SLC6A1 variants identified in epilepsy patients reduce γ-aminobutyric acid transport

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1 INTRODUCTION

SLC6A1 encodes the electrogenic sodium and chloride-coupled γ-aminobutyric acid (GABA) transporter, GAT-1, which is responsible for the reuptake of the inhibitory neurotransmitter GABA from the synapse.1 Previous reports identified heterozygous SLC6A1 variants in patients with myoclonic-atactic epilepsy (also called myoclonic-astatic epilepsy or Doose syndrome) and other generalized epilepsies.2-6 Although it has been hypothesized that SLC6A1 epilepsy mutations are likely to be loss-of-function, the functional effects of these reported variants have not yet been experimentally determined.

In this study, we report eight SLC6A1 variants identified from an unselected cohort of 460 epilepsy patients referred for genetic testing. Using splicing and GABA transport assays, we demonstrated that these variants reduce or abolish the function of the GAT-1 GABA transporter.
2 MATERIALS AND METHODS

2.1 Next generation sequencing and Sanger confirmation

Next generation sequencing and Sanger confirmation were performed as previously published. Briefly, DNA samples from 460 epilepsy patients underwent targeted resequencing of approximately 4800 genes associated with human disease. Variants were called within the coding exons and ±10 bp into the introns using NextGENe (SoftGenetics, State College, Pennsylvania). Variants were filtered for population frequency using the Genome Aggregation Database. The institutional review board of Emory University approved this study.

2.2 GABA transport assay

Each patient variant was generated by site-directed mutagenesis of the rat GAT-1 cDNA (which shares 98% amino acid identity with human GAT-1) in the vector pBluescript SK− (Stratagene, San Diego, California) as described previously.

HeLa cells were cultured in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum, 200 U/mL penicillin, 200 μg/mL streptomycin, and 2 mmol/L glutamine. Infection with recombinant vaccinia/t7 virus vTF7-3 and subsequent transfection with plasmid DNA in the expression vector pBluescript SK− were performed as previously described.

GABA transport assays were performed as previously published. Briefly, using the subsaturating [3H]-GABA concentration of 22.3 nmol/L, transport of radioactive GABA was performed for 10 minutes. The uptake of GABA by each mutant was normalized to that of wild-type (WT) GAT-1, as indicated in the legend of Figure 2. Statistical evaluation of GAT-1 transporter activity used a one-way analysis of variance with a post hoc Dunnett multiple comparison test, where \( P < 0.05 \) was considered significant.

2.3 Minigene splicing assay

A 1450-bp fragment containing the last 276 bases of SLC6A1 intron 7 through the first 263 bases of intron 10 (NM_003042.3) was polymerase chain reaction (PCR) amplified from human genomic DNA using Phusion HotStart II Polymerase (Invitrogen; 5′-CACCTCCCTGTCACCA CATGCAATAC-3′, 5′-CTGCCATCTTTCTAGCTCCA TAC-3′). The fragment was cloned into a pENTR/D-TOPO vector (Invitrogen) and verified by restriction digestion using AscI and SacII. The SLC6A1 fragment was then gateway-cloned into the pDESTsplice minigene splicing vector and verified by restriction digestion using HindIII, XhoI, and SacII. The c.850-2A>G variant was subsequently introduced into the SLC6A1 fragment by site-directed mutagenesis using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California). Sanger sequencing confirmed the presence of the c.850-2A>G variant as well as the absence of any unwanted substitutions.

Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. HEK293 cells were transfected with 1.25 μg of pDESTsplice vector using Lipofectamine 3000 (Invitrogen). The pDESTsplice vector contained one of two inserts: (1) WT SLC6A1 (Figure 2B) or (2) SLC6A1 c.850-2A>G (splice mutant; Figure 2C). Transfections were performed in triplicate. RNA was extracted using the PureLink RNA Mini Kit (Invitrogen) 24 hours posttransfection. Before first-strand cDNA synthesis, RNA was treated with DNase at 37°C for 30 minutes.

First-strand cDNA synthesis was performed using SuperScript III Reverse Transcriptase with oligo(dT) primers (Invitrogen), and subsequent cDNA was amplified using primers to exons 8 and 10 of SLC6A1 (5′-GATCA TCTGTTCACCTCGG-3′, 5′-GAGAAGATGACGAATC CTGGC-3′).

