$ mice were crossed with the *APP/PSEN1* line (Warner et al., 2015). Data were scored according to abnormal EEG patterns associated with an observable seizure-related behavior (MJs and TCSs) and those that presented the same abnormal EEG patterns but without a clearly defined behavioral correlate (e.g., SWDs in place of MJs, and tonic clonic–like seizure [TCLS] rather than TCSs. Fig. 3G–J). In some cases, the latter may represent absence seizures; however, these

Table 3
Benjamini-Hochberg–corrected *p* values for gene array data testing differences between high ascorbate–supplemented and low ascorbate–supplemented $Gulo^{-/-}$ mice

Gene label	Gene name	Individual *t*-tests *p*-values	Benjamini-Hochberg *p*-value	Benjamini-Hochberg significance
Slc1a1	**EAAC1**	**0.0037**	**0.0409**	Significant
Slc1a2	**GLT-1**	**0.0046**	**0.0409**	Significant
Snca	Alpha synuclein	0.0123	0.0702	Not significant
Slc1a3	GLAST	0.0156	0.0702	Not significant
Slc17a6	VGLUT2	0.1540	0.5544	Not significant
Slc17a7	VGLUT1	0.2570	0.6120	Not significant
Slc1a6	EAAT4	0.3440	0.6120	Not significant
Slc7a11	XCT	0.3440	0.6120	Not significant
Cacna1a	Calcium voltage-gated channel subunit Alpha1 A	0.3610	0.6120	Not significant
P2rx7	Purinergic receptor P2X, Ligand-Gated Ion channel, 7	0.3660	0.6120	Not significant
Bdnf	Brain-derived neurotrophic factor	0.3740	0.6120	Not significant
Adora1	Adenosine receptor A1	0.4390	0.6585	Not significant
Slc6a13	GABA transporter 2	0.5080	0.6789	Not significant
Nsf	Vesicular-fusion protein NSF	0.5280	0.6789	Not significant
Slc6a1	GABA transporter 1	0.6010	0.7007	Not significant
Slc38a1	Amino acid transporter A1	0.6228	0.7007	Not significant
Slc32a1	Vesicular GABA transporter	0.8140	0.8619	Not significant
Slc6a11	GABA transporter 3	0.9360	0.9360	Not significant

Genes labelled in bold are significant ($p < 0.05$) when corrected for multiple comparisons.

are harder to quantify accurately in the mouse so were not given a separate category.

In the final 15 minutes of baseline EEG measurements, significantly more SWDs were observed in low-ascorbate *Gulo*$^{-/-}$ mice compared with those receiving high ascorbate [t (16) = 3.51, p = 0.0029; Fig. 3A]. Low-ascorbate mice also had more MJs on average (Mann Whitney U = 17, p = 0.044; Fig. 3B), although the difference comes more from the number of mice experiencing events rather than the number of events (2 of 10 high-ascorbate mice had 1 MJ, whereas 5 of 8 low-ascorbate mice had 1 MJ, and 1 low-ascorbate mouse had 3 MJs), and thus a nonparametric test was employed to account for unequal variances. In the first 15 minutes following kainic acid exposure low-ascorbate mice exhibited significantly more SWDs [t (16) = 2.56, p = 0.0209; Fig. 3C], MJs [t (16) = 2.75, p = 0.0141; Fig. 3D], TCSs [t (16) = 3.31, p = 0.0044 Fig. 3E], and TCLSs [t (16) = 2.70, p = 0.0159 Fig. 3F], indicating significant changes in electrical signaling. We had previously observed that effects of the drug appeared to last longer in low-ascorbate mice, so we also examined the final 15 minutes of EEG data (45–60 minutes after kainic acid administration). Equivalent numbers of SWDs, MJs, TCSs, and TCLSs at this point (Ps > 0.05, *data not shown*) indicated that the effect of the kainic acid was to cause an accelerated and thus elongated response to the drug, rather than to extend its effect.

