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A missense mutation in *SLC6A1* associated with Lennox-Gastaut syndrome impairs GABA transporter 1 protein trafficking and function

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Abstract

Background: Mutations in *SLC6A1* have been associated mainly with myoclonic atonic epilepsy (MAE) and intellectual disability. We identified a novel missense mutation in a patient with Lennox-Gastaut syndrome (LGS) characterized by severe seizures and developmental delay.

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Contributions

Genetic data were generated and analyzed by JW and SY. Functional evaluations were performed by KC, JE, WS, GN, JS and J.Q.K. Structural modeling was performed by JW and DX. WJ, SY, and JQK., wrote the paper. All authors reviewed the compiled manuscript.

Ethics statement

Parents of each patient provided signed informed consent using a protocol approved by the Ethics Review Committee of Guangzhou Medical University.

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Methods: Exome Sequencing was performed in an epilepsy patient cohort. The impact of the mutation was evaluated by ^{3}H γ-aminobutyric acid (GABA) uptake, structural modeling, live cell microscopy, cell surface biotinylation and a high-throughput assay flow cytometry in both neurons and non neuronal cells.

Results: We discovered a heterozygous missense mutation (c700G to A [pG234S) in the *SLC6A1* encoding GABA transporter 1 (GAT-1). Structural modeling suggests the mutation destabilizes the global protein conformation. With transient expression of enhanced yellow fluorescence protein (YFP) tagged rat GAT-1 cDNAs, we demonstrated that the mutant GAT-1(G234S) transporter had reduced total protein expression in both rat cortical neurons and HEK 293T cells. With a high-throughput flow cytometry assay and live cell surface biotinylation, we demonstrated that the mutant GAT-1(G234S) had reduced cell surface expression. ^{3}H radioactive labeling GABA uptake assay in HeLa cells indicated a reduced function of the mutant GAT-1(G234S).

Conclusions: This mutation caused instability of the mutant transporter protein, which resulted in reduced cell surface and total protein levels. The mutation also caused reduced GABA uptake in addition to reduced protein expression, leading to reduced GABA clearance, and altered GABAergic signaling in the brain. The impaired trafficking and reduced GABA uptake function may explain the epilepsy phenotype in the patient.

Keywords

Lennox-Gastaut Syndrome; mutation; GABA transporter 1; trafficking; ^{3}H GABA uptake; epilepsy

Introduction

Gamma-Aminobutyric acid (GABA) transporter 1 (GAT-1), encoded by *SLC6A1*, is one of the major γ-aminobutyric acid (GABA) transporters in the brain and a major component of GABA signaling. GABA signaling is a neurotrophic signaling that regulates neural stem cell proliferation and is critical for early brain development [1, 2]. Impaired GABAergic signaling is a converging pathway of pathophysiology in genetic epilepsy associated with a number of genes[3]. Previously, many mutations in ion channel genes have been associated with various epilepsy syndromes[4–7]. Recently, many mutations in non-ion channel genes have also been reported to be associated with a spectrum of epilepsy syndromes [8–14], some of which have similar clinical phenotypes as those with ion channel gene mutations. Whether the mutations are in ion channel genes or non-ion channel genes, they are likely to cause defects in possible common converging pathways, such as the GABAergic pathway, which are critical for seizure generation. GAT-1 is a major GABA transporter subtype of sodium- and chloride-dependent transporters and is localized in GABAergic axons and nerve terminals. Unlike $GABA_A$ receptors that directly conduct postsynaptic GABAergic currents, GAT-1 influences GABAergic synaptic transmission by clearance and re-uptake of GABA from the synapse [15].

Recently, genetic sequencing has identified over a hundred mutations in *SLC6A1* associated with a spectrum of epilepsy syndromes with myoclonic atonic epilepsy (MAE) and

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intellectual disability (ID) as two prominent features [16, 17]. It is suggested that loss-of-function underlies the disease mechanism. However, the pathophysiology underlying *SLC6A1* mutations is unclear and the detailed functional assay in the mutant GAT-1 is lacking. How a mutation in GAT-1 affects protein biogenesis and trafficking and how the altered protein contributes to epilepsy have never been investigated.

In this study, we report the impact of a novel mutation G234S in GAT-1 associated with Lennox-Gastaut syndrome from the disease history to the mutant protein trafficking and biogenesis. Lennox-Gastaut syndrome is a severe epilepsy syndrome with developmental delay, which has previously been associated with other genes including $GABA_A$ receptor subunit, *GABRB3* [18] and *KCNB1* [19]. Using protein structure modeling and machine learning, a high throughput assay, flow cytometry, and live cell surface biotinylation, as well as confocal microscopy for subcellular localization and ^3H radioactive GABA uptake assay, we identified that the mutant GAT-1 had reduced global protein stability, reduced transporter biogenesis, and reduced cell surface expression and GABA uptake. The study revealed a novel mechanism underlying how a defective GAT-1 can affect GABAergic signaling and contribute to epilepsy phenotype.

Materials and methods

Subjects

The patient with idiopathic epilepsy and his unaffected father were recruited at the Epilepsy Centre of the Second Affiliated Hospital of Guangzhou Medical University. The collected clinical data included age at seizure onset, seizure types and frequency, response to antiepileptic drugs (AEDs), general and neurological examination, developmental evaluation, and family history. Brain magnetic resonance imaging (MRI) was performed to exclude symptomatic epilepsy. Video electroencephalography (EEG) was performed and the results were reviewed by two qualified electroencephalographers. Epileptic seizures and epilepsy syndromes were diagnosed and classified according to the criteria of the Commission on Classification and Terminology of the International League Against Epilepsy (1989, 2001, and 2010).

Genetic analysis

Blood samples of the patient and his father were collected. His mother was unavailable. Genomic DNA was extracted from the blood using the QIARamp mini kit (Qiagen, Hilden, Germany). Exome sequencing was performed on an Illumina Hiseq 2000 sequencer by BGI, Shenzhen. Exome was captured from fragmented genomic DNA samples using the SureSelect Huamn All Exon 50 Mb kit (Aglient Technologies, Santa Clare, CA) for enrichment, and paired-end 90-base massively parallel sequencing was performed with more than 125 times average depth and more than 98% coverage of the target region. Raw image files were processed with Illumina Basecalling Software 1.7 for base calling with default parameters.