PCR products were visualized by gel electrophoresis. Bands were extracted using the PureLink Quick Gel Extraction Kit (Invitrogen) and Sanger sequenced to verify the splice products.

3 RESULTS

3.1 SLC6A1 variants identified in individuals with epilepsy

To identify disease-associated SLC6A1 variants, we examined all coding exons of SLC6A1 (NM_003042.3) from 460 unselected epilepsy patients referred for gene panel analysis at EGL Genetics. We identified eight heterozygous variants: five missense, one nonsense, one splice-site, and one in-frame deletion (Figure 1, Table 1). All substitutions affected highly conserved residues (Figure 1) and were predicted to be damaging by in silico tools (Table 1). Additionally, all identified variants were absent from the Genome Aggregation Database, a population database that includes approximately 277 200 alleles from whole-exome and genome sequencing but excludes individuals with severe pediatric diseases. Five of the identified variants were novel, whereas three (p.G550R, p.F270S, c.850-2A>G) had been previously reported (Table 1). The p.G550R variant carried by Patient 8 was previously identified in an autism patient by Wang et al. Coincidentally, two of the patients in the current study were included in a recent
publication by Johannesen et al. Specifically, Patient 3 (p.F270S) and Patient 5 (c.850-2A>G) correspond to Patient 10 and Patient 32, respectively, in the study by Johannesen et al.4

3.2 | GABA transport is reduced by variants identified in epilepsy patients

Variant location in Figure 1 is based on the published crystal structure of LeuT, the bacterial homolog of Na+/Cl−-dependent neurotransmitter transporters.14 Six of the variants affect the transmembrane helices (TMs) of GAT-1 or their extracellular boundaries, and two variants are in the third extracellular loop connecting TMs 5 and 6 (see Figure 1 and Discussion). Introduction of the identified SLC6A1 variants into the rat GAT-1 sequence resulted in a reduction or complete loss of [3H]-GABA transport activity (Figure 2A). Small levels of residual activity were seen for transporters containing p.F270S, p.Y445C, and p.I272del variants (2%, 6%, and 13% of WT activity, respectively), whereas p.G94E, p.W496Ter, and p.G550R abolished GABA transport activity. p.W235R had the smallest impact; however, it still reduced GABA transport to 27% of WT activity.

3.3 | c.850-2A>G affects the splicing of SLC6A1

The SLC6A1 c.850-2A>G variant alters the canonical splice acceptor site of intron 8. In silico analysis using Human Splicing Finder v3.0 (Aix Marseille University and Inserm, Marseille, France) predicted that this variant would eliminate the acceptor site. In HEK293 cells, expression of the c.850-2A>G splice-site variant caused exon 9 to be incorrectly spliced out of the RNA transcript (Figure 2D). Sanger sequencing of PCR bands confirmed that the c.850-2A>G variant caused exons 8 and 10 to be spliced together, excluding exon 9 (Figure 2E). Loss of exon 9 would be predicted to decrease GAT-1 expression through the introduction of a premature stop codon.

4 | DISCUSSION

Variants in SLC6A1 were first identified in patients presenting with myoclonic-atonic epilepsy, which is characterized by a range of seizure types including myoclonic, myoclonic-atonic, atonic, and absence seizures. Additionally,
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<th>Bilateral upper extremity tremor, mild tandem gait ataxia</th>
<th>Mild speech delay, episodes of falling and eye deviation</th>
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<td>([^3]H)-GABA uptake(^f)</td>
<td>0%</td>
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<td>2%</td>
<td>13%</td>
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<td>6%</td>
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ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; CADD, Combined Annotation Dependent Depletion; GABA, \(\gamma\)-aminobutyric acid; gnomAD, Genome Aggregation Database; HIV, human immunodeficiency virus; MAE, myoclonic-atactic epilepsy; NA, not available; ODD, oppositional defiant disorder; SIFT, Sorting Intolerant From Tolerant.

\(^a\)Previously published as Patient 10 in Johannesen et al.\(^4\)

\(^b\)Previously published as Patient 32 in Johannesen et al.\(^4\)

\(^c\)Based on reference sequence NM_003042.3.

\(^d\)Number of times observed in the gnomAD database.

\(^e\)PolyPhen-2 and SIFT score missense variants.

