HHS Public Access

Author manuscript

Neurobiol Aging. Author manuscript; available in PMC 2019 November 01.

Published in final edited form as:

Neurobiol Aging. 2018 November ; 71: 241–254. doi:10.1016/j.neurobiolaging.2018.08.002.

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

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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, 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 for brain ascorbate in maintaining protection during glutamatergic hyperexcitation events, including seizures. The study further supports a role for mild, sub-clinical seizures in cognitive decline in Alzheimer's Disease.

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FEH, JQK, JAK and TAW all designed research; FEH, DJM, SD, TAW, JAK, DAS, ESK, LMM, DCC, CWB, AJE all performed research, FEH analyzed data and wrote the paper.

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Conflicts of Interest. The authors have no competing conflicts of interest to report.

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Keywords

Vitamin C; Ascorbate; GLT1; glutamate; seizure; behavior

INTRODUCTION

Unprovoked seizures are 5–10-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). Non-convulsive, sub-clinical 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 3xTg-AD model of Alzheimer's disease, but not in wild-type 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 via SVCT2 (sodium dependent vitamin C transporters; Figure 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., 1998,Trotti, et al., 1997) 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 (CSF) 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

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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 (3–4 fold greater) when *APP/PSEN1* mice also had decreased ascorbate (30–50% lower in brain compared to wild-type), whether by knockout of *SVCT2* or the enzyme gulolactone oxidase (*gulo*) (Dixit, et al., 2015,Harrison, et al., 2010c,Warner, et al., 2015). Increased mortality is observed prior to 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 wild-type litter mates (Steinbach, et al., 1998) and show more myoclonic jerks 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.

MATERIALS AND METHODS

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). Wild-type-equivalent levels of ascorbate in tissues are maintained by providing de-ionized drinking water with 1.0 g/L ascorbate and 20μL 0.5μM 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 wild-type without risking development of scurvy (Harrison, et al., 2010b,Harrison, et al., 2008,Ward, et al., 2013).

Wild-type 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 5–6 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

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crossed with the *SVCT2*$^{+/-}$ mouse line (Dixit, et al., 2015) to yield litters of four genotypes; wild-type, *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 (IACUC) and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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), Pentylenetetrazol (PTZ) (#P6500; 40 mg/kg), and Ceftriaxone disodium salt hemi(heptahydrate) (#C5793; 200 mg/kg) were all obtained from Sigma-Aldrich, (St. Louis, MO). Drugs were either made fresh daily (Ceftriaxone, Pilocarpine, Methyl scopolamine) or reconstituted daily from stock kept at −2° for up to 1 month (Kainic acid). Pilocarpine was given 30 min 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 mls/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 out of the range reported in literature from comparable studies in mice since our goal was to avoid initiating severe seizure activity (and mortality).

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 post 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 removed either immediately following 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 myoclonic jerk as reported in the 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° and 'large' if the accompanying tail flick was >45°. Recordings were made in 5-min. time blocks, for 15 mins. 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 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 two experimenters, at least one of whom was blind to the experimental conditions of the mice. Following each session,

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reports were compared to ensure inter-rater reliability. Where differences existed between recorded behavior onset times, an average of the two latencies was used.

For **Experiment 4b** immobility time was measured using Force Plate Actimeters (FPA; 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 o f low mobility" were defined as lack of movement for 5s. FPAs also provide a measure of distance travelled within the 42×42 cm chamber. A trained coder, blinded to mouse treatment group 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 Figure 4b). Baseline level was adjusted as needed according to changes in activity level.

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 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.

Headmount affixation surgeries and EEG measurements (Experiment 3).— Mice were affixed with a prefabricated headmount (Pinnacle Technology Inc.) comprised of three channels; 2 EEG 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 in order 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 which was fastened to the skull with stainless steel screws (Small Parts, Miami Lakes, FL). 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 two 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 since the larger size of male mice at 12 weeks of age makes them better able to tolerate the surgery and head-mount 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

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recordings were conducted to assess baseline brain electrical activity over a 24-h 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 off-line. For baseline EEG recordings, seizure-related activity (both EEG and corresponding video) was monitored during the final 15 mins. of the 24-hour recording period immediately prior to kainic acid treatments. For EEG recordings with seizure induction the first and final 15 mins. of 60-min. recordings following kainic acid administration were scored. Synchronized 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 (12:00–15:00h). We analyzed 15-min. time bins for better comparison of data pre-and post-kainic acid, rather than assessing smaller time periods across the whole 24 hour time block e.g. (Arain, et al., 2012), since the purpose of the extended un-treated 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 (myoclonic jerks) following previously determined guidelines (Fig. 4)(Akman, et al., 2010,Arain, et al., 2015,Arain, et al., 2012,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 (myoclonic jerk-like activity) were quantified separately when observed without a detectable associated behavior with specific seizure-related event (Warner, et al., 2015).

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 hippocampus in 3xTg-AD mice (200 mg/kg, 2 months (Zumkehr, et al., 2015)) and in cortex and striatum of R6/2 and wild-type mice (200 mg/kg, 5 days (Sari, et al., 2010)).