The raw data was aligned to the human reference genome (GRCh37) using SOAP aligner ((http://soap.genomics.org.cn/). Stepwise filtering was performed to obtain potential

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pathogenic variants: 1) Population-based filtration removed variants with a minor allele frequency (MAF) ≥ 0.005 in public databases including dbSNP, the 1000 Genomes Project, ExAC ExAC-East Asian Population and ESP, and a BGI in-house database. 2) Functional impact-based filtration prioritized functional variants (protein-altering) that are missense, nonsense, indel, frameshift, and splicing variants. Potential pathogenic variants were obtained if predicted as damaging by SIFT (http://sift.jcvu.org), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2), and MutationTaster (http://www.mutationtaster.org/). After filtration, we further analyzed the phenotypes of the patient and evaluated their concordance with previously reported phenotypes of the mutated genes. Finally, the pathogenicity of the candidate variant was assessed according to ACMG, for which population data, functional damaging and information from other databases were considered[20].

Sanger sequencing was employed to validate the candidate variant on ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). The primers were 5'-TCTCCTCCCTCCACTGTTTGAC-3' and 5'-TGAGTTCCAGGGAGGCAAAG-3'. We recruited 296 Chinese healthy volunteers as normal controls.

Ethics statement

The appropriate consent form has been obtained from each patient using a protocol approved by the Ethics Review Committee of Guangzhou Medical University.

The cDNAs for coding GABA transporter 1

The plasmid cDNA encoding EYFP tagged rat GAT-1 transporter was sub-cloned into the expression vector pcDNA3.1(+). The GAT-1(G234S) mutation was generated using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing.

Measurement of surface and total expression of GAT-1 using flow cytometry

The protocol used for measurement of surface and total expression of GAT-1 using flow cytometry as well as ^{3}H GABA uptake has been described previously [8, 9]. Briefly, HEK293T cells were transfected using polyethylenimine (PEI) reagent (40 kD, Polysciences) at a DNA (μg): PEI (μl) ratio of 1:2.5 and harvested 48 hours after transfection. To express wildtype (GAT-1) and mutant GAT-1(G234S), a total of 3 μg of subunit cDNAs was transfected into a 60mm^{2} dishes. The transfected HEK293T cells were removed from the dishes by trypsinization and then re-suspended in FACS buffer (phosphate buffered saline (PBS) supplemented with 2% FBS and 0.05% sodium azide). Following washes with FACS buffer and permeabilization with Cytofix/cytoperm (BD Biosciences, CA) for 15 minutes, cells were incubated with rabbit polyclonal anti-GAT-1 antibody (1:200) (Synaptic System, catalog no. 274 102) for 2 h. Cells were then washed with FACS buffer and then incubated with fluorophore Alexa-555 conjugated goat anti-rabbit secondary antibody (1:400) for 1 h at 4°C. Cells were then washed with FACS buffer and the cell surface Alexa-555 fluorophore intensity was determined using a 3-laser LSR II machine at Vanderbilt Flow Cytometry Core. The acquired data was analyzed using FlowJo 7.1 (Tree Star, Inc., OR).

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Live cell surface biotinylation and Western blot analysis—Cell surface receptor biotinylation and Western blots were performed as reported previously [21]. Briefly, HEK293T cells were seeded in 100mm^2 dishes 2 days before transfection to avoid cell detachment. Live, transfected cells were washed with phosphate buffered saline containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4) and then incubated with sulfo-NHS biotin for 1 hour at 4° C. The sulfo-NHS biotin was quenched with PBS containing 0.1 mM glycine. Cells were lysed in RIPA buffer (20 mM Tris, 20 mM EGTA, 1 mM DTT, 1 mM benzamidine), supplemented with 0.01 mM PMSF, 0.005 μg/mL leupeptin, 0.005 μg/mL pepstatin for 1 hour at 4° C. The extracted supernatant was then incubated with immobilized streptavidin for 1 hour at room temperature. The biotinylated proteins were eluted from the streptavidin by incubation with 2× sample buffer at 37°C for 30 minutes. Membranes were incubated with primary rabbit polyclonal antibodies against GAT-1 (Alomone, 1:200) or mouse monoclonal GFP (Millipore, Billerica, MA, 1:200).

Neuronal cultures and transfection in neurons—Rat cortical neuronal cultures and transfection were prepared as previously described [22, 23]. Rat hippocampal neurons were cultured from embryonic day 18 old rat pups. The neurons were plated at a density of 2X10^5 for Western blot in plating media that contained DMEM 420ml, F12 40ml, fetal bovine serum 40ml, penicillin and streptomycin 1ml and L-Glutamine (200mM) 0.2ml for 4 hrs and then maintained in Neurobasal media that contained B27 supplement (50:1), L- Glutamine (200mM) and penicillin and streptomycin 1ml. Neurons were transfected with 15 μg cDNA at day 5–7 in culture with calcium phosphate and were harvested 8–10 days after transfection. Four 100 mm^2 dishes of neurons were transfected with each subunit in each experiment to ensure enough proteins for immunoblotting assay due to low transfection efficiency in neurons.

Radioactive ^{3}H labeling GABA uptake assay—The radioactive ^{3}H labeling GABA uptake assay in HeLa cells was modified from previous studies [24]. Briefly, HeLa cells were cultured in in 100-mm^2 dishes in Dulbecco modified Eagle medium with 10% fetal bovine serum, 1% penicillin/streptomycin. Two days before GABA uptake, cells were transfected with equal amount of wildtype or mutant GAT-1(G234S) cDNA (9 μg. Before the flux, the cells were transferred to 35-mm dished coated with poly-lysine (0.1 mg/ml) for 2 hours. For the uptake, the cells were first incubated with pre-incubation solution for 15 min and then incubated with identical solution containing 1μCi/ml ^{3}H GABA and 10 uM unlabeled GABA for 15 min at room temperature. After thee washes in ice-cold solution, the cells were lysed with 500 ul of 0.25 N NaOH for 1hr, followed by addition of 250 ul acetic acid glacial. Cell lysates (300 ul) were added to 5 ml scintillation fluid and radioactivity was determined using a Packard β-scintillation QuantaSmart counter. The influx of GABA, expressed in pmol/ug protein/min, was averaged from at least triplets for each condition and for each transfection. The average counting was taken as n = 1. The untransfected condition was taken as baseline flux, which was subtracted from both the wild-type and the mutant conditions. The pmol/μg protein/min in the mutant was then normalized to the wild-type from each experiment which was arbitrarily set as 100%.