These data confirm that low ascorbate can alter the brain's response to excitotoxic challenge. We also observed some unexpected differences in EEG patterns between the groups at baseline and before administration of kainic acid. These indicated differences that would likely not be observed through any outward behavioral differences but could contribute to the explanation of increased susceptibility to seizures in low ascorbate conditions.

3.4. Ceftriaxone increases latency-to-onset of kainic acid–induced seizure in WT mice

Given the central role of the glutamate uptake-ascorbate release exchange mechanism of astrocytes in the clearance of glutamate from the synapse, we next sought to assess whether ceftriaxone, a β-lactam antibiotic reported to upregulate GLT-1, could offer any protection against glutamate excitotoxicity. Inadequate clearance of glutamate can result in accumulation of extracellular glutamate, which increases the likelihood of excitotoxic damage. Five-day treatment with ceftriaxone (200 mg/kg) led to approximately 30% increase in expression of GLT-1 in the cortex and striatum in both WT and R6/2 mice (Sari et al., 2010). Ceftriaxone treatments also restored a deficiency in striatal ascorbate release associated with a Huntington's disease phenotype in the R6/2 line (Miller et al., 2008, 2012). In the present study, WT mice were treated with ceftriaxone (200 mg/kg, i.p.) daily for 14 days. Ninety minutes after the final ceftriaxone injection, mice were then treated with kainic acid (10 mg/kg). In WT mice, this low dose of kainic acid led to head bob behaviors in only a small number of mice, although almost all mice exhibited other signs of the drug including flattening, tail extensions, and immobility. Mice pretreated with ceftriaxone spent less time immobile after kainic acid administration than saline treated mice [t (18) = 3.18, p = 0.0052; Fig. 4A], indicating that pretreatment with ceftriaxone had indeed protected WT mice against occurrence of mild seizure activity.

To further probe the potential role for ceftriaxone and ascorbate levels to mitigate kainic acid–induced hyperexcitability, the previous experiment was repeated in *Gulo*$^{-/-}$ mice on high or low ascorbate supplements. On the 10th to the 14th day of ceftriaxone treatments, mice were treated with 10 mg/kg kainic acid concurrent with ceftriaxone injections, and on the final day, mice were then placed immediately into FPAs to measure movement. We confirmed that quantification of bouts of low mobility was an appropriate measure to differentiate among treatment groups by testing naïve *Gulo*$^{-/-}$ mice maintained on high and low ascorbate, tested in the FPA chambers without any preceding injections. None of these mice recorded any bouts of low mobility, although there was a slight trend for the low-ascorbate animals to travel less distance than the high-ascorbate controls (p > 0.05; Fig. 4D–F). In this experiment, we found that administration of kainic acid increased the number of bouts of low mobility and spikes corresponding to MJs and decreased distance traveled compared to non–kainic acid treated mice, but there were no significant differences according to either ascorbate level or ceftriaxone on these automated measures (p's > 0.05; Fig. 4D–F). Under these conditions, GLT-1 was significantly upregulated in low-ascorbate mice ($F_{1,\ 27}$ = 22.582, p < 0.001; Fig. 4G). There was a further main effect of ceftriaxone treatment in increasing GLT-1 expression ($F_{1,\ 27}$ = 4.946, p = 0.035; Fig. 4I), although this was driven by a difference in the low-ascorbate mice (p = 0.019) with no clear increase in the high-ascorbate mice (p = 0.499). xCT expression was also upregulated in low-ascorbate mice ($F_{1,\ 13}$ = 17.258, p < 0.001; Fig. 4H–I), with no additional effect of ceftriaxone ($F_{1,\ 13}$ = 0.361, p = 0.558). The effect of ceftriaxone in the absence of kainic acid was not assessed in *Gulo*$^{-/-}$ mice.

