Recurrent *SLC1A2* Variants Cause Epilepsy via a Dominant Negative Mechanism

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SLC1A2 is a trimeric transporter essential for clearing glutamate from neuronal synapses. Recurrent de novo *SLC1A2* missense variants cause a severe, early onset developmental and epileptic encephalopathy via an unclear mechanism. We demonstrate that all 3 variants implicated in this condition localize to the trimerization domain of *SLC1A2*, and that the Leu85Pro variant acts via a dominant negative mechanism to reduce, but not eliminate, wild-type *SLC1A2* protein localization and function. Finally, we demonstrate that treatment of a 20-month-old *SLC1A2*-related epilepsy patient with the SLC1A2-modulating agent ceftriaxone did not result in a significant change in daily spasm count.

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Glutamate is the predominant central nervous system excitatory neurotransmitter, and its synaptic concentration is kept under tight control to prevent neuronal damage from excessive activation of glutamate receptors.[1,2] Of the 5 glutamate transporters, the trimeric transporter *SLC1A2* (also known at GLT-1 and EAAT2) and *SLC1A3* mediate the bulk of glutamate clearance from the synaptic cleft via their expression in astrocytes. Pan-knockout or astrocyte-specific knockout of *Slc1a2* in mice results in neuronal excitotoxicity, epilepsy, and premature death.[3,4] Despite the severe epilepsy phenotype of homozygous *Slc1a2* knockout mice, mice heterozygous for the *Slc1a2* deletion demonstrate no apparent clinical phenotype.[3] Furthermore, heterozygous deletion of *SLC1A2* in humans (as is frequently seen in the WAGR deletion syndrome) is only rarely associated with epilepsy.[5] In contrast to individuals with heterozygous *SLC1A2* deletions, 3 heterozygous *SLC1A2* missense variants have recently been implicated in severe early onset epilepsy (Online Mendelian Inheritance in Man database: #617105).[6–8] The mechanism by which these *SLC1A2* missense variants cause epilepsy remains unclear, significantly hindering the ability to apply glutamate and *SLC1A2*-modulating therapies to treat these patients.

Patients and Methods

Identification of SLC1A2 *Variant*

Singleton whole exome sequencing (Prevention Genetics, Marshfield, WI) revealed a pathogenic heterozygous c.254T>C (p.Leu85Pro) variant in the *SLC1A2* gene (NM_004171.3), which was classified as de novo using parental testing.

Cell Culture, Transfection, Plasma Membrane Isolation, and Immunoblotting

HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. To isolate plasma membrane-bound proteins, cells were treated with 1.5mg/ml sulfo-NHS-SS-biotin for 1 hour at 4°C, then washed with phosphate-buffered saline (PBS) containing 1mM $MgCl_2$, 0.1mM $CaCl_2$, and 100mM glycine, rinsed with PBS, then lysed with 150mM

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NaCl, 5mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50mM Tris-HCl, pH 7.4 containing protease inhibitors. Insoluble debris was cleared by centrifugation, and protein equivalent amounts of cleared lysates were incubated with streptavidin-agarose beads overnight at 4°C, followed by 3 washes with 100mM NaCl, 5mM EDTA, and 40mM Tris-HCl, pH 7.4, 2 washes with 500mM NaCl and 50mM Tris-HCl, pH 7.4, and 1 wash with 50mM Tris-HCl, pH 7.4. Beads were resuspended in 2× Laemmli buffer and heated to 95°C. Elute was resolved on SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membrane prior to immunoblotting. Antibodies for immunoblots include α-SLC1A2 (PA5-17099; Thermo Scientific, Waltham, MA), rabbit polyclonal antiactin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish peroxidase–conjugated goat antirabbit IgG (W4011; Promega, Madison, WI). Optical densitometry determination was made using ImageJ.

Cycloheximide Treatment

Transfected HEK293 cells were treated with Dulbecco modified Eagle medium media containing 20μg/ml cycloheximide for different amounts of time, then harvested with radioimmunoprecipitation assay buffer and assayed by immunoblotting as above.