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Protein Structural modeling

Tertiary structures of both the wide-type and G234S mutated protein GAT-1 were predicted by I-TASSER[25] and MAESTROweb [26]. Details in structural differences between the wide-type and the mutant GAT-1 were illustrated using the modelled structure by DynaMut [27]. Analysis of self-aggregation or co-aggregation was conducted using PASTA 2.0 [28].

Results

Patient with Lennox-Gastaut syndrome (LGS)

The proband is a 14-year-old boy, who had his first seizure at 9.5-year-old under no obvious predisposing causes. It was a generalized tonic-clonic seizure (GTCS) lasting 2–3 min. Similar seizures subsequently occurred once a month or even 3 times a day. Then, he suffered from daily tonic seizures that occurred more than 10 times per day and started with a quick nod, followed by elevation of both upper limbs, lasting for 2–3 sec. Additionally, the patient had complex partial seizures that occurred once or several times per month and occasionally myoclonic-atonic seizures. His brain MRI was normal. Ictal video EEG recordings showed 15–20 Hz generalized polyspikes during tonic seizure (Figure 1A). Interictally, diffuse irregular spikes, polyspike and slow waves (1.5–2.5Hz), slow waves, and multifocal spike or slow waves predominantly in the frontal and temporal lobe were monitored during wakefulness and sleep (Figure 1B). The patient was born to non-consanguineous Chinese parents and a healthy mother after full-term pregnancy and a spontaneous delivery. He had an appropriate body size and had no physical malformations. The patient had normal development before seizure onset. After the occurrence of frequent tonic seizures, he had moderate intellectual disability (ID) and speech slowing. There was no history of epilepsy, development disorders or other neurological disorders in his parents, siblings, or other relative. A diagnosis of late-onset LGS was considered. The patient had not received regular therapy before 13.5 years old. He was on valproate (VPA) with a dose of 11.9 $mg \cdot kg^{-1} \cdot d^{-1}$, but the seizures were still observed. Then, he was on lamotrigine (LTG) with a dose of 1.59 $mg \cdot kg^{-1} \cdot d^{-1}$, and the GTCS disappeared, but the tonic seizures did not change (Table 1).

Mutation analysis identified G234S variation in GAT-1

Since the genomic DNA of his mother was unavailable, we just performed whole exome sequencing in the proband and his unaffected father. We initially obtained all rare and high-confidence candidate variants through population and functional impact-based filtration; then according to the clinical concordance evaluation between the previously reported phenotypes of the mutated genes and phenotypic characteristic of the patient, a novel heterozygous missense variation of *SCLC6A1* (Ref Seq accession number NM_001348250: c.700G>A/p.Gly234Ser) was identified in the patient. *SLC6A1* has previously been reported to be an epilepsy gene and associated with myoclonic-atonic epilepsy (MIM#616421). His unaffected father did not harbor this variation. Moreover, this variation did not present in the general population of the 1000 Genomes Project, ExAC, ExAC-EAS. It was predicted to be “damaging” by SIFT (score = 0.033) and “deleterious” by Mutation Taster (score = 0.999). PCR-Sanger sequencing also confirmed the c.700G>A variant in the patient but not in his unaffected father (Figure 2A and 2B) and our 296 normal controls.

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Protein structural modeling suggests that G234S switch in GAT-1 protein destabilize global protein conformation—Multiple mutations have been identified in the GAT-1 protein (Figure 3A) [8, 17, 29]. Protein sequence alignment indicates that G234 in GAT-1 is a conserved residue located at the junction between the 4–5 intracellular loop and the 5th transmembrane domain (residues from 239 to 256). Homology modelling on G234S mutation in protein GAT-1 was conducted using I-TASSER [30] with homology template PDB id 4m48, as shown in Figure 3C. Residue 234 is colored red, where Glycine is mutated to Serine.

Mutation from Glycine to Serine at residue 234 may trigger several conformational variations on GAT-1 protein folding. Residue 234 is located at the 2nd intracellular loop [17], which is near the turn of two helices exposed on the surface in protein tertiary structure (Supplementary video 1). The additional hydroxyl in Serine brought polar in side chain polarity comparing with wide type Glycine. These polarity changes may disturb the equilibrium of the protein shape and cause protein structure destabilization. This hypothesis is also supported by predicting ΔΔG upon mutation using machine learning based methods SDM[31], mCSM, DUET [32], INPS [33, 34], DynaMut [27] and MAESTROweb (Laimer, et al., 2016). Details in structural differences (Figure 3D) between the wide-type Glycine (upper) and mutated Serine (lower) are illustrated using the modelled structure by DynaMut interatomic interactions predictions. The modeling using PASTA 2.0 [28] did not suggest any protein self-aggregation or co-aggregation from the perspective of energy changes. However, all the machine learning and modeling tools suggest the mutant residue reduces the transporter protein stability compared with the wildtype protein (Supplementary Table 1).

GAT-1(G234S) had reduced total protein in both HEK 293T cells and rat cortical neurons—Altered protein stability and enhanced protein degradation are common phenomena caused by mutations in various genes. We first determined the total expression of the mutant GAT-1(G234S) by transfected HEK 293T cells with wild-type or mutant GAT-1 cDNAs (Figure 4A) for 48hrs. Both wildtype GAT-1 or the mutant GAT-1(G234S) mainly migrated at 108 KDa, 96 KDa and 90 KDa, which is consistent with previous findings[35, 36]. However, when expressed in rat cortical neurons, only one main band was detected in both the wildtype and the mutant GAT1(G234S) condition (Figure 3B). Compared to the wildtype, the GAT-1(G234S) had reduced total protein expression (wt =1 and 0.75 ± 0.032 for G234) in both HEK 293T cells (Figure 4C) and in rat cortical neurons (wt =1 and 0.635± 0.003 for G234) (Figure 4D). This suggests a similar reduction of the total protein level in the mutant GAT-1(G234S) in neurons and non neuronal cells.