\(^f\)[\(3^\text{H}\)]-GABA uptake relative to wild-type.
patients with myoclonic-atonic epilepsy also have variable degrees of intellectual disability, developmental delay, and in some cases, autism spectrum disorder, and other behavioral disorders.\textsuperscript{2,4–6,15} Recently, Johannesen et al\textsuperscript{4} showed that variants in \textit{SLC6A1} are more broadly associated with generalized epilepsies, with absence seizures and intellectual disability being common phenotypes. In the present study, we identified eight \textit{SLC6A1} variants in individuals with epilepsy and evaluated their functional effects.

Using a radioactive GABA transport assay, the five missense variants, one in-frame deletion, and one nonsense variant identified in this study were found to reduce GABA transport, with three variants (p.G94E, p.W496Ter, p.G550R) completely abolishing GABA transport. The lack of transport activity associated with p.G94E is unsurprising given the role that this glycine residue plays in the bending of TM2 during substrate translocation.\textsuperscript{16} Similarly, p.G550R likely leads to defective transport due to destabilization of the TM12 region by the introduction of the positively charged arginine residue. Residual transporter activity ranging from 2\% to 27\% compared to WT was observed for the p.F270S, p.I272del, p.Y445C, and p.W235R variants. p.F270S and p.I272del are located in extracellular loop 3, which connects TM5 and TM6 that are part of the scaffold and core domains, respectively.\textsuperscript{14} These domains move relative to each other during substrate translocation, and perturbations of the extracellular loop 3 linker likely affect this movement.\textsuperscript{16} Finally, both p.Y445C and p.W235R involve the replacement of aromatic residues near transmembrane boundaries. Aromatic residues typically stabilize transmembrane domains;\textsuperscript{17} therefore, replacement of these residues may affect protein stability.

Using a minigene splicing assay, we also confirmed that the c.850-2A>G variant disrupts the canonical splicing of \textit{SLC6A1}, resulting in the exclusion of exon 9 from the gene structure.
mRNA transcript (Figure 2C and 2D). This result is predicted to introduce a premature stop codon, which would likely result in nonsense-mediated decay and possibly explain the fainter mutant PCR product observed in Figure 2D.

As an increasing number of SLC6A1 variants are identified, the ability to quantify the impact of variants on GABA transport will provide the opportunity to explore genotype-phenotype correlations. Based on the clinical information available to us, there were no clear differences in clinical presentation between patients with variants causing complete loss of GABA transport activity and those with residual activity (Table 1). For example, Patient 2 with the p.W235R variant, which retained 27% of WT activity, presented with intractable absence epilepsy, moderate intellectual disability, and autism spectrum disorder. This presentation was similar to Patient 7, carrying the p.W496Ter variant that completely abolished transport activity.

We hypothesize that reduced GAT-1 function could influence neuronal excitability via multiple mechanisms. GAT-1 dysfunction is expected to reduce GABA clearance, leading to increased GABA levels, both at the synapse and extrasynaptically. Increased GABA levels could lead to the overstimulation of extrasynaptic GABA_A and GABA_B receptors, which are responsible for slow and sustained (tonic) inhibitory responses. Cope et al showed that GABA_A-mediated tonic inhibition is increased in thalamocortical neurons in GAT-1 knockout mice. Increased GABA_A-mediated tonic inhibition can lead to neuronal hyperpolarization and burst pattern firing in thalamocortical neurons, which can promote the generation of spike-wave discharges. Similarly, prolonged activation of GABA_B receptors is known to stimulate low voltage-activated (T-type) Ca^{2+} channels, which can cause recurrent excitation within the thalamocortical system through successive Na^+ spikes. Previous studies show that GABA_B receptor activation causes absence seizures in mice and rats and that pre-treatment with a GABA_B antagonist can decrease the duration of chemically induced absence seizures. Reduced GAT-1 function could also decrease the amount of intracellular GABA available for release to activate GABA_A-mediated synaptic (phasic) signaling. Decreased GABA_A-mediated synaptic signaling is already associated with variants in several GABA_A receptor subunits in genetic epilepsies. Together, these results suggest that reduced GAT-1 function might lead to epilepsy through overactivation of extrasynaptic GABA_A and GABA_B receptors, and reduction in GABA_A synaptic signaling.

In summary, we identified eight SLC6A1 variants, five of which are novel, in an unselected cohort of 460 epilepsy patients, representing a 1.7% diagnostic yield. Functional analyses of these variants identified reduced GABA transport as a common underlying disease mechanism.

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DISCLOSURE

The authors have no conflicts of interest to report. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

REFERENCES


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