L-[^{3}H]-Glutamate Uptake

HEK293 cells were transiently transfected in triplicate, and after 24 hours each well was washed 3 times with uptake buffer (140mM NaCl, 2.5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 1.2mM K_2HPO_4, 10mM glucose, 10mM hydroxyethylpiperazine ethane sulfonic acid, pH 7.4), then incubated for 10 minutes at room temperature with 100μl of uptake buffer containing 100μM L-glutamate and 0.05μCi of L-[^{3}H]-glutamate. Cells were then washed 3 times with ice-cold uptake buffer, then treated with 100μl of MicroScint-20 (PerkinElmer, Waltham, MA) for 1 hour at room temperature. L-[^{3}H]-Glutamate uptake was determined using TopCount microplate scintillation and luminescence counter (PerkinElmer), and counts per minute (cpm) values were transformed into flux rates and averaged and normalized as follows:

$$\frac{counts(cpm)/well \times [substrate](pM)}{total\ counts(cpm/liter) \times incubation\ time(\min)}$$

Ceftriaxone Trial

An innovative therapy protocol was approved by the Boston Children's Hospital Institutional Review Board (IRB). After observing baseline epileptic spasm frequency via both caregiver-observed spasm count and continuous video-electroencephalography (EEG) for 24 hours, intravenous (IV) ceftriaxone was initiated at 80mg/kg/day. The patient was observed for 2 days after ceftriaxone initiation with continuous video-EEG, then discharged after placement of a peripherally inserted central catheter for home ceftriaxone infusion. On days 7 and 14 of ceftriaxone therapy, she was admitted for planned monitoring of spasm counts using continuous video-EEG. Per the IRB protocol, treatment was stopped after 14 days of ceftriaxone therapy. Daily spasm counts were collected by the caregiver during treatment and for 12 days after ceftriaxone discontinuation.

Results

Clinical Spectrum of SLC1A2-Related Epilepsy

We present an expanded clinical phenotype of *SLC1A2*-related epilepsy based on 7 independent cases, including 1 new de novo dominant case, 5 previously reported de novo dominant cases,[6–8] and 1 previously reported recessive case.[9] All 6 de novo dominant cases developed early onset epilepsy with symptom onset between 2 days and 6 weeks of life (Supplementary Table). Seizures typically presented as focal motor events (often tonic or myoclonic), and progressed to multiple seizure types including epileptic spasms, myoclonic seizures, focal and generalized tonic seizures, and tonic–clonic seizures. Seizures were systematically refractory to multiple medications. Brain magnetic resonance imaging was normal for the first several months of life, but around 5 months of life began to show evidence of delayed myelination, thinning of the corpus callosum, and cerebral cortical atrophy. EEG patterns demonstrated multifocal and generalized epileptiform discharges with abnormal background activity, including modified and classic hypsarrhythmia. Common clinical features included severe global developmental delay (6/6 cases), cortical visual impairment (3/6 cases), axial hypotonia (6/6 cases), spasticity and/or joint contractures (5/6 cases), and kyphoscoliosis (2/6 cases). Of note, the case of recessive *SLC1A2*-related epilepsy appears milder, with seizure onset at 2 years of age that was controlled with a single medication and mild developmental delay.

Recurrent De Novo SLC1A2-Related Epilepsy Variants Localize to the Trimerization Domain of SLC1A2

Five of the 6 de novo dominant cases result from recurrent alterations at *SLC1A2* Gly82 (Gly82Arg) and Leu85 (Leu85Pro), whereas Pro289Arg has only been observed in 1 case. All 3 of these amino acids are highly conserved and colocalize within the same region of the SLC1A2 trimerization domain, far from the glutamate binding site (Fig 1). Both Gly82 and Leu85 are located in a close helix–helix contact between transmembrane helix (TMH)