GAT-1(G234S) had reduced cell surface protein expression with both flow cytometry assay and live cell surface biotinylation

Reduced total protein could reduce the surface protein expression and consequently reduce the function of the transporter. We first used a high throughput assay flow cytometry to evaluate the surface expression of GAT-1 transporter. The wildtype and the mutant GAT-1 cDNAs were transfected into HEK 293T cells for 48 hrs before harvest. The cells were unpermeabilized and stained with either mouse anti-GFP (data not shown) or rabbit anti-

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GAT-1 (Figure 5A). We then performed live cell surface biotinylation and determined the surface GAT-1 protein (Figure 5B). There was a moderate but consistent reduction of GAT-1(G234S) compared with the wildtype (wt=1 vs 0.74±0.028) (Figure 5C). Consistent with the data from flow cytometry assay, in the surface biotinylation, the relative surface presence of the mutant GAT-1 had a similar reduction (wt=1 vs. 0.68±0.03) (Figure 5E) as observed by flow cytometry (Figure 5D). In summary, in *vitro* functional studies have demonstrated that the *SLC6A1* mutation (c.700G>A/p.Gly234Ser) has a damaging effect on the transporter protein trafficking by reducing the total and the cell surface transporter protein expression.

GAT-1(G234S) had reduced GABA uptake with ^{3}H-GABA transport assay.

The reduced cell surface expression of the mutant GAT1(G234S) could consequently impair the function of mutant transporter GAT-1(G234S). However, the mutant transporters that reached cell surface could also have altered function. We then determined the function of the wildtype and the mutant GAT-1(G234S) in HeLa cells by ^{3}H-GABA uptake assay. The major reason to use HeLa cells was due to the adherent nature of Hela cells. The flux was conducted in an isosmotic saline containing 1μCi/ml and 10μM cold GABA at room temperature for 15 min. The counts per minute (CPM) were converted to pmol/ug protein/min by normalizing to the standard CPM, protein concentration, and time for flux. The measurements in the mutant transporter were then normalized to the wild-type which was taken as 100%. Compared with the wild-type condition, the GAT-1(G234S) had reduced ^{3}H-GABA influx (wt = 100% vs mutant = 33.54% ± 6.4%). It is worth noting that the reduction of GABA uptake (~70%) was more than the reduction (20–30%) of the total and cell surface protein expression. This suggests that the mutant GAT-1(G234S) transporter may have impaired function of GABA uptake in addition to the impaired protein trafficking.

Discussion

Mutations in SLC6A1 are associated with a spectrum of epilepsy syndromes including LGS

It has been previously reported that MAE and ID are the two prominent phenotypes for SLC6A1 mutations. More recently, studies on clinical manifestations associated with *SLC6A1* variants indicate that variants in *SLC6A1* can give rise to variable epilepsy and neurodevelopmental syndromes, ranging from focal epilepsy to generalized epilepsy as well as intellectual disability without epilepsy [37]. Our study supports the hypothesis that mutation in SLC6A1 could give rise to a wide spectrum of clinical phenotypes. We here report that *SLC6A1* mutation is associated with LGS, which expands the phenotype spectrum associated with SLC6A1 mutations and further supporting previous hypothesis[17]. It is unclear that some patients only manifested myoclonic atonic epilepsy as reported before[16]. However, the seizure phenotypes in the reported patient are myoclonic-atonic seizures, atonic seizures and absence seizures, which have been previously reported in other *SLC6A1* mutations, suggesting a similar neural circuitry may be affected. The mean age of onset in a previous report[16] is about 3 years of age. The patient in this study had first seizure onset at age of 9.5 years old. It is possible that different mutations may impair the function of transporter to different degrees. In GAT-1(G234S) mutant transporter, the

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GABA uptake function is 33.5% of the wildtype while other mutations may cause a complete loss of function of the mutant transporter. In addition to GABA uptake ability, other factors such as the amount of the steady state amount of the mutant protein and genetic background may also play a role but needs further investigation.

It is worth noting that LGS has also been associated with mutations in other genes such as *GABRB3 and KCNB1* [33] [19]. It is not surprising that mutations in genes encoding both $GABA_A$ receptor subunits and GABA transporter 1 are associated with the same clinical epilepsy phenotype. It is plausible that $GABA_A$ receptors and GABA transporters like GAT-1 work in concert to ensure an appropriate level of GABAergic neurotransmission. Therefore, mutations affect either $GABA_A$ receptors or GABA transporters such as GAT-1can impair GABAergic signaling and give rise to a similar clinical presentation. However, it merits further study to elucidate the similarity and difference of mutations in both genes from functional evaluations to clinical phenotypes.

Mutant GAT-1(G234S) protein associated with LGS had impaired protein trafficking due to reduced global protein stability.

Homology modelling could build useful models for proteins without experimental resolved structures [38, 39], but these template-based methods may not be highly accurate for the atomic level changes at high resolution. Machine learning based structure stability prediction could deduce mutation resulted structural outcomes based on learning from existing knowledge. According to the consensus of most methods, the G234S point mutation on GAT-1 protein probably causes destabilization of global protein conformation. GAT-1 (G234S) is located at the 2^{nd} intracellular loop [17], which is near the turn of two helices exposed on the surface in protein tertiary structure (Supplementary video 1) and close to previously identified mutations. With appropriate modeling and machine learning based predictions, we hypothesize that GAT-1 (S234) has reduced global protein stability compared with GAT-1 (G234).