Experiment 4a utilized wild-type mice. Mice were treated daily with ceftriaxone (200 mg/kg, N=13) or saline (N=15) for 14 days. Of these two 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, that were given either ceftriaxone (200 mg/kg, i.p.) or saline for 14 days. On days ten through fourteen, all mice received kainic acid (10 mg/kg, i.p.). Behavioral observations in Force Plate Actimeter chambers began immediately following kainic acid injections, and mice were sacrificed within 60 mins. of kainic acid treatment. Mice were perfused with 10 ml. of cold saline, and one hemi-brain 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

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paraffin embedded and sectioned. The other hemi brain was dissected into hippocampus and cortex and snap frozen before being kept at −80°.

Western Blot (Experiments 4a & 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 (LifeTechnologies, USA). Membranes were incubated with GLT-1 (AB1783, 1:4,000) and GFAP (MAB360, 1:2,000; both Millipore, Bedford, MA), xCT (NB-300–318, Novus Biologicals, Littleton, CO, USA), and Actin (D35E4, 1:5,000; Santa Cruz, USA). Appropriate secondary antibodies were selected from Anti-Goat IgG (A5420, 1:5,000), Anti-Guinea Pig IgG (A7289, 1:5,000) and Anti-Rabbit IgG (A0545, 1:5,000) all from Sigma-Aldrich (USA)

Immunohistochemistry (Experiment 4b).—Slide preparation and staining was completed by the Vanderbilt Translational Pathology Shared Resource Core Facility. Paraffin-embedded hemi-brains 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; 1:300 or anti-GFAP (Cat.# ab16997, abcam, Cambridge, MA; 1:500 dilution) for one. The Bond Polymer Refine detection system was used for visualization. Slides were the dehydrated, cleared and cover-slipped. For **Flurojade C** staining 10 micrometer 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 non-specific fluorescence. Slides were stained with 0.001% FluroJade performed for 30 mins in a light-tight box, followed by washing in distilled water. Once dry, sections were cover-slipped using Dapi Mounting media.

Quantification of GFAP-labelled 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 (BLOB) 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.

Brain Ascorbate levels (Experiments 1, 4b).—Ascorbate was measured in cerebellum which is an accurate reflection of other more critical brain areas including 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).

Behavioral methods.—The four genotypes included in the present study were: Wild-type (WT; $n = 17$), *APP/PSEN1* ($n = 8$), *SVCT2*$^{+/-}$ ($n = 9$), and *SVCT2*$^{+/-}$ *APP/PSEN1* ($n = 9$).

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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.

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 min session 24 hours prior to water maze training.

Rotarod.—Motor learning and coordination were tested using an accelerating rotarod (Ugo Basile model 7650; Stoelting Co., Wood Dale, IL). The rotarod accelerates from 6–50 RPM during the 300 s 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 three trials per session.

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. (i) 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 four trials (maximum 60 s) per day for three days. The visible platform phase accounts for sensorimotor differences that may affect spatial learning (i.e. visual acuity, swim speed). (ii) Hidden platform testing was conducted with the platform submerged 1 cm below the surface of the water. Mice received four acquisition trials per day for five days. To assess retention of the platform location, a 60 s probe trial was conducted with the platform removed. The first probe trial was given 24 hrs 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. (iii) Mice were treated with kainic acid 1–2 hrs after the first probe trial. Probe 2 was given 24 hrs following the first probe trial. (iv) Mice were then trained to locate a new hidden platform location with four 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) 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).

Experimental Design and Statistical Analysis.—Animal numbers for all experiments are provided in Table 2. Differences between two groups (e.g. high versus low ascorbate in *Gulo*$^{-/-}$ mice, ceftriaxone versus saline in wild-type mice) were analyzed in GraphPad Prism

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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 two groups requiring non-parametric testing Mann-Whitney U or Kruskall-Wallis tests were used.

Where there were two 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 X 2 Analysis of Variance (ANOVA). Tests that occurred across multiple days of testing (e.g. water maze acquisition) were analyzed using Repeated Measures (RMANOVA) 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, e.g. the effect of *APP/PSEN1* genotype within either *SVCT2*$^{+/+}$ or *SVCT2*$^{+/-}$ groups. (These comparisons do not allow for direct comparison between wild-type and *SVCT2*$^{+/-}$ *APP/PSEN1* groups).

For gene array data genes which had at least two 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 sub-set of genes, to protect against false discovery rates.

RESULTS

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 wild-type 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 pentylentetrazole (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 mins. Mice for which a head bob was not observed (N=6, high ascorbate) were given a latency score of 35 min. (not 60 min. to avoid skewing the results, even though this may slightly under-estimate 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 one mouse (high ascorbate) also suffered more severe seizure events encompassing shakes or tremors, but which did not include rearing/falling, barrel rolls, or tonic-clonic seizures. 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 min. ± 1.63, Low 10.89 min. ± 1.84, **t(12)=1.27, *P*=0.23**, *not shown*).

Three out of six (50%) high ascorbate mice and five out of nine low ascorbate mice (55.6%) mice suffered a major seizure event lasting greater than 5s. 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 min, 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*), four or fewer tics were observed per mouse within the 15 min. scoring time frame. We confirmed that ascorbate levels in brain (cerebellum) were decreased by at least 50% in the low ascorbate mice (N=15) compared to 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.