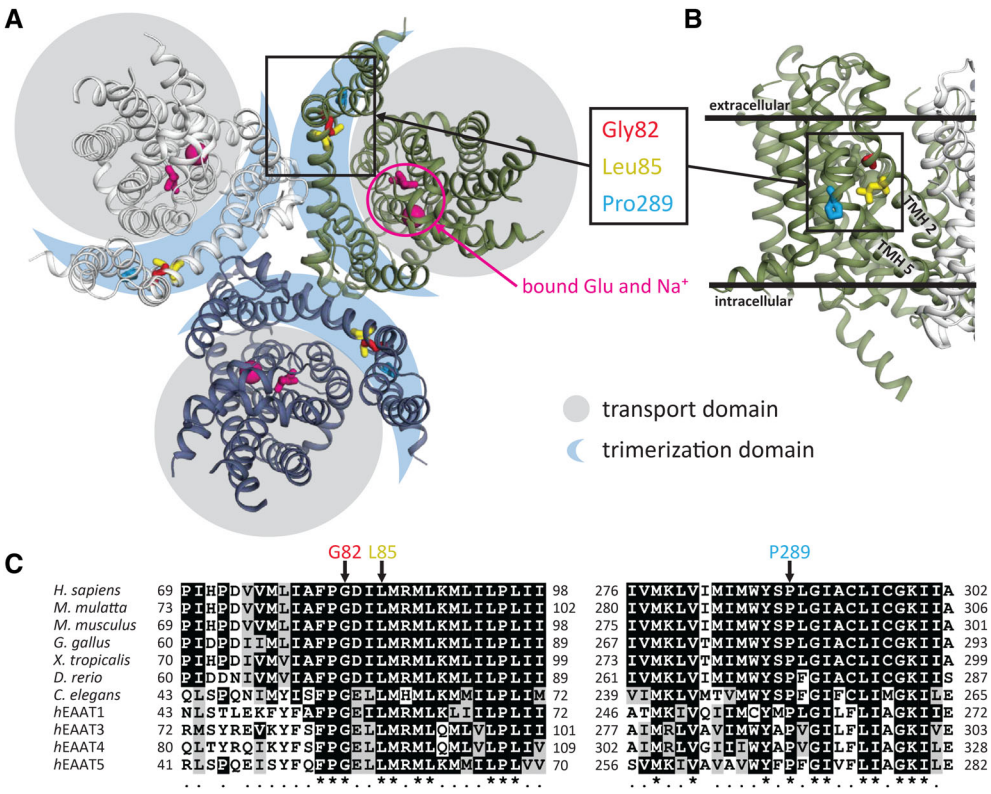

FIGURE 1: Structural relationship and evolutionary conservation of de novo *SLC1A2* variants associated with epileptic encephalopathy. (A) Crystal structure of thermostable *SLC1A3* (Protein Data Bank ID: 5LLU) marked using conserved residues homologous to *SLC1A2* Gly82, Leu85, and Pro289 positions, and the glutamate substrate binding pocket. (B) Close-up view of transmembrane helix (TMH) 2 and 5 in reference to the inner and outer layers of the cytoplasmic membrane as well as the trimerization domain. (C) ClustalW 2.1 sequence alignment of *SLC1A2* orthologs and different EAAT proteins for the *SLC1A2* protein regions containing G82, L85, and P289. [Color figure can be viewed at www.annalsofneurology.org]

2 and 5, whereas Pro289 is required to create a kink in TMH5. All 3 of these variants are predicted to disrupt TMH2 or TMH5.

Leu85Pro Variant Significantly Reduced SLC1A2 *Activity*

To evaluate the effects of these de novo dominant variants on SLC1A2 function, we studied the Leu85Pro variant in a well-established in vitro model of transiently expressed SLC1A2 in HEK cells.[10] Both wild-type (WT) SLC1A2 ($SLC1A2_{wt}$) and Leu85Pro SLC1A2 ($SLC1A2_{L85P}$) proteins were readily expressed in HEK cells and were able to form trimeric plasma membrane-bound proteins (Fig 2). However, compared to $SLC1A2_{wt}$, $SLC1A2_{L85P}$ formed less monomeric (37 ± 9.4% of WT) and trimeric (47 ± 8.9% of WT) plasma membrane-bound SLC1A2 protein, yet maintained a preserved trimer-to-monomer ratio. The protein half-life did not differ between $SLC1A2_{wt}$ and $SLC1A2_{L85P}$, demonstrating that the Leu85Pro variant is associated with decreased plasma membrane-bound SLC1A2 protein, but not an overt defect in trimerization or increased protein degradation. Furthermore, whereas cells transfected with $SLC1A2_{wt}$ demonstrated robust glutamate transport activity, cells transfected with $SLC1A2_{L85P}$ had significantly reduced glutamate uptake, indicating that $SLC1A2_{L85P}$ has significantly reduced functional activity despite its ability to form stable trimeric plasma membrane-bound protein. However, when both $SLC1A2_{wt}$ and $SLC1A2_{L85P}$ were transfected together, the glutamate transport activity was reduced below 50%, suggesting a dominant negative effect of the mutant allele over the $SLC1A2_{wt}$.