Mutant GAT-1(G234S) had reduced total protein expression

We previously demonstrated that the mutant $GABA_A$ receptor subunits were retained inside ER and were removed from the cells by ER-associated degradation, and this is a major pathogenicity for $GABA_A$ receptor subunit gene mutations. Because GAT-1 is a transmembrane protein, it is likely that at least some mutations in GAT-1 cause protein instability and impair trafficking. Although it has not been directly demonstrated, our data indicates that the mutant GAT-1(G234S) is subject to the similar intracellular protein processing as for many $GABA_A$ receptor subunit gene mutations due to a conserved protein quality control machinery inside cells [23, 40].

GAT-1(G234S) results in reduced functional GAT-1 at cell surface and comprises the function of the transporter on GABA uptake

Based on the extensive studies on $GABA_A$ receptor function and trafficking, we identified that the surface-level protein could at least partially serve as a surrogate for functional protein. In this study, we compared the surface protein levels and GABA uptake assay, our data indicates that GAT-1(G234S) impaired GABA reuptake in addition to impaired protein

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trafficking, which could explain the associated epilepsy phenotype in the patient carrying the GAT-1(G234S) mutation. With both, flow cytometry and live cell surface biotinylation, we identified that the surface presence of the mutant GAT-1 (G234S) was reduced compared with the wildtype GAT-1. Although the reduction was modest, it was very consistent across experiments and different assays. As consistently observed in $GABA_A$ receptors, only transporter proteins that have reached the cell surface are functional. With 3H GABA uptake assay, we demonstrated those mutant transporters that reached cell surface may have compromised capacity for GABA uptake. This further reduced the overall function in the mutant condition.

Role of reduced GAT-1function in epilepsy and the implications of GAT-1 inhibition in antiseizure drugs (ASDs)

GABA transporter-1 is a major target for ASDs. For example, Tiagabine (TGB) is an inhibitor of GAT-1 and is widely used in focal epilepsy. Another ASD, Vigabatrin (VGB), increases GABA concentration by inhibiting GABA transaminase and reducing GABA clearance. How can mutations in GAT-1 cause epilepsy even though inhibiting GAT-1 function or increasing the GABA level treats epilepsy? Findings from GAT-1 knockout mice have provided some important clues. For example, there is a large increase in a tonic postsynaptic hippocampal $GABA_A$ receptor-mediated conductance with unchanged or decreased amplitude of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) [41, 42]. However, how a *SLC6A1* mutation affects GABAergic signaling and brain development has never been addressed. Thus, it is of critical importance to dissect the pathophysiology of GAT-1 mutations, the impact on brain development, and the therapeutic implications of inhibiting GABA clearance in epilepsy and ID.

In summary, based on both genetic study and functional assay, this variant is likely to be a pathogenic mutation. Because the mutations in GAT-1 have just been recently associated with epilepsy, there is no study on how a mutation in GAT-1 can affect its biogenesis and protein trafficking. The study provided the first evidence that a mutation in GAT-1 associated with epilepsy causes impaired protein trafficking, which is in line with previous hypothesis that mutations in *SLC6A1* are associated with epilepsy and may cause loss of function. The 3H GABA uptake assay indicate the mutant GAT-1(G234S) had reduced function in addition to reduced surface protein expression. Future study with the mutation-bearing mouse model and patient-derived neuronal cells will further our understanding of how the variations in *SLC6A1* give rise to epilepsy and impair cognition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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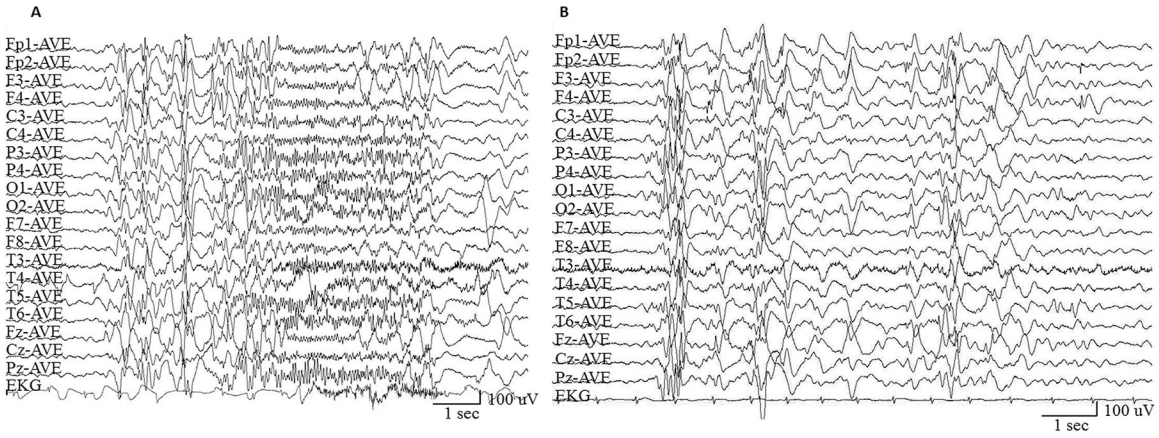

Figure 1. Electroencephalogram of proband carrying GAT-1(G234S) mutation.

(A). Ictal video EEG recordings showed 15–20 Hz generalized polyspikes during tonic seizure. **(B).** Interictal video EEG recording demonstrated diffuse irregular spikes, polyspike and slow waves (1.5–2.5Hz). Slow waves and multifocal spikes are predominantly in the frontal and temporal lobe during both wakefulness and sleep.