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 hippocampus in both glutamatergic and GABAergic systems using pre-made microarray plates (GABA and Glutamate PCR array, Qiagen). Expression patterns were only considered for genes with a Ct value of <32, and data. Data were normalized to expression of Hsp90ab which was determined by the software to provide the most stable expression via a non-normalized calculation out of the five possible housekeeping genes (Actb, B2m, Gapdh, Gusb, Hsp90ab1). We observed moderate changes in gene expression according to ascorbate treatment in five genes from the Glutamatergic array including the three 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**)). Since our hypothesis was that changes would be observed in ‘transport and trafficking genes’ we subjected the 18 genes in that sub-set, 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.

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

Given the differences in gene expression in 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 (myoclonic jerks (MJ), and tonic clonic seizures, (TCS)), and those that presented the same abnormal EEG patterns but without a clearly defined behavioral correlate (e.g. spike wave discharge (SWD) in place of myoclonic jerks, and tonic clonic-like seizure (TCLS) rather than tonic-clonic seizures. Fig. 3G-J). In some cases, the latter may represent absence seizures, however, these 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 SWD were observed in *Gulo*$^{+/-}$ low ascorbate mice compared to those receiving high ascorbate (**t(16)=3.51, *P*=0.0029;** Fig. 3A). Low ascorbate mice also had more myoclonic jerks 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 (two out of ten high ascorbate mice had one MJ, whereas five out of eight low ascorbate mice had 1 MJ, and one low ascorbate mouse had 3 MJ) and thus a non-parametric test was employed to account for unequal variances. In the first 15 minutes following kainic acid exposure low ascorbate mice exhibited significantly more SWD (**t(16)=2.56, *P*=0.0209;** Fig. 3C), MJ (**t(16)=2.75, *P*=0.0141;** Fig. 3D), TCS (**t(16)=3.31, *P*=0.0044** Fig. 3E) and TCLS (**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 mins. of EEG data (45 to 60 mins. post kainic acid administration). Equivalent numbers of SWD, MJ, TCS and TCLS at this point (*P*s>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 prior to 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.

4. Ceftriaxone increases latency-to-onset of kainic acid-induced seizure in wild-type 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

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accumulation of extracellular glutamate which increases the likelihood of excitotoxic damage. 5-days treatment with ceftriaxone (200 mg/kg) led to approximately 30% increase in expression of GLT-1 in cortex and striatum in both wild-type 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., 2012,Miller, et al., 2008). In the current study wild-type 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 wild-type 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 pre-treated with ceftriaxone spent less time immobile following kainic acid than saline treated mice (**$t(18)=3.18$, $P=0.0052$;** Fig. 4A), indicating that pre-treatment with ceftriaxone had indeed protected wild-type 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 days of ceftriaxone treatments mice were treated with 10 mg/kg kainic acid concurrent with ceftriaxone injections, and on the final day were then placed immediately into Force Plate Actimeters 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 myoclonic jerks, and decreased distance travelled compared to non-kainic acid treated mice, but there were no significant differences according to either ascorbate level or ceftriaxone on these automated measures (**Ps>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 hippocampus of seven to eight mice per group for cleaved caspase 3, GFAP, and FlurojadeC as markers for apoptotic cells, astrogliosis and neurodegeneration respectively, to establish long-term damage in response to kainic acid. FluroJadeC-positive

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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 group (***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).

5. Single kainic acid-induced seizures induce 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 above in *Gulo*$^{-/-}$ low ascorbate mice, *SVCT2*$^{+/-}$*APP/PSEN1* mice also had modest, but non-significant increases in absence seizures and myoclonic jerks during baseline EEG measurements (Warner, et al., 2015). To investigate whether altered seizure activity or susceptibility may contribute to cognitive ability, 4–5 month-old wild-type, *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 prior to onset of significant baseline cognitive impairment in order 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 wild-type (**$F_{1, 37}$ = 10.11, *P*=0.003,** Fig. 5A). Although *SVCT2*$^{+/-}$*APP/PSEN1* mice travelled 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 using the rotarod test. Increasing latency to fall from the rotarod across the two 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, *Ps*>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 three 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 five training days (**$F_{4, 156}$=34.44, *P*<0.001**) with no significant differences based on genotype (**$Fs_{1, 39}$<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 (*P*s>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.

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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 mins. so that all mice could be included in analyses. One $SVCT2^{+/-}$ and one $SVCT2^{+/-}$ *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 wild-type mice (**Kruskall-Wallis statistic = 16.02, *P*<0011.** Fig. 5D). The combination genotype was not more affected than either single mutation alone, but wild-type mice had longer latencies than all 3 groups (**Dunn's multiple comparison's 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 (**$Fs_{1,38} < 2.86$, *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 wild-type (**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 three 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* X 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 re-learning 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 travelled further before finding the platform.

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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-supplemented littermates. This finding was supported by microarray data that identified changes in two glutamate transporter genes in low ascorbate *Gulo*$^{-/-}$ mice but no equivalent number of 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 to 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 cortex and striatum of wild-type 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 post-synaptic receptors and greater Ca^{2+} influx into the post-synaptic neuron (hyper-stimulation). 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 Huntington's disease patients, (Hassel, et al., 2008) and in ethanol-

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treated rats (Melendez, et al., 2005) despite no changes 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 to 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 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 Force Plate Actimeters, 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 wild-type 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 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 seizure on water maze retention and relearning were modest, changes were observed after a single, relatively low dose of kainic acid. 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 old. 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 seizure Stage 3 only in the *SVCT2*$^{+/-}$*APP/PSEN1* group. The latency to the head bob behavior was also similar across the three 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 3xTg-AD following 2 months of ceftriaxone (Zumkehr, et al., 2015). Nine days of ceftriaxone treatment (50–200 mg/kg) in wild-type (Balb-c) mice increased hippocampal GLT-1 but did not improve performance on the water maze (Karaman, et al., 2013). In contrast, eight 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 LTP (Omrani, et al., 2009)

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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 post-mortem brain samples from Huntington's disease patients increased expression of GLT-1 was observed in prefrontal cortex (Hassel, et al., 2008). Glutamate uptake measured in post mortem tissue was decreased by 43% compared to controls which was attributed to irreversible oxidative inhibition of the transporter. Similarly, glutamate uptake is impaired in 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 non-medical 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 healthcare 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. While 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.