Finally, we established that ascorbate was indeed decreased in the cortex in low groups ($F_{1,\ 30}$ = 36.308, p < 0.0001; Fig. 4J) with no effect of ceftriaxone on ascorbate level (p = 0.129). Global ascorbate levels reflect overall stored levels in whole tissue, including neuronal and glial cells; however, it is not a direct measure of acute availability and neither can it differentiate between storage capacity in either cell type. We also performed immunostaining in the hippocampus of 7 to 8 mice per group for cleaved caspase 3, GFAP, and Fluoro-Jade C as markers for apoptotic cells, astrogliosis, and neurodegeneration, respectively, to establish long-term damage in response to kainic acid. Fluoro-Jade C–positive cells were observed in low numbers overall, and there were no differences among the groups (F's < 0.309, p's > 0.583, data not shown). We observed very few caspase 3–positive cells in hippocampal sections from all groups (<4 per section), and this did not differ among groups (F's < 1.529, p's > 0.228, data not shown). We also observed no clear differences among groups in GFAP staining in either number or area coverage (F's < 0.763, p's > 0.391, data not shown).

3.5. Single kainic acid–induced seizure induces cognitive deficits in young APP/PSEN1 mice

We have previously reported that both *SVCT2*$^{+/-}$ and *APP/PSEN1* mice are more susceptible to pharmacologically induced seizures (Warner et al., 2015). Similar to the results reported previously in low-ascorbate *Gulo*$^{-/-}$ mice, *SVCT2*$^{+/-}$*APP/PSEN1* mice also had modest but nonsignificant increases in absence seizures and MJs during baseline EEG measurements (Warner et al., 2015). To investigate whether altered seizure activity or susceptibility may contribute to cognitive ability, 4- to 5-month-old WT, *SVCT2*$^{+/-}$, *APP/PSEN1*, and *SVCT2*$^{+/-}$*APP/PSEN1* mice were assessed for spatial memory in the water maze before and after seizure induction with a single dose of kainic acid (10 mg/kg). We chose to use young mice before onset of significant baseline cognitive impairment to specifically test the effect of the kainic acid, without any potential further cognitive deficit being masked by other pathologically induced changes in cognition. We first measured locomotor activity in the mice. A small increase in activity was observed in *APP/PSEN1* mice versus WT ($F_{1,\ 37}$ = 10.11, p = 0.003, Fig. 5A). Although *SVCT2*$^{+/-}$*APP/PSEN1* mice traveled the farthest distance indicating that the combination of mutations worsens the hyperactivity phenotype, there was no significant main effect of *SVCT2* genotype manipulation on activity (p's > 0.08). Neuromuscular ability was also verified