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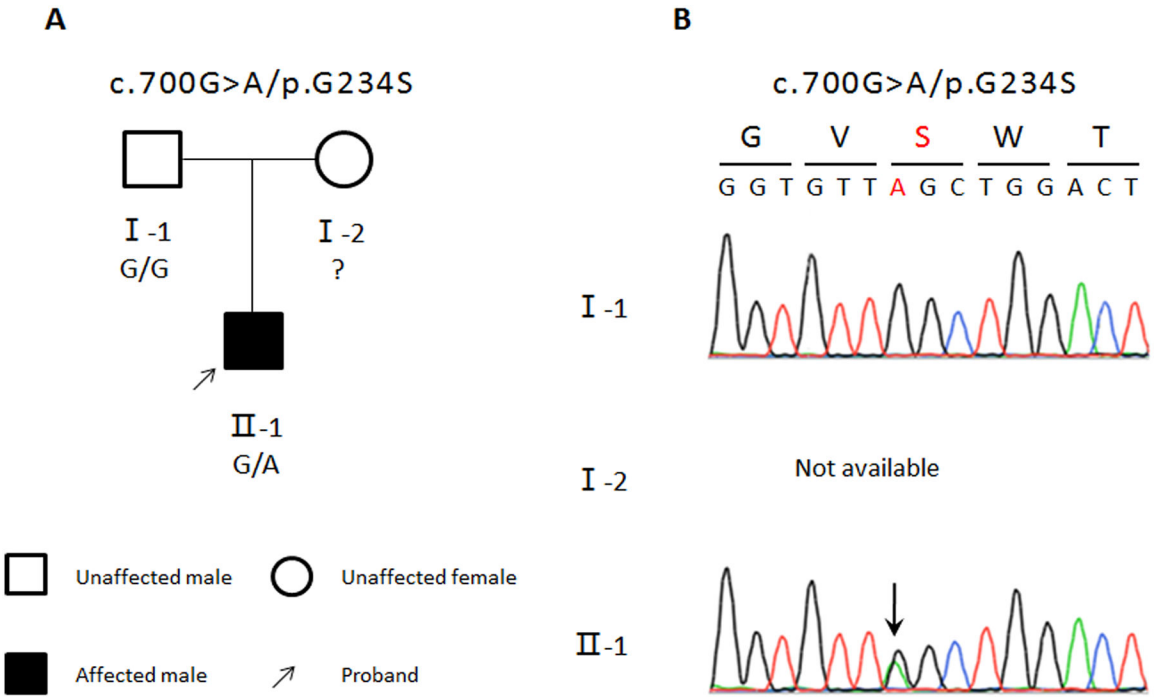

Figure 2. Pedigree and a novel *SLC6A1* missense mutation.

(A) Pedigree and the genotype. A missense mutation was found only in the proband of this family. Pedigree and the genotype of each subject of the family. The proband with late-onset Lennox-Gastaut syndrome had the mutation. His unaffected father did not have the mutation. Because the gDNA of his mother was not available, the origin of the mutation was unknown. (**B**) Chromatogram of PCR-Sanger sequencing. DNA sequences of the proband and his father were shown. Arrow indicated a G-to-A substitution.

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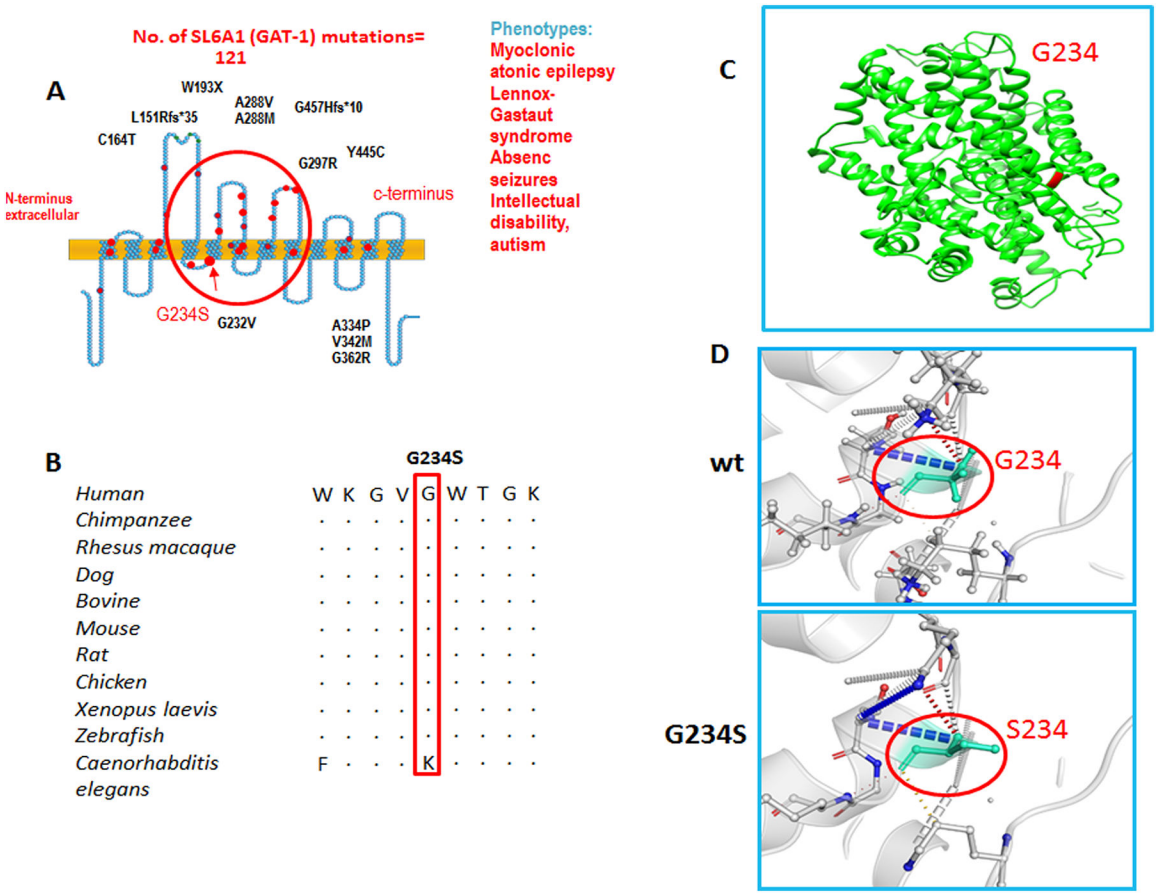

Figure 3. Modeling of the mutant GAT-1 protein and interspecies conservation of the amino acid G234S.