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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 JQK. We 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. We would like to acknowledge the assistance and technical support of Navila Sharif, Robert Zhang, and Meghan Montgomery. We would further like to thank Kylie Muccilli for assistance with creating figures and Dr. Christine Konradi and Dr. Julie Loundes-Taylor for assistance with statistical analyses.

ABBREVIATIONS (non standard)

Gulo	gulonolactone oxidase
SVCT2	Sodium Dependent Vitamin C transporter – type 2
GLT-1	glutamate transporter 1 (EAAT2, excitatory amino acid transporter 2)
GLAST/EAAT1	Glutamate Aspartate Transporter
EAAC1/EAAT3	excitatory amino acid carrier 1

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3–5 HIGHLIGHTS BULLETS

- Low ascorbate increases sensitivity to kainic acid
- Low ascorbate altered expression of GLT-1, EAAC1 and xCT
- Ceftriaxone decreased effects of kainic acid in wild-type mice
- Mild seizures have a greater effect on cognition in low ascorbate and APP/PSEN1 mice

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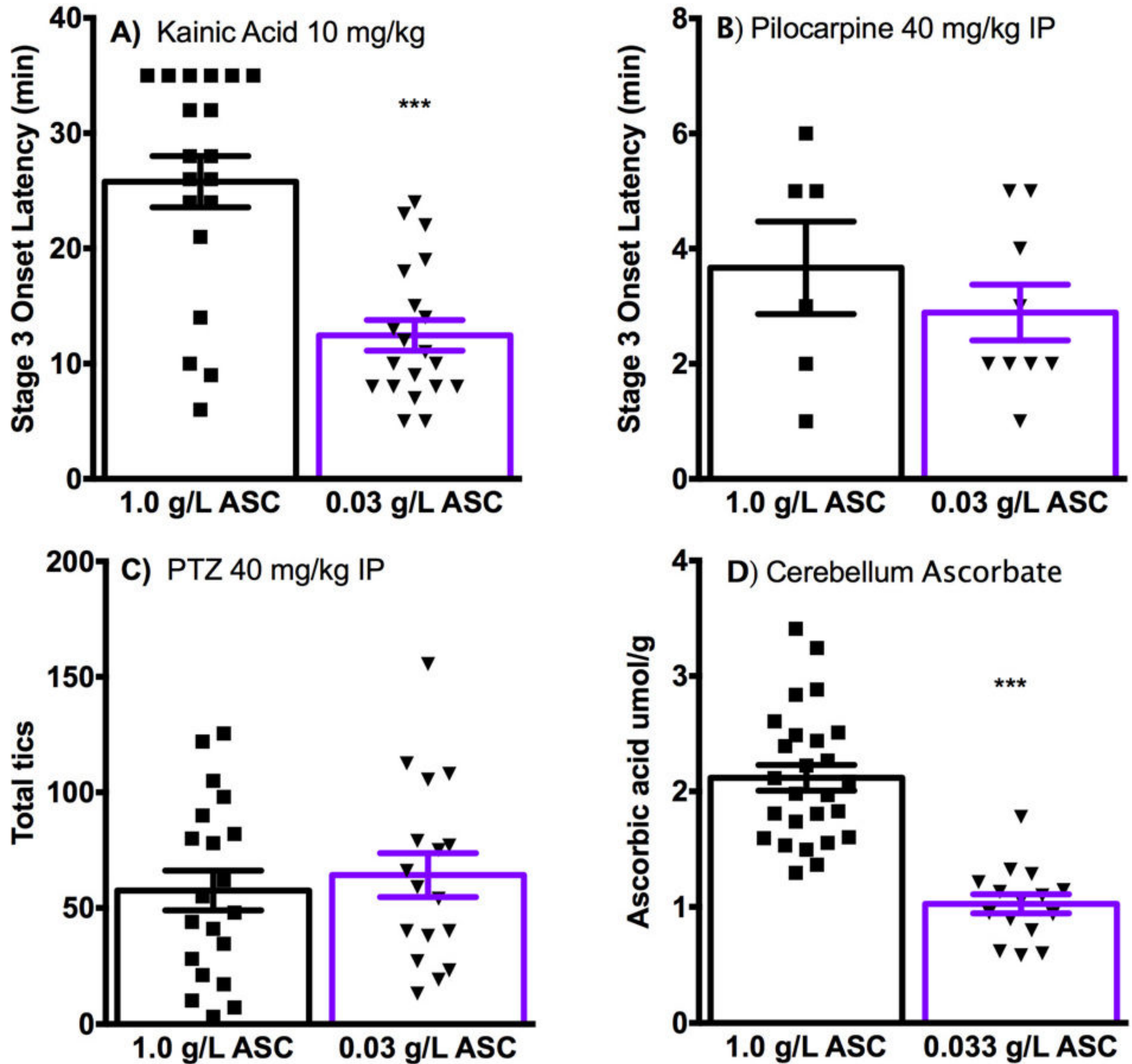

Figure 1. Glutamate uptake-ascorbate release exchange mechanism in astrocytes.

