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BRIEF COMMUNICATION

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

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Summary

Previous reports have identified *SLC6A1* variants in patients with generalized epilepsies, such as myoclonic-atonic epilepsy and childhood absence epilepsy. However, to date, none of the identified *SLC6A1* variants has been functionally tested for an effect on GAT-1 transporter activity. The purpose of this study was to determine the incidence of *SLC6A1* variants in 460 unselected epilepsy patients and to evaluate the impact of the identified variants on γ-aminobutyric acid (GABA) transport. Targeted resequencing was used to screen 460 unselected epilepsy patients for variants in *SLC6A1*. Five missense variants, one in-frame deletion, one nonsense variant, and one intronic splice-site variant were identified, representing a 1.7% diagnostic yield. Using a [^{3}H]-GABA transport assay, the seven identified exonic variants were found to reduce GABA transport activity. A minigene splicing assay revealed that the splice-site variant disrupted canonical splicing of exon 9 in the mRNA transcript, leading to premature protein truncation. These findings demonstrate that *SLC6A1* is an important contributor to childhood epilepsy and that reduced GAT-1 function is a common consequence of epilepsy-causing *SLC6A1* variants.

KEYWORDS

γ-aminobutyric acid transport, absence epilepsy, epilepsy genetics, GAT-1, myoclonic-atonic epilepsy

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-atonic 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.

K.A.M. and K.M.B. contributed equally to this paper.

2 | MATERIALS AND METHODS

2.1 | Next generation sequencing and Sanger confirmation

Next generation sequencing and Sanger confirmation were performed as previously published.[7] 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.[8,9]

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.[10]

GABA transport assays were performed as previously published.[11] Briefly, using the subsaturating [^{3}H]-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′-CACCTCCTGTCACCACATGCAATAC-3′, 5′-CTGCCATCTTTCTAGCTCCATAC-3′). The fragment was cloned into a pENTR/D-TOPO vector (Invitrogen) and verified by restriction digestion using *Asc*I and *Sac*II. The *SLC6A1* fragment was then gateway-cloned into the pDESTsplice minigene splicing vector[12] and verified by restriction digestion using *Hind*III, *Xho*I, and *Sac*II. 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′-GATCATCCTGTTCTTCCGTGG-3′, 5′-GAGAAGATGACGAATCCTGCG-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.[13] Coincidentally, two of the patients in the current study were included in a recent