(A) Schematic representation of GAT-1 protein topology and locations of GAT-1 variants identified in patients associated with a spectrum of epilepsy syndromes. It is predicted that GAT-1 contains 12 transmembrane domains. G234S is located at the junction of the second intracellular loop and the 5th transmembrane domain of the GAT-1 protein. The positions of variants are based on the published LeuT crystal structure. (**B**) Amino acid sequence homology shows that glycine (G) at residue 234 is highly conserved in SCL6A1 in human (Accession NO. NP_003033.3) and across species. (**C**) Tertiary structures of both the wildtype and G234S mutant protein GAT-1 are predicted by I-TASSER. Residue 234 is highlighted as red and Glycine is mutated to Serine. (**D**) From interatomic interactions predictions by DynaMut, wild-type (upper) and G234S mutation (bottom) residues are colored in light-green and are also represented as sticks alongside with the surrounding residues. Halogen bonds are depicted in blue. Hydrogen bonds are colored in red. Machine learning methods as Supplementary Table 1 predicted this mutation destabilized the global conformation of the GAT-1 protein.

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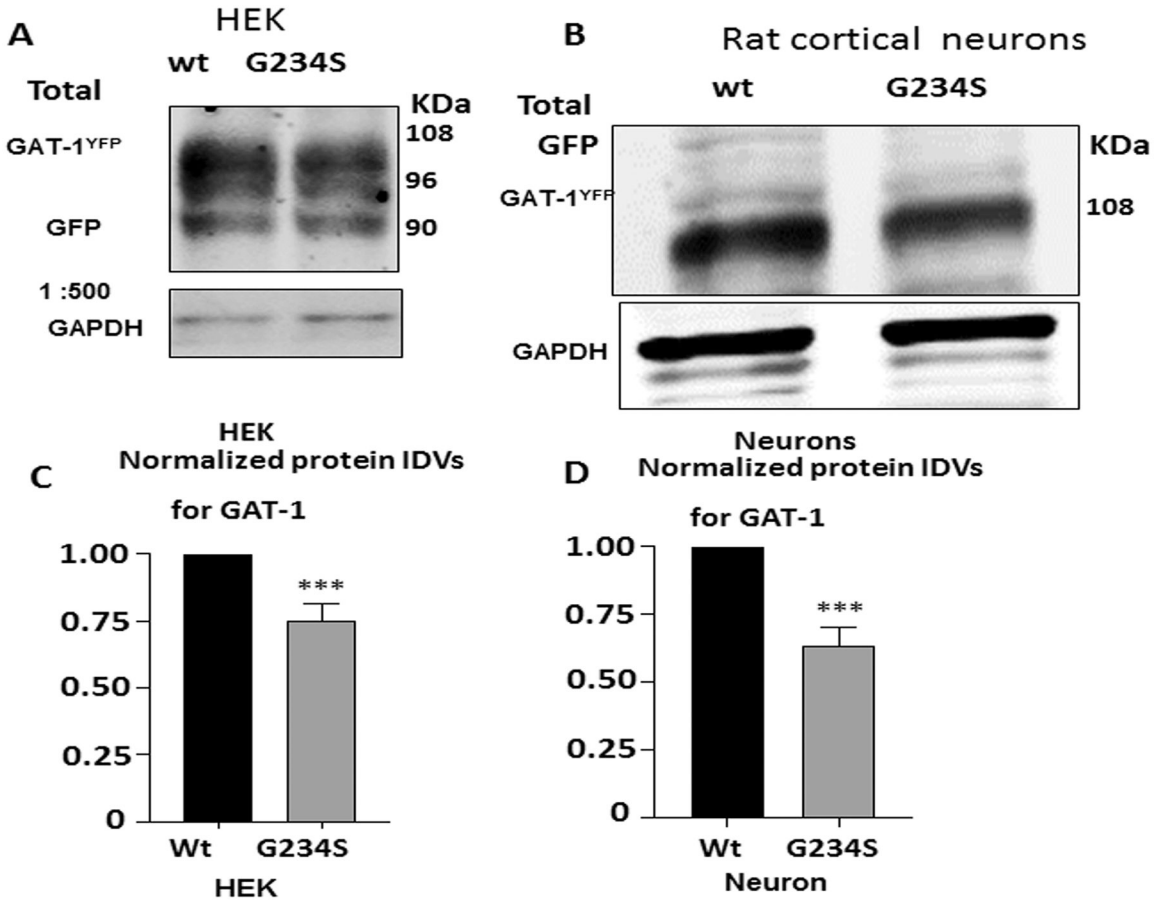

Figure 4. The total level of GAT-1 (G234S) protein was reduced in HEK 293T cells and rat cortical neurons.

A-B. HEK293T cells were transfected with GAT-1^{YFP} (3μg) for 48 hrs. (**A**) Total lysates were analyzed by SDS-PAGE and western blot. The membranes were blotted with mouse anti-GFP antibody. **(B**) Rat cortical neurons were transfected with the wildtype or the mutant GAT-1(G234S)^{YFP} cDNAs at day 7 days old in cultured dish. The total lysates were harvested from rat cortical neurons expressing the wildtype GAT-1^{YFP} (wt) or mutant GAT-1(G234S)^{YFP} (G234S) transporters after 8 days of transfection. The total lysates were then analyzed by SDS-PAGE. In HEK 293T cells (**A**), three protein bands were detected in both the wildtype and the mutant conditions. In rat cortical neurons (**B**), only a single strong band was detected in both the wildtype and the mutant conditions. In A and B, 1:500 means the ratio of the GFP antibody in buffer (1μg of GFP in 500 μl 1XPBS). (**C, D**). The total protein integrated density values (IDVs) were measured. The abundance of the mutant (GAT-1(G234S) transporter was normalized to the wildtype condition. In **C**, the total protein abundance was measured by adding up all the three bands run between 90–110 KDa. In both **C** and **D**, the total protein IDVs of either the wildtype or the mutant was normalized to its loading control. The abundance of the mutant transporter was then normalized to the wildtype. (***$p < 0.001$ vs. wt, n=4 different transfections).