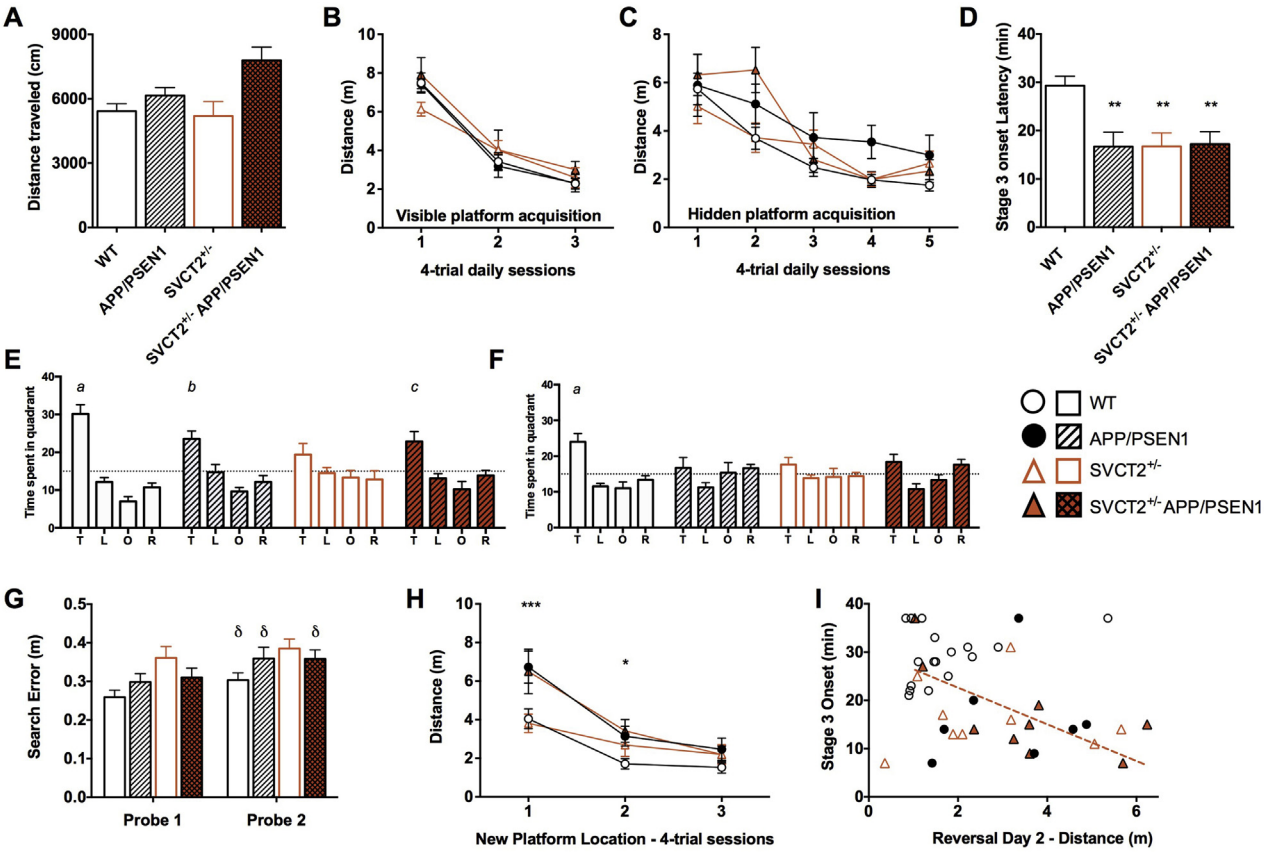

Fig. 5. Single kainic acid–induced seizure can impact spatial learning and memory in young mice. (A) Increased locomotor activity levels in *APP/PSEN1* and *SVCT2*$^{+/-}$*APP/PSEN1* mice. The 4 genotypes did not differ on acquisition of the water maze task for either (B) visible or (C) hidden platform acquisition. (D) Shorter latencies to show head bob behaviors (stage 3 of Racine scale) following kainic acid (10 mg/kg) treatment indicated greater sensitivity in all 3 mutant genotypes compared with WT mice. One mouse died after seizure initiation and so is included in cued and hidden platform acquisition data only. Time spent in target "T" versus nontarget quadrants (left [L], opposite [O], and right [R]) during (E) first and (F) second probe trials. (G) Comparison of search error, average distance from the platform during probe trials performed before and after kainic acid injection indicated that all mice performed more poorly on the second test, and this difference was significant in WT, *SVCT2*$^{+/-}$, and *SVCT2*$^{+/-}$ *APP/PSEN1* mice. (F) Reversal learning of a new hidden platform position following kainic acid was impaired in *APP/PSEN1* mice on days 1 and 2. (G) There was a significant correlation between seizure stage 3 onset latency and recall of previously learned platform position for *SVCT2*$^{+/-}$*APP/PSEN1* mice only, dotted line. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ genotype different from WT; $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ from chance performance (dashed line); $^{\delta}p < 0.05$, Probe 2 different from Probe one within genotype. WT N = 17, *SVCT2*$^{+/-}$ N = 9, *APP/PSEN1* N = 8, and *SVCT2*$^{+/-}$APP/PSEN1 N = 9. SVCT2, sodium-dependent vitamin C transporter, type 2; WT, wild-type.

using the rotarod test. Increasing latency to fall from the rotarod across the 2 sessions indicated that motor learning had occurred in all mice ($F_{1,\ 38} = 5.35$, $p = 0.026$, *data not shown*). No genotype differences in motor learning were found indicating no major motor weakness in the mice at this age (F's < 0.72, p's > 0.40).