The two tethered systems (red box) of glutamate uptake via GLT-1, and ascorbate release through volume regulated anion channels (VRAC) are highlighted. Under normal conditions, as glutamate enters the astrocyte, it causes cellular swelling. This change results in the opening of volume regulated anion channels (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 Sodium Dependent Vitamin C Transporter, type 2 (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 hyper-stimulation of post-synaptic receptors, and contribute to localized oxidative stress, with the potential to further damage GLT-1 function.

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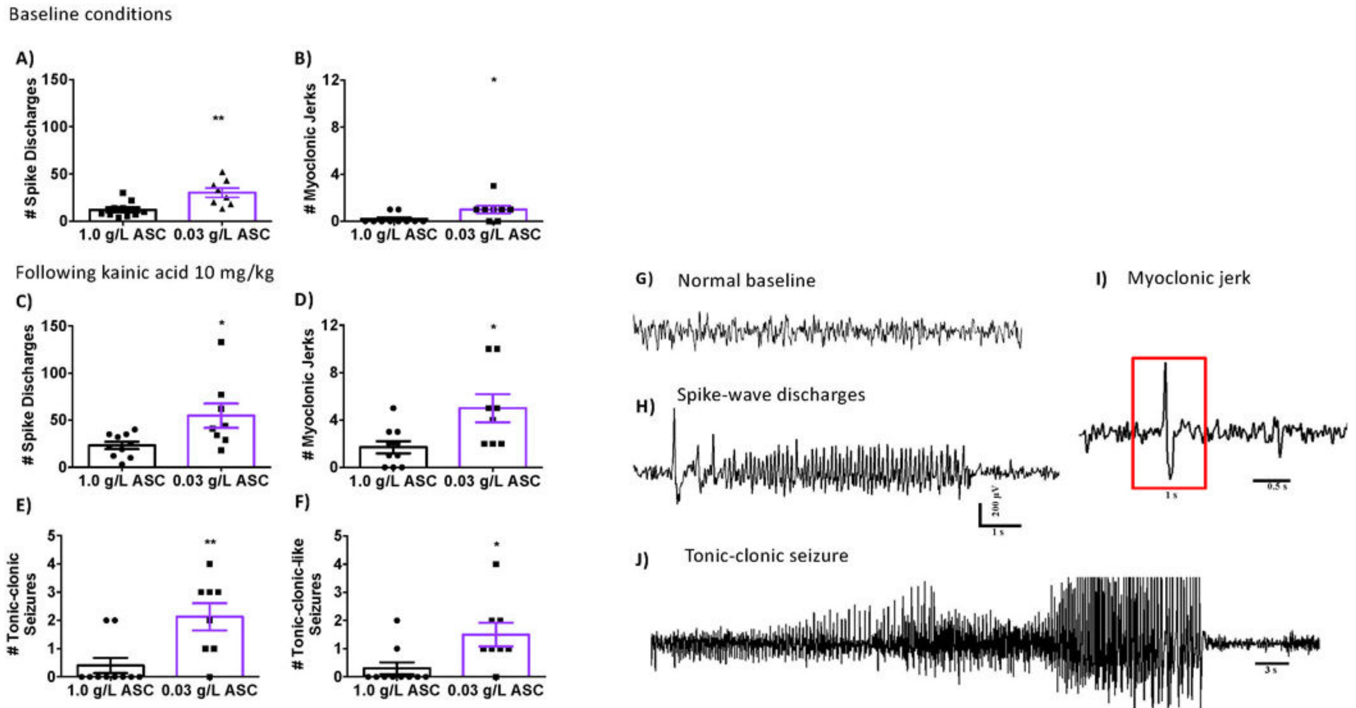

Figure 2. Increased seizure susceptibility to kainic acid but not pilocarpine or PTZ in low ascorbate-supplemented *Gulo*$^{-/-}$ mice.

Gulo$^{-/-}$ mice were scored according to severity of behavioral response following treatment with (A) kainic acid (10 mg/kg) N=19 high ascorbate (1.0 g/L), N=20 low ascorbate (0.03 g/L), (B) pilocarpine (40 mg/kg) N=6 high ascorbate, N=9 low ascorbate, or (C) PTZ (40 mg/kg) N=20 high ascorbate, 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 number of small and large full body tics were scored. (D) Brain ascorbate was measured in cerebellum N=26 High, N=15 Low. Data analyzed by unpaired t-test with Welch's correction where variances differed significantly between groups. *** $P<0.001$ different from high ascorbate condition. ASC, Ascorbate.