$SLC1A2_{L85P}$ Acts via a Dominant Negative Mechanism to Reduce $SLC1A2_{wt}$ Activity

To directly evaluate whether $SLC1A2_{L85P}$ acts via a loss-of-function or dominant negative mechanism, we compared the activity of SLC1A2 between cells expressing only $SLC1A2_{wt}$ or both $SLC1A2_{wt}$ and $SLC1A2_{L85P}$, using different DNA ratios. Identical amounts of $SLC1A2_{wt}$ DNA was used in both transfections, enabling us to quantify the effect of $SLC1A2_{L85P}$ on $SLC1A2_{wt}$ protein function. Notably, glutamate transport activity in cells expressing both $SLC1A2_{wt}$ and $SLC1A2_{L85P}$ was reduced by 33% compared to cells expressing only $SLC1A2_{wt}$ (67 ± 10.8% of WT; see Fig 2E), demonstrating that $SLC1A2_{L85P}$

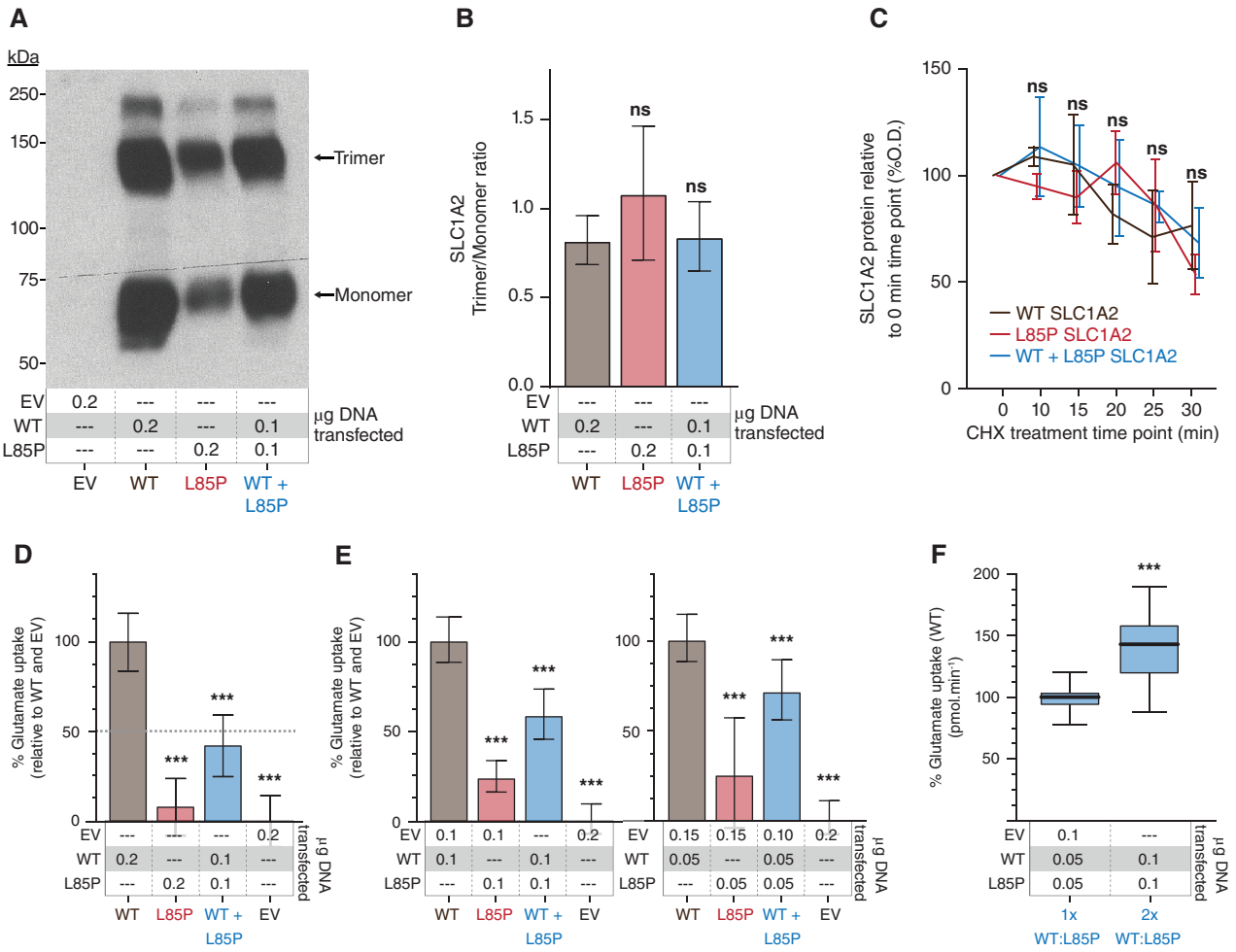

FIGURE 2: SLC1A2 Leu85Pro is a dominant negative variant that interferes with wild-type (WT) *SLC1A2* function. (A) Plasma membrane expression of $SLC1A2_{wt}$ and $SLC1A2_{L85P}$ in transiently transfected HEK cells. EV = empty vector. (B) Trimer/Monomer ratio of plasma membrane SLC1A2 from cells transfected with $SLC1A2_{wt}$, $SLC1A2_{L85P}$, or both $SLC1A2_{wt}$ and $SLC1A2_{L85P}$. Mean ± standard deviation (SD) of 3 independent experiments is represented. ns = not significant ($p > 0.05$). (C) SLC1A2 protein expression levels in total lysates from cells transfected with $SLC1A2_{wt}$, $SLC1A2_{L85P}$, or both $SLC1A2_{wt}$ and $SLC1A2_{L85P}$ after incremental treatment with cycloheximide (CHX). Values were obtained in triplicate and corrected against actin loading and normalized to the value of the untreated condition (sample 0). Values represent mean ± SD. ns = nonsignificant differences in the values obtained at each time point ($p > 0.05$). (D, E) Uptake of L-[^{3}H] glutamate by HEK cells transiently transfected with the indicated plasmids. Data from 4 independent experiments (n = 20–65) are expressed as percentage of average $SLC1A2_{wt}$ uptake (pmol · min^{-1}) controlled for EV uptake, and are represented as the mean ± SD. ***Significant difference ($p < 0.001$) in level of $SLC1A2_{wt}$ versus the indicated sample. (F) Uptake of L-[^{3}H] glutamate by HEK cells transiently transfected with equal amounts of $SLC1A2_{wt}$ and $SLC1A2_{L85P}$ plasmids, as well as twice as much of each plasmid. Data from 3 independent experiments expressed as percentage of average uptake (pmol · min^{-1}) of the leftward experiment are represented as the mean ± SD. ***Significant difference ($p < 0.001$) calculated using unpaired Student test. [Color figure can be viewed at www.annalsofneurology.org]

acts in a dominant negative manner to reduce, but not eliminate, $SLC1A2_{wt}$ glutamate transporter function. The dominant negative effect of $SLC1A2_{L85P}$ on $SLC1A2_{wt}$ function is likely mediated via an effect on SLC1A2 folding, trafficking, or functional disruption of mixed trimeric proteins, given the ability of $SLC1A2_{L85P}$ to form stable trimeric protein.

Effect of Ceftriaxone on Seizure Frequency in a Patient with SLC1A2*-Related Epilepsy*

Given our observation in vitro that transfection with an increasing amount of both $SLC1A2_{wt}$ and $SLC1A2_{L85P}$ resulted in increased *SLC1A2* function (see Fig 2F), we hypothesized that increasing both $SLC1A2_{L85P}$ and $SLC1A2_{wt}$ expression in vivo may help partially alleviate the glutamate transporter defect caused by $SLC1A2_{L85P}$. To test this, we evaluated the SLC1A2-modulating agent ceftriaxone on a 20-month-old with de novo dominant Leu85Pro *SLC1A2*-related epilepsy. Ceftriaxone is an US Food and Drug Administration–approved antibiotic that crosses the blood–brain barrier and increases *SLC1A2* expression and function within several days in neural tissue.[11–15] After IRB approval, the patient was initiated on IV ceftriaxone at 80mg/kg/day for 14 days. Caregiver-assessed daily spasms