Spatial learning was assessed using a Morris water maze. During the cued phase of testing, all mice learned the location of the marked visible platform, as evidenced by decreasing swim distance to the platform across 3 training days ($F_{2,\ 78} = 90.55$, $p < 0.001$; Fig. 5B). All genotypes performed similarly, indicating that they had the physical ability to complete the task and were able to learn the association between reaching the platform and being returned to their home cage. All mice learned the location of the hidden platform similarly, as evidenced by decreased distance covered to escape across 5 training days ($F_{4,\ 156} = 34.44$, $p < 0.001$) with no significant differences based on genotype (F's$_{1,\ 39} < 4.01$, p's > 0.05; Fig. 5C). Retention of the platform location was affected by low ascorbate as *SVCT2*$^{+/-}$ mice showed poorer search accuracy, spending a smaller percentage of time in the target quadrant during the probe trial ($F_{1,\ 39} = 4.303$, $p = 0.045$, data not shown) and had a greater search error (mean distance from the platform; $F_{1,\ 39} = 5.49$, $p = 0.024$; Fig. 5E) with only a modest effect of *APP/PSEN1* genotype (Ps > 0.068).

Following the probe trial, mice were treated with 10 mg/kg kainic acid. All mice reached stage 1 (immobility) and stage 2 (forelimb and/or tail extension, rigid posture) seizures. Beyond stage 2, there were significant differences in severity and susceptibility based on ascorbate level (*SVCT2* genotype) and *APP/PSEN1* mutations. All *APP/PSEN1* (8/8, 100%), and *SVCT2*$^{+/-}$ (9/9, 100%), mice and all but 1 *SVCT2*$^{+/-}$ *APP/PSEN1* mouse (8/9, 89%) were observed exhibiting head bobs or repetitive behaviors indicative of stage 3, whereas only 13/17 (76%) of WT mice reached stage 3. Mice that did not reach stage 3 were given the maximum recorded value of 37 minutes, so that all mice could be included in analyses. One *SVCT2*$^{+/-}$ mouse and 1 *SVCT2*$^{+/-}$ *APP/PSEN1* mouse reached stage 4. One *APP/PSEN1* mouse reached stage 5 and died during this stage. When examining the onset latency of stage 3 seizures, both mice with low ascorbate and *APP/PSEN1* mutations reached this stage faster than WT mice (Kruskal-Wallis statistic = 16.02, $p < 0011$; Fig. 5D). The combination genotype was not more affected than either single mutation alone, but WT mice had longer latencies than all 3 groups (Dunn's multiple comparisons SVCT2$^{+/-}$ $p = 0.0084$, APP/PSEN1 $p = 0.011$, SVCT2$^{+/-}$APP/PSEN1 $p = 0.0069$).

A second probe trial was conducted 24 hours after kainic acid treatment, and there were no group differences in search error during this trial (F's$_{1,\ 38} < 2.86$, p's > 0.10; Fig. 5E, left). However, all

4 genotypes performed more poorly on the second probe trial (post–kainic acid, Fig. 5E, right). Paired *t*-tests for each genotype indicated that these differences were significant for WT [t (16) = −2.16, $p = 0.046$], *APP/PSEN1* [t (6) = −3.17, p = 0.019], and *SVCT2*$^{+/-}$*APP/PSEN1* [t (8) = −3.035, $p = 0.016$], but not *SVCT2*$^{+/-}$ mice [t (8) = −0.88, $p = 0.40$] in which probe 1 performance was poorer than the other groups. Mice were given an additional training (with platform) trial following the initial probe, and therefore the poorer performance most likely indicates some disruption of memory rather than memory extinction following the previous probe trial. No differences in swim speed were found according to genotype during either of the probe trials (*F*'s < 0.1.75, *p*'s > 0.19).