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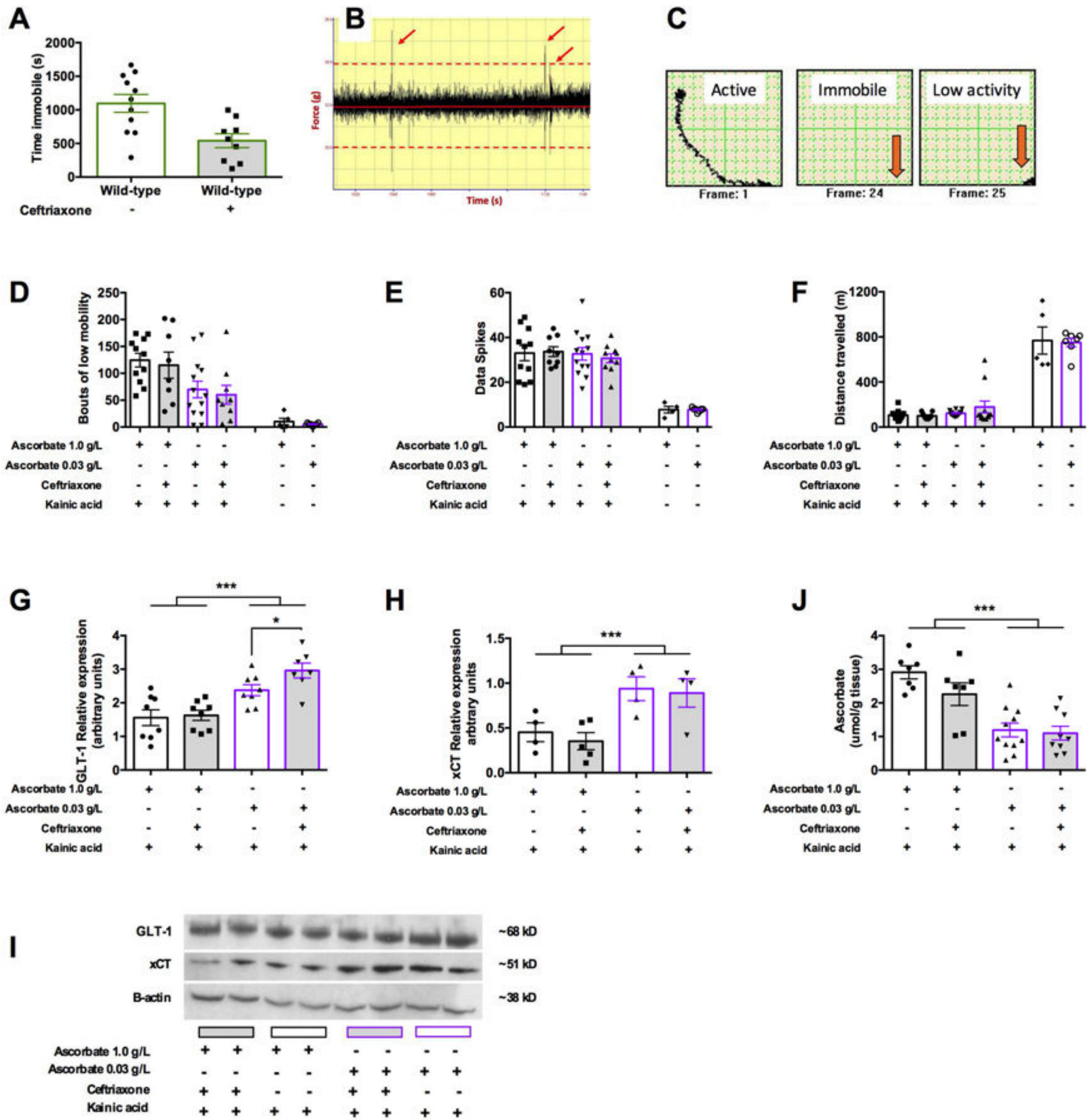

Figure 3. Greater abnormal EEG and seizures activities in low ascorbate *Gulo*$^{-/-}$ mice at baseline and following kainic acid.

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 prior to-and 60 mins. following treatment with 10 mg/kg kainic acid. At baseline more (A) spike wave discharges, and (B) myoclonic jerks were observed in mice on low ascorbate treatments. Following kainic acid low ascorbate mice experienced more (C) spike discharges, (D) myoclonic jerks, (E) tonic-clonic seizures, and (F) tonic clonic-like seizures than control, high-ascorbate mice. (G-I) Representative EEG recordings show (G) slow spike-wave discharges (SWDs), (H) myoclonic jerks and (I) generalized tonic clonic seizures from the *Gulo*$^{-/-}$ mice supplemented with low ascorbate (ASC). Unpaired t-tests, * $P<0.05$, ** $P<0.01$ from high ascorbate controls.