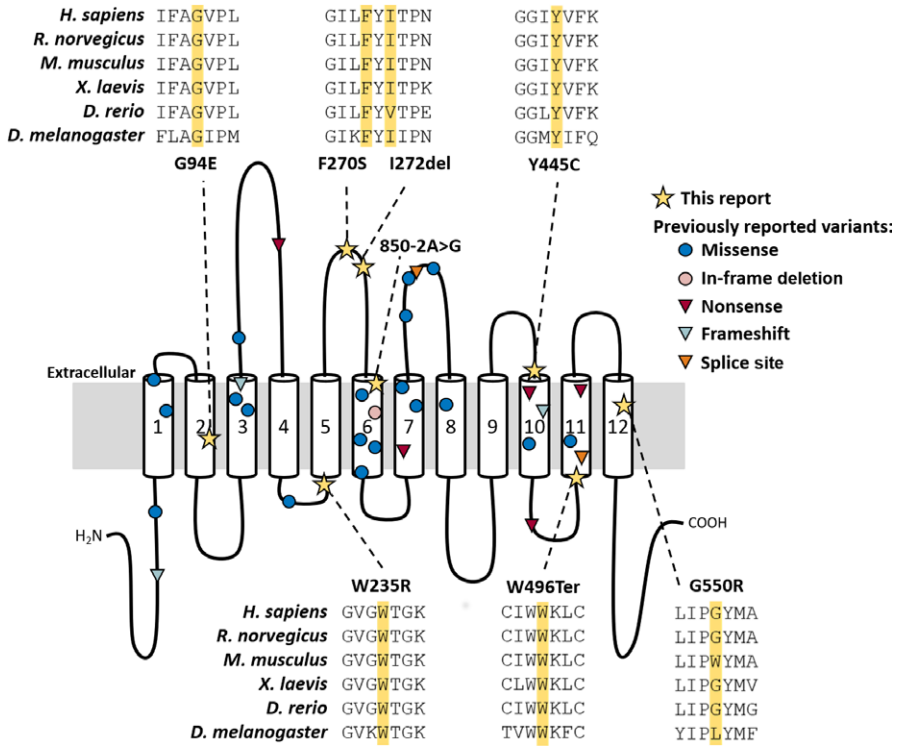

FIGURE 1 Location and conservation of GAT-1 variants identified in epilepsy patients. Schematic representation of the GAT-1 protein is shown. Numbered cylinders represent the 12 transmembrane domains. Stars indicate approximate locations of the eight variants identified in the present study. Circles indicate previously published missense (dark blue) and in-frame deletion (pink) variants, and truncating variants are indicated by triangles: nonsense (red), frameshift (light blue), and splice-site (orange).[2–6,13,15,26–31] Variant positions are based on the previously published LeuT crystal structure.[14] The affected amino acid residues from the variants identified in this study are highlighted in yellow in the protein alignments. The following GAT-1 protein sequences were used in the alignments: *Homo sapiens*, NP_003033.3; *Rattus norvegicus*, NP_0077347.1; *Mus musculus*, AAH59080.1; *Xenopus laevis*, AAI70214.1; *Danio rerio*, NP_001007363.1; *Drosophila melanogaster*, NP_651930.2

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 [^{3}H]-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,

TABLE 1 *SLC6A1* variants identified from 460 individuals with epilepsy

	Patient 1	Patient 2	Patient 3[a]	Patient 4	Patient 5[b]	Patient 6	Patient 7	Patient 8
Sex	Female	Female	Male	Male	Female	Female	Male	Male
Variant[c]	c.281G>A (p.G94E)	c.703T>C (p.W235R)	c.809T>C (p.F270S)	c.815_817delTCA (p.I272del)	c.850-2A>G	c.1334A>G (p.Y445C)	c.1487G>A (p.W496Ter)	c.1648G>A (p.G550R)
Inheritance	Unknown	Unknown (adopted)	De novo	De novo	De novo	Unknown	Unknown	Unknown
gnomAD[d]	0	0	0	0	0	0	0	0
CADD	29	24.5	24.5	—	23.5	26.6	43	25
PolyPhen-2[e]	Probably damaging	Probably damaging	Benign	—	—	Probably damaging	—	Probably damaging
SIFT[e]	Deleterious	Deleterious	Deleterious	Deleterious	—	Deleterious	—	Deleterious
Epilepsy syndrome	NA	Intractable absence epilepsy	MAE; intractable primary generalized epilepsy	MAE; intractable primary generalized epilepsy	Generalized epilepsy	Generalized epilepsy	Generalized epilepsy	Generalized epilepsy
Seizure types	NA	Generalized tonic–clonic, absence, atypical absence, tonic	Absence, eyelid myoclonias, atonic head nodding	Intractable absence, history of focal, generalized tonic and atonic	Focal and generalized	NA	Absence	Absence, tonic–clonic
Intellectual disability	NA	Moderate	Moderate	Moderate	NA	NA	NA	NA
Behavioral	NA	ASD	ADHD, ODD/ aggressive behavior	Normal	NA	NA	ASD, behavioral problems	NA
Other features	NA	Hypogammaglobulinemia, precocious puberty, insomnia, in utero drug and HIV exposure	Hypotonia, insomnia, hypoxia at birth	Bilateral upper extremity tremor, mild tandem gait ataxia	Mild speech delay, episodes of falling and eye deviation	NA	Macrocephaly, mild hypotonia	NA
[^{3}H]-GABA uptake[f]	0%	27%	2%	13%	—	6%	0%	0%

ADHD, attention-deficit/hyperactivity disorder; ASD, autism spectrum disorder; CADD, Combined Annotation Dependent Depletion; GABA, γ-aminobutyric acid; gnomAD, Genome Aggregation Database; HIV, human immunodeficiency virus; MAE, myoclonic-atonic 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}H]-GABA uptake relative to wild-type.