Reversal learning of a new platform location began 48 hours after kainic acid treatment. All mice learned the new platform location across 3 days of training as indicated by decreasing path lengths ($F_{2, 76} = 42.88$, $p < 0.001$). Overall, mice with *APP/PSEN1* mutations took more trials to learn the new location ($F_{1, 38} = 12.204$, $p < 0.001$), although the genotype differences were only observed on days 1 ($p < 0.001$) and 2 ($p = 0.028$) of reversal learning (*APP/PSEN1* × day $F_{2, 76} = 4.99$, $p < 0.01$; Fig. 5F). There were no main effects of *SVCT2* genotype and no interactions (*F*'s < 0.71, *p*'s > 0.49).

To determine if kainic acid–induced seizure susceptibility was related to relearning deficits, the latency to onset of stage 3 seizures was correlated with distance traveled during day 2 of relearning. Day 2 was chosen to examine memory for the new platform position 24 hours after initial training to the new location. Stage 3 seizure onset was significantly and negatively correlated with distance to locate the new platform location on day 2 of relearning in the *SVCT2*$^{+/-}$*APP/PSEN1* mice only [Pearson $r(9) = -0.36$, $p = 0.030$; Fig. 5G]. This result indicates that mice with faster onset of stage 3 seizures, both *SVCT2*$^{+/-}$ and *APP/PSEN1*, had poorer learning or recall of a new platform position, as they traveled further before finding the platform.

4. Discussion

Previous data have suggested a potential link with ascorbate treatment and reduced seizure susceptibility or improved outcomes (Kim et al., 2016; MacGregor et al., 1996; Schneider Oliveira et al., 2004). These effects are typically attributed to the general antioxidant properties of ascorbate, such that targeting oxidative stress may improve outcomes in some models of epilepsy (Pauletti et al., 2017). Nevertheless, such studies are critically limited by the ability of most rodents to synthesize their own ascorbate in the liver through the action of the *gulonolactone L-oxidase* gene, and the tight control of brain ascorbate levels by the SVCT2 transporter (Harrison and May, 2009). The novelty of the present study lies in the ability to investigate differences in seizure susceptibility in an ascorbate-*deficient* condition. Low-ascorbate *Gulo*$^{-/-}$ mice showed a stronger response to a low dose of kainic acid than their adequately ascorbate-supplemented littermates. This finding was supported by microarray data that identified changes in glutamate transporter genes in low-ascorbate *Gulo*$^{-/-}$ mice, but no equivalent changes in GABAergic genes. Following these discoveries, it was shown that altered EEG signaling was detected even at baseline in the low ascorbate–treated mice. These mild differences should not be considered as seizure activity, nor are they likely, alone, to necessarily increase seizure occurrences. Nevertheless, in the presence of an additional stressor, such as disease, this difference may be implicated in the greater effects observed in low-ascorbate mice. Indeed, these patterns of aberrant electrical signaling were magnified to a greater extent in response to kainic acid in the low-ascorbate mice. The faster onset of disturbed signaling results in a longer duration of abnormal activity and thus a greater potential for generating damage over time.

Presumed improvement in glutamate clearance via treatment with ceftriaxone led to the novel result of a decreased response to kainic acid compared with control-treated mice. Ceftriaxone is protective against the convulsant effects of PTZ in rats (Jelenkovic et al., 2008) and decreased astrogliosis and epilepsy in a rat model of traumatic brain injury (Goodrich et al., 2013). We demonstrated increased GLT-1 and xCT expression in the low-ascorbate mice, which may be a response to oxidative stress changes or represent a compensatory mechanism. GLT-1 expression increased in low-ascorbate mice in response to ceftriaxone, but the same effect was not observed in the high-ascorbate mice. This is in contrast to ceftriaxone upregulation of GLT-1 in the cortex and striatum of WT and R6/2 mice (Sari et al., 2010) and may indicate regional differences in regulation of glutamate transporters. These changes occurred in the absence of any overall change in number of astrocyte coverage indicating a specific effect rather than a change relating to more generalized astrogliosis in response to oxidative stress. Prolonged high glutamate in the synapse results in further glutamate binding to postsynaptic receptors and greater Ca^{2+} influx into the postsynaptic neuron (hyperstimulation). It can also allow glutamate to diffuse to surrounding areas, thus potentially expanding the affected area and potential for damage. Neurons with low intracellular ascorbate are likely already under oxidative stress or at least more vulnerable to excitotoxic damage. GLT-1 functional efficiency was not directly tested in this study; nevertheless, deficient glutamate uptake was observed in postmortem brain tissue taken from patients with Huntington's disease (Hassel et al., 2008) and ethanol-treated rats (Melendez et al., 2005), although no changes were observed in glutamate transporter levels in either case.