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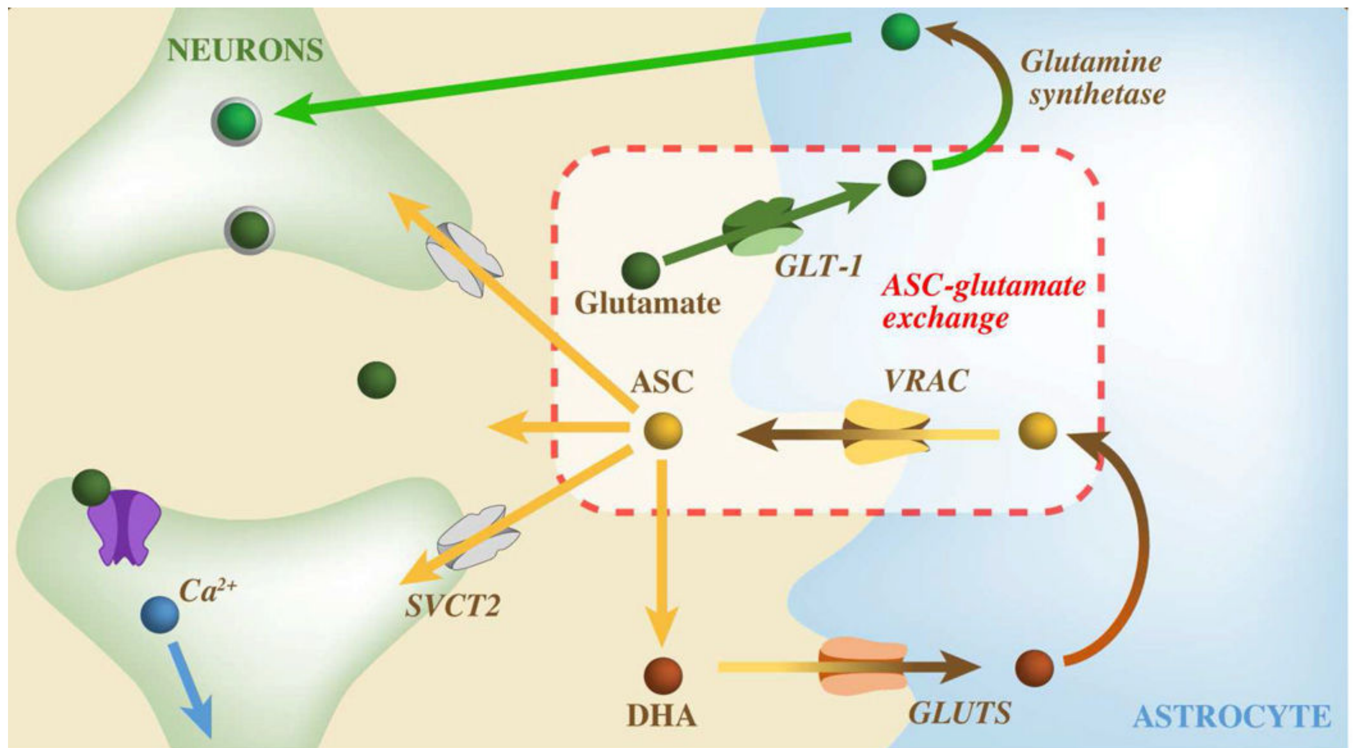

Figure 4. Ceftriaxone upregulates GLT-1 but does not protect against kainic acid-induced behavioral changes.

A) Wild-type mice pre-treated with 200 mg/kg ceftriaxone daily for 14 days spent less time immobile in the 30 mins following treatment with 10 mg/kg kainic acid. Saline N=11, ceftriaxone N=9. **B-C)** Illustration of activity data output from FPA chambers showing **B)** spikes representative of myoclonic jerks (red arrows) and **C)** Movement in the chambers in the initial few minutes of testing following kainic acid administration. **D-F)** High (1.0 g/L) and low (0.03 g/L) ascorbate treated *Gulo*$^{-/-}$ mice pre-treated with 14 days CFX (200 mg/kg) did not differ in three automated or semi-automated measures of activity in the Force Plate Actimeters **D**) Bouts of low mobility, **E**) Spikes reflecting possible myoclonic jerks, and **F**) Distance travelled. (High SAL N=9, High ceftriaxone n=11, Low SAL N=11, Low ceftriaxone N=13). **G,I)** GLT-1 expression was significantly increased in low ascorbate *Gulo*$^{-/-}$ mice with a further increase low ascorbate mice according to ceftriaxone treatment (N=7–9 per group, from 4 separate blots). **H,I)** xCT expression was increased in low ascorbate *Gulo*$^{-/-}$ mice but did not changes in response to ceftriaxone (N=4–5 per group). **J)** Brain ascorbate levels reflected dietary supplementation regimens. * $P<0.05$, ***$P<0.001$ differences between groups as marked.