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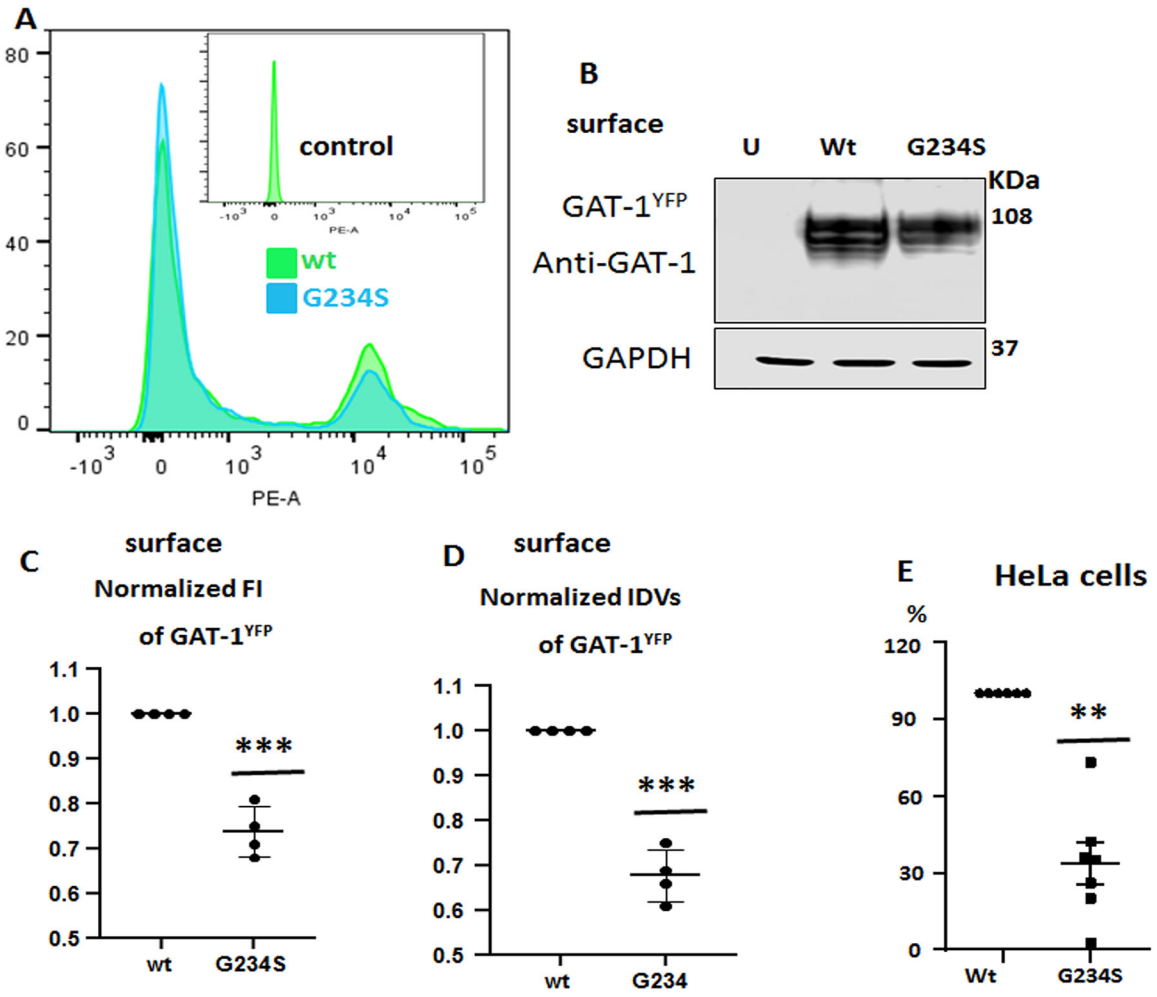

Figure 5. Reduced cell surface levels and impaired GABA uptake of the mutant GAT-1(G234S)

(**A**) The flow cytometry histograms depict surface expression levels of GAT-1 from HEK293T cells were transfected with wildtype GAT-1^{YFP} (wt), or the mutant GAT-1(G234S)^{YFP} cDNAs or control (insert). Cell surface wild type and mutant GAT-1 stained with polyclonal anti-GAT-1 antibody that was fluorescently conjugated with Alexa Fluor-555. (**B**) Surface protein was isolated by biotinylating live cells expressing wildtype GAT-1^{YFP} (wt), or the mutant GAT-1(G234S)^{YFP} and analyzed by SDS-PAGE. The membranes were visualized by polyclonal anti-GAT-1 protein. (**C**) The normalized relative fluorescence intensity (FI) (**C**) or the surface protein intensity values (IDVs) (**D**) were normalized to those obtained with wildtype GAT-1 while the FI or protein IDVs in the wildtype was arbitrarily taken as 1 in each experiment (***$p < 0.001$ vs. wt, n=4 different transfections). (**E**). The GABA uptake assay was carried out with ^{3}H radioactive GABA transport in HeLa cells transiently expressing the wildtype or the mutant GAT-1^{YFP} for 48 hrs. GABA flux was measured after 15 min transport at room temperature (**$p < 0.01$ vs. wt, n=6 for wildtype and 7 for the mutant which represents number of transfections).

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Table 1.

Clinical features of the patients with *SLC6A1* variant.

Patient ID	**LG172**
Variant	c.700G>A (NM_001348250)
Protein change	p. G234S
Origin	Unknown (*De novo* or Maternal)
Sex	Male
Current age	14 years
Age at seizure onset	9.5 years
Seizure type at onset	GTCS
Seizure frequency at onset	1 time per month
Further seizure types	Tonic, GTCS, CPS, myoclonic-atonic seizure
Ictal EEG	PSW (tonic)
Interictal EEG	SSW
Seizure outcome	Seizure uncontrolled
Duration before seizure free	-
Intellectual disability	Moderate ID
Language	Impaired; slow speech
Neurological exam	Normal
MRI findings	Normal
Epilepsy	Late-onset Lennox-Gastaut syndrome
SIFT (score)	Damaging (0.033)
Polyphen2 (score)	Benign (0.043)
MutationTaster(score)	Disease causing (0.999)
Frequency in ExAC	-

EEG = electroencephalography; PSW =polyspike and wave complex; SSW= slow spike and wave complex; CPS= complex partial seizure; GTCS = generalized tonic-clonic seizure; ID= Intellectual Disability; MRI = magnetic resonance imaging.