xCT is the catalytic subunit of the cystine-glutamate exchanger. As a membrane-bound antiporter located predominantly on glia, it exchanges intracellular glutamate for extracellular cysteine (Baker et al., 2002) and may actually be responsible for the majority of extracellular glutamate (compared with vesicular glutamate) release (Baker et al., 2002). Both xCT and GLT-1 are regulated by exposure to ceftriaxone although they have separate roles in ceftriaxone action (Knackstedt et al., 2010; LaCrosse et al., 2017; Rao et al., 2015; Sari et al., 2009). Specifics of the relationship between GLT-1 and xCT, and ceftriaxone, may also vary across brain areas and in relation to the nature of the neural challenge. A significant effect of ceftriaxone has been observed for each in modulating addiction-extinction-reinstatement patterns in the nucleus accumbens (LaCrosse et al., 2017; Rao et al., 2015; Sari et al., 2009) for upregulating GLT-1 expression in the cortex and striatum in a mouse model of Huntington's disease (Sari et al., 2010). Despite upregulation of GLT-1 and xCT following ceftriaxone administration, we did not detect differences in response to kainic acid in the FPAs, although this task was clearly sensitive to the activity-decreasing effects of kainic acid. It is possible that the dosing regimen used was insufficient in this study, and because the *Gulo*$^{-/-}$ model is already more sensitive to glutamatergic challenge than typical WT animals, the task is insufficiently sensitive to capture differences according to transporter upregulation. Simple upregulation of some aspects of the glutamate transport system alone may be insufficient to improve behavioral outcomes following direct glutamatergic challenge, particularly in conditions of increased oxidative stress through low ascorbate.

Finally, the functional importance of even a single mild seizure was illustrated in the Morris water maze. Previously, treatment with antiepileptic drugs was shown to rescue cognitive and synaptic impairments in *APP/PSEN1* mice (Zhang et al., 2014). Young, *SVCT2*$^{+/-}$ (low ascorbate) and *APP/PSEN1* mice that were more

sensitive to kainic acid also had poorer relearning of a new platform position. Although the overall magnitude of the effects of seizures on water maze retention and relearning were modest, changes were observed after a single, relatively, low dose of kainic acid administration. They were also more severe in *APP/PSEN1* mice, indicating particular relevance to Alzheimer's disease progression. Water maze data were collected from mice younger than 6 months. Thus, amyloid accumulation in the form of plaques is unlikely to be a major factor. An expected cumulative effect of *APP/PSEN1* and *SVCT2* mutations was not observed, although there was a correlation between retention of new platform position and latency to stage 3 seizures only in the *SVCT2*$^{+/-}$*APP/PSEN1* group. The latency to the head bob behavior was also similar across the 3 mutant groups. It is possible that in older mice with additional chronic pathological changes, a cumulative effect may be observed. A modest improvement in water maze performance was observed in 10-month-old 3×Tg-AD after 2 months of ceftriaxone treatment (Zumkehr et al., 2015). Nine days of ceftriaxone treatment (50–200 mg/kg) in WT (Balb-c) mice increased hippocampal GLT-1 but did not improve performance on the water maze (Karaman et al., 2013). In contrast, 8 days of ceftriaxone treatment (200 mg/kg) in Sprague-Dawley rats led to impaired novel object recognition (Matos-Ocasio et al., 2014). Ceftriaxone increased hippocampal GLT-1 in rats, which was associated with decreased long-term potentiation (Omrani et al., 2009) indicating the potential to impair learning and memory. It may be that benefits of such treatment depend on the extent of any pre-existing impairment. Future work should test the potential for ceftriaxone to rescue memory impairment that may be specifically related to occurrence of multiple seizures. However, given the small impact of ceftriaxone on kainic acid–induced seizure behaviors, this may need to be attempted in conjunction with additional intervention measures for optimal effect. Further comparisons between low and high ascorbate-supplemented mice, in the context of Alzheimer's disease pathology, will also be beneficial to tease out this relationship.