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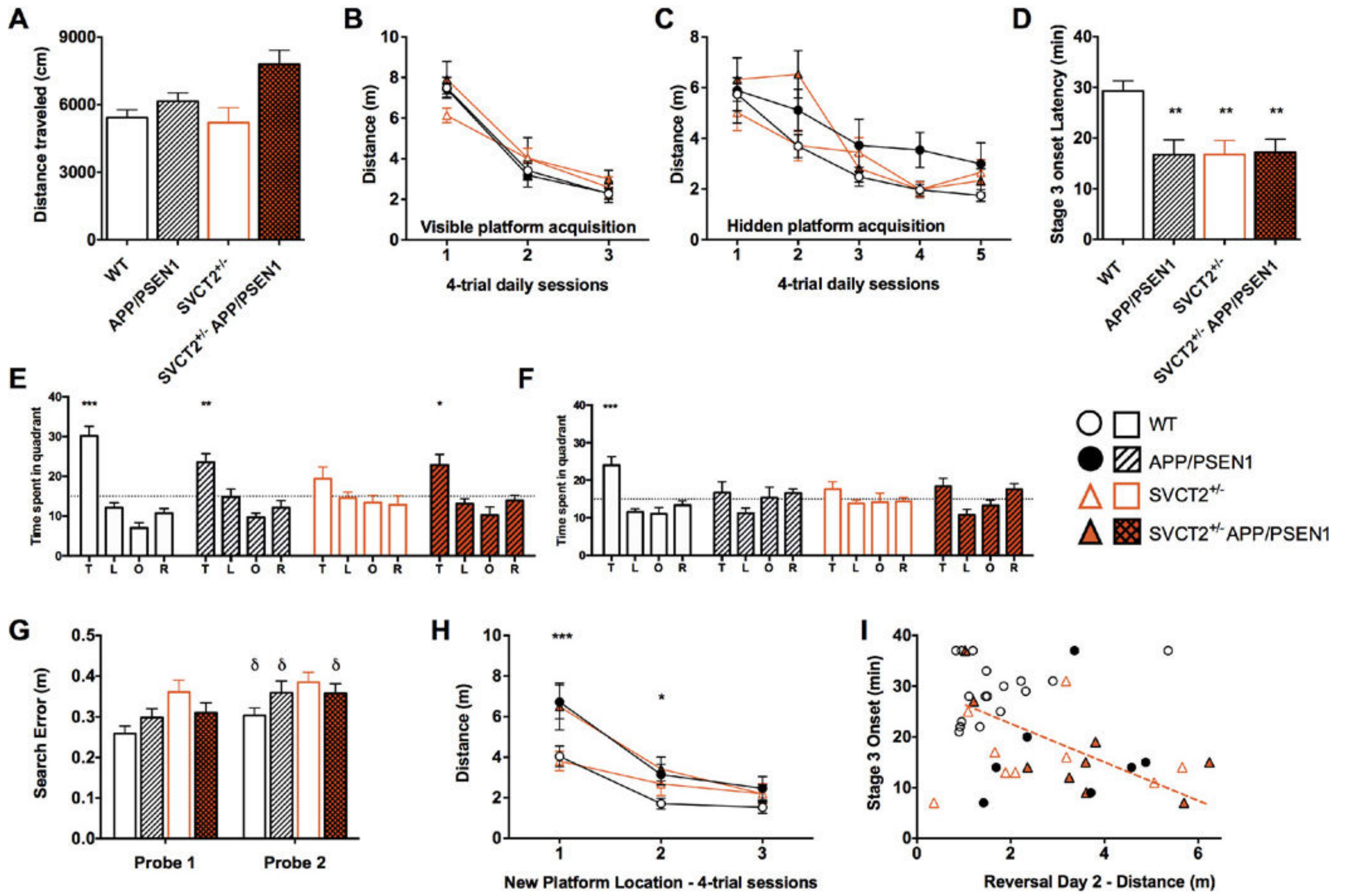

Figure 5. Single kainic acid-induced seizures can impact spatial learning and memory in young mice.

(A) Increased locomotor activity levels in *APP/PSEN1* and *SVCT2*$^{+/-}$*APP/PSEN1* mice. The four 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 three mutant genotypes compared to wild-type mice. One mouse died following seizure initiation and so is included in cued, and hidden platform acquisition data only. Time spent in target "T" versus non-target 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 pre-and post-kainic acid injection indicated that all mice performed more poorly on the second test, and this difference was significant in wild-type, *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. *, *** $P<0.05$, $P<0.001$ 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. Wild-type (WT) N=17, *SVCT2*$^{+/-}$N=9, *APP/PSEN1* N=8, *SVCT2*$^{+/-}$APP/PSEN1 N=9.

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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 jerk
	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 >5s
PTZ	Full body tic (small)	
	Full body tic (large)	

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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 Kainic acid, Low ascorbate	7M, 12F 9M, 11F
		PTZ, High ascorbate PTZ, Low ascorbate	12M, 8F 10M, 8F
		Pilocarpine, High ascorbate Pilocarpine, Low ascorbate	5M 9M
2) Gene expression	*Gulo*$^{-/-}$	High ascorbate Low ascorbate	3M, 3F 3M, 3F
3) EEG [1]	*Gulo*$^{-/-}$	High ascorbate Low ascorbate	10M 8M
4) Ceftriaxone studies [2]	Wild-type	Saline Ceftriaxone	4M5F 5M6F
	Gulo$^{-/-}$	High ascorbate, Saline High ascorbate, Ceftriaxone Low ascorbate. Saline Low ascorbate, Ceftriaxone	9M, 2F (11) 6M, 3F (8) 6M, 7F (14) 3M, 6F (9)
5) Behavioral studies [3]	*SVCT2*$^{+/-}$ X *APP/PSEN1*$^{+/-}$	*Wild-type* *APP/PSEN1* *SVCT2*$^{+/-}$ *SVCT2*$^{+/-}$ *APP/PSEN1*	17F 8F 9F 9F

No mice were excluded from analyses, except for health concerns as listed below. These mice are not included in group numbers given above:

[1] Death after or during surgery since *Gulo*$^{-/-}$ mice are more sensitive to the effects of isoflurane anesthesia (N=2 low ascorbate).

[2] Mice were excluded if they exhibited illness due to repeated injections, e.g. weight loss or swelling, internal bleeding at sacrifice (N=3 high ascorbate). This experiment was repeated in two separate cohorts of animals.

[3] One mouse escaped from the locomotor activity apparatus and so was excluded from analyses. M, Male; F, Female

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Table 3.

Benjamini-Hochberg-corrected P values for gene array data testing differences between high and low supplemented *Gulo*$^{-/-}$ mice.

Gene label	Gene name	Individual t-tests P-values	Benjamini-Hochbgerg 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