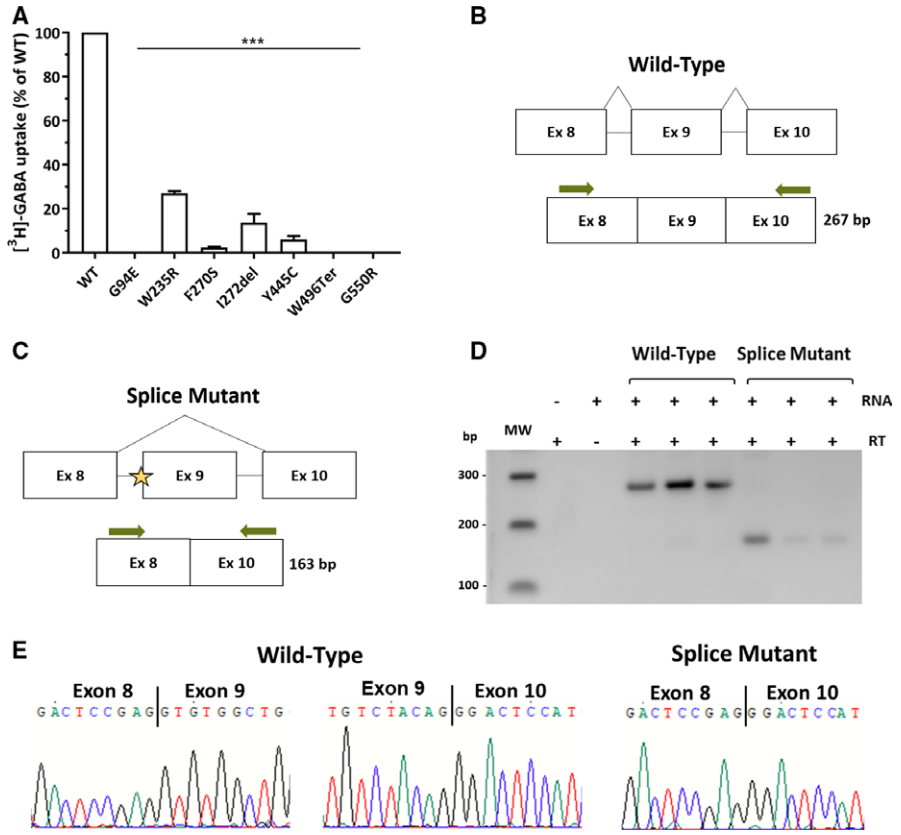

FIGURE 2 *SLC6A1* epilepsy variants have deleterious effect on GAT-1 function. A, GAT-1 wild-type (WT) and missense variants identified in epilepsy patients were transiently expressed in HeLa cells. Sodium-dependent [^{3}H]–γ-aminobutyric acid (GABA) transport was measured at room temperature for 10 minutes. Results represent mean ± SD of three separate transfections performed in quadruplicate. Means of variant transport were compared to WT using a one-way analysis of variance with a post hoc Dunnett multiple comparison test (***$P < 0.001$). B-C, Schematics showing expected splicing pattern of vectors transfected into HEK293 cells. The expected mRNA structure is indicated below the gene structure. Green arrows indicate the location of primers used to amplify cDNA. B, Splicing pattern for WT *SLC6A1*. C, Predicted splicing pattern for the *SLC6A1* c.850-2A>G splice mutant. The star indicates the location of the c.850-2A>G variant. D, Gel image showing splice products. RT indicates the presence (+) or absence (−) of reverse transcriptase during cDNA synthesis. RNA indicates the presence (+) or absence (−) of RNA during cDNA synthesis. The 100-bp molecular weight (MW) marker (Promega, Madison, Wisconsin) with band sizes is marked on the left. E, Sanger sequencing traces of gel extracted bands showing exon boundaries

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.[2,4–6,15] Recently, Johannesen et al[4] showed that variants in *SLC6A1* are more broadly associated with generalized epilepsies, with absence seizures and intellectual disability being common phenotypes. In the present study, we identified eight *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 (Figure 2A). Three variants (p.G94E, p.W496Ter, p.G550R) completely abolished 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.[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.[14] These domains move relative to each other during substrate translocation, and perturbations of the extracellular loop 3 linker likely affect this movement.[16] Finally, both p.Y445C and p.W235R involve the replacement of aromatic residues near transmembrane boundaries. Aromatic residues typically stabilize transmembrane domains;[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 *SLC6A1*, resulting in the exclusion of exon 9 from the

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.[18] 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.[19,20] Cope et al[20] 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.[21] 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.[19,22] Previous studies show that $GABA_B$ receptor activation causes absence seizures in mice and rats and that pretreatment with a $GABA_B$ antagonist can decrease the duration of chemically induced absence seizures.[23,24] 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.[25] 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.

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