Other neurodegenerative disorders are also associated with glutamate toxicity, oxidative stress, and seizures. In postmortem brain samples taken from patients with Huntington's disease, increased expression of GLT-1 was observed in the prefrontal cortex (Hassel et al., 2008). Glutamate uptake measured in postmortem tissue was decreased by 43% compared with controls, which was attributed to irreversible oxidative inhibition of the transporter. Similarly, glutamate uptake is impaired in the striatum of R6/2 mice, a model for Huntington's disease in which seizures are also observed. Ceftriaxone treatment in these mice diminished many behavioral abnormalities (hand clasping, explorations, and repetitive movements) and increased glutamate uptake (Miller et al., 2008). In a rat model of induced traumatic brain injury, expression of GLT-1, GLAST, and EAAC1 were all altered (Zou et al., 2013), indicative of slower glutamate clearance. Both expression patterns of glutamate transporters and cognitive deficits were at least partially attenuated following long-term treatment with levetiracetam, which can upregulate both GLT-1 and GLAST (Zou et al., 2013). Finally, patients carrying single-nucleotide polymorphisms of glutamate transporter SLC1A1 (EAAC1) were significantly more likely to experience seizures within 3 years of an initial traumatic brain injury (Ritter et al., 2016).

Given the current inability to reverse Alzheimer's disease–related damage to cognition, identifying and potentially treating other factors that contribute to memory loss in the disease could have a significant impact on the patient population. Many seizures in at-risk populations likely go unreported due to failure of caregivers (especially nonmedical family members) to observe symptoms or because the behavioral manifestations are subtle. Such mild events, particularly if repeated, could have major impact on cognition over time. The role of ascorbate has yet to be fully elucidated, but these data suggest that even mild deficiency can significantly impact at-risk groups. Future work should focus on identifying these mild seizure events and determining their effect on cognition in human populations. EEG is a directly translatable technique that can be used in human populations to detect the subtle changes in ictal discharge patterns that may indicate a person is at risk for seizures. As health care moves toward individualized medicine, it may also be useful to identify individuals with mutations in GLT-1 and other glutamate transporters, which would place them at risk. Although adequate ascorbate intake is possible through diet alone in the majority of individuals, vulnerable groups may require ascorbate supplementation in addition to normal dietary intake to ensure repletion of this vital antioxidant molecule.

Disclosure statement

The authors have no actual or potential conflicts of interest.

Acknowledgements

Funding for these studies was provided by AG038739 from NIA/NIH to FEH and I01 CX001610-01 from the VA. Behavioral testing was performed in the Vanderbilt Mouse Neurobehavioral Laboratory, which is supported by the EKS NICHD of the NIH under Award #U54HD083211. The work was also supported by research grants from CURE, Dravet syndrome foundation, and NIH (NS 082635) to J-QK. CWB received support from T32-AG058524 from NIA/NIH.

The authors acknowledge the Translational Pathology Shared Resource supported by NCI/NIH Cancer Center Support Grant 5P30 CA68485-19 and the Vanderbilt Mouse Metabolic Phenotyping Center Grant 2 U24 DK059637-16. The authors would like to acknowledge the assistance and technical support of Navila Sharif, Robert Zhang, and Meghan Montgomery. The authors would further like to thank Kylie Muccilli and Rafael Perez for assistance with creating figures and Dr Christine Konradi and Dr Julie Loundes-Taylor for assistance with statistical analyses.

FEH, J-QK, JAK, and TAW all designed research; FEH, DJM, SD, TAW, JAK, DAS, ESK, LMM, DCC, CWB, and AJE all performed research; FEH analyzed data and wrote the article.

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