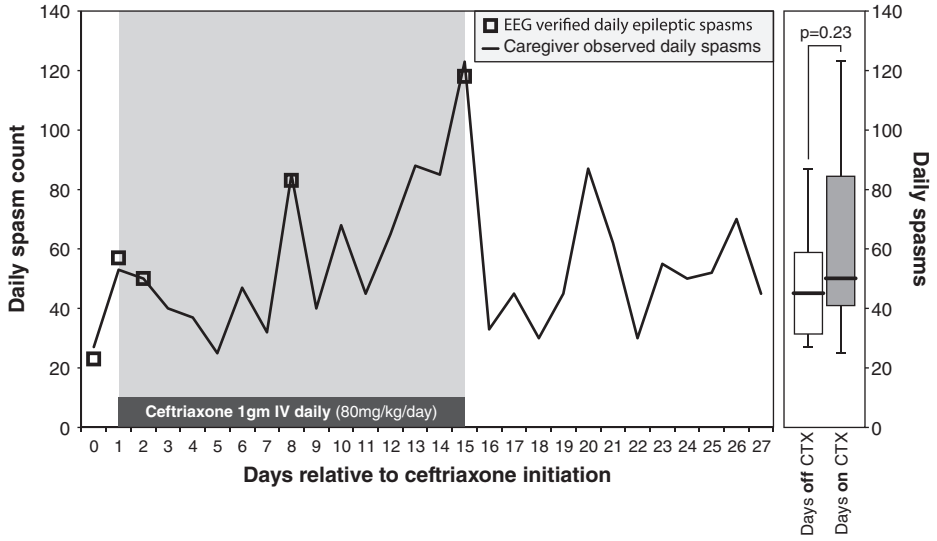

FIGURE 3: The *SLC1A2*-modulating agent ceftriaxone did not significantly alter daily spasm count in a 20-month-old heterozygous for Leu85Pro *SLC1A2*. (Left) Daily spasm counts before, during, and after a 14-day trial of intravenous (IV) ceftriaxone (CTX) in a 20-month-old girl with a heterozygous Leu85Pro *SLC1A2* variant causing *SLC1A2*-related epilepsy. Electroencephalogram (EEG)-verified daily epileptic spasms are indicated. (Right) Box-and-whisker plots showing the median, quartile, and minimum/maximum daily spasm counts across the 13 days without ceftriaxone treatment and the 15 days with ceftriaxone treatment. Probability value was calculated using the 2-tailed Welch *t* test.

counts were recorded for 28 days and demonstrated good agreement with continuous video-EEG monitoring (Fig 3). Ceftriaxone therapy did not result in a significant change in the daily spasm count (median 50 with vs 45 without ceftriaxone; $p = 0.23$, 2-tailed Welch t test; see Fig 3), although subjectively the caregiver thought that the patient's overall level of alertness improved during ceftriaxone therapy. EEG background was unchanged, and treatment was not associated with any major adverse events. Overall, these findings indicate that the SLC1A2-modulating agent ceftriaxone did not improve daily epileptic spasm frequency over the short term in a 20-month-old with Leu85Pro *SLC1A2*-related epilepsy.

Discussion

We demonstrate that the recurrent de novo Leu85Pro *SLC1A2* variant causes a severe, early onset developmental and epileptic encephalopathy via a dominant negative mechanism that reduces, but does not eliminate, $SLC1A2_{wt}$ protein localization and function. Based on these findings, as well as the lack of seizures in humans and mice with heterozygous *SLC1A2* loss-of-function variants, it appears that there is a critical dosage of functional SLC1A2 protein (somewhere between 0 and 50% of WT) under which seizures develop. It is possible that the differences in seizure severity between the dominant and recessive forms of this condition (see Supplementary Table) result from differences in the amount of remaining functional SLC1A2 protein, with the recessive variants resulting in a higher amount of remaining functional SLC1A2 protein. Finally, although ceftriaxone is one of the best studied SLC1A2-modulating agents across mice and humans, our report represents the first trial of ceftriaxone in a human with a condition resulting from a direct alteration in SLC1A2. Although we observed no benefit of a 2-week trial of ceftriaxone in this patient, additional studies are warranted. It is likely, based on progressive neuroimaging abnormalities in this disorder, that there is cumulative neuronal damage over time from excessive activation of glutamate receptors. As such, earlier initiation of SLC1A2-modulating agents may be efficacious to overcome the cumulative dominant negative effects of the variant *SLC1A2* allele.

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Author Contributions

A.B.S, L.H.R., J.Pi., P.A.R., A.B., J.Pe., C.M.E.A, C.H., S.M., and A.R. contributed to the ceftriaxone trial study concept and design. A.B.S, J.P-G., M.A.H., and L.H.R. contributed to the in vitro study concept and

design. A.B.S, L.H.R., and A.R. contributed to the ceftriaxone trial data acquisition and analysis. J.P-G., A.B.S, G.G., D.F., G.A., M.T., L.H.R., and M.A.H. contributed to the in vitro data acquisition and analysis. A.B.S, J.P-G., M.A.H., and L.H.R. drafted the manuscript and figures.

Potential Conflicts of Interest

Nothing to report.

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