Original article

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GAT-1 (rs2697153) and GAT-3 (rs2272400) polymorphisms are associated with febrile seizures and temporal lobe epilepsy*

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ABSTRACT – *Aim.* The purpose of this study was to determine a possible association between two GABA transporter (GAT) single-nucleotide polymorphisms (SNPs), rs2697153 G>A in *SLC6A1* (GAT-1) and rs2272400 C>T in *SLC6A11* (GAT-3), and drug-resistant temporal lobe epilepsy (TLE).
Methods. DNA was isolated from 138 TLE patients (from the neocortex) and 94 non-epileptic controls (from blood/buccal swaps), and amplified by polymerase chain reaction and subjected to restriction fragment length polymorphism assays. A subgroup of patients with a positive history of febrile seizures (FS+) and traumatic brain injury (TBI+) were investigated in a separate analysis. *P* values were obtained using the Chi-Square test and Fishers exact test.
Results. The GAT-1 SNP was different between patients and controls ($p<0.05$); the AA genotype was observed in 40% of the cases vs 23% of the controls ($p<0.05$). Thirty-one patients were FS+ and the GAT-3 CT genotype was observed significantly more frequently in the FS+ group (14%) than in the FS- group (1%; $p<0.01$). Thirteen patients were TBI+, and genotyping for GAT-1 and GAT-3 in these patients did not result in statistical differences between TBI+ and TBI- groups.
Conclusions. The findings suggest that TLE is associated with GAT-1 and GAT-3 SNPs. More specifically, GAT-3 c1572T seems to be associated with TLE in patients with FS+. However, the pathophysiological consequences of these SNPs remain to be elucidated.

Key words: GABA transporter, single nucleotide polymorphism, febrile seizures, temporal lobe epilepsy

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Temporal lobe epilepsy (TLE) is one of the most prevalent types of focal epilepsy (Téllez-Zenteno and Hernández-Ronquillo, 2012) and occurs usually in late childhood to early adulthood. TLE seizures are frequently drug-resistant (Engel, 2001). They are classified according to semiology (Fisher *et al*., 2017) or pathology (Wyler *et al*., 1992). Hippocampal sclerosis (HS) is the most prevalent pathological substrate (Jay *et al*., 1993) and is associated with a relatively uniform clinical presentation.

The exact aetiology remains controversial; current hypotheses focus on certain susceptibility genes (Stögmann *et al*., 2002) and a failure of inhibitory neurotransmission and/or potentiation of excitatory neurotransmission. Two mechanisms that occur within the hippocampus are hallmarks of TLE pathophysiology: hilar mossy cell loss leading to decreased inhibition of granular cells of the dentate gyrus (Sloviter, 1994), and granular cell sprouting which increases excitatory feedback on the dentate gyrus (Babb, Brown, *et al*., 1984; Babb, Lieb, *et al*., 1984; Adzhubei *et al*., 2010; Schmeiser *et al*., 2017). As inhibition is mainly effectuated through γ-aminobutyric acid (GABA), this neurotransmitter is likely to play a pivotal role in epilepsy (Treiman, 2001; Mody and Pearce, 2004; Guazzi and Striano, 2019).

After release into the synaptic cleft, GABA activity is terminated by reuptake into glial cells and GABA-ergic neurons via a family of electrogenic, sodium- and chloride-dependent transporters (Kaila *et al*., 1992; Cammack, Rakhilin and Schwartz, 1994; Petroff, 2002). During this voltage-dependent transport, two sodium ions and one chloride ion are exchanged for one GABA molecule (Radian and Kanner, 1983). After reuptake in the neuron, GABA is re-utilized as transmitter located in synaptic vesicles. After reuptake in the astroglial cell, GABA is metabolized (Waagepetersen, Sonnewald and Schousboe, 2003). Molecular cloning has revealed four distinct GABA transporters, termed GABA transporter-1 to -3 (GAT-1 to -3) and betaine/GABA transporter (BGT-1). In the hippocampus, extracellular GABA is taken up primarily by both presynaptic neuronal GAT-1 and astrocytic GAT-3. Studies in TLE patients have shown changes in extracellular GABA concentrations, related to alterations in GAT quantity and/or function (During and Spencer, 1993; During, Ryder and Spencer, 1995; Williamson, Telfeian and Spencer, 1995; Mathern *et al*., 1999; Hoogland *et al*., 2004). A reduced number of GATs and/or GAT dysfunction is consequently suspected in the hippocampus of TLE patients. Recent data have confirmed previous observations that GAT expression is spatially reduced, in an isoform-specific manner, in HS (Schijns *et al*., 2015). Even though it is clear that GABA-ergic neurotransmission is disturbed, it is unclear why. Regarding epileptogenesis, only two risk factors have been clearly identified: traumatic brain injury (TBI) and febrile seizures (FS). It is not clear, however, why a minority of patients develop epilepsy after TBI or FS, and why a significant part of the TLE-FS$^+$ subgroup shows HS.

Perhaps a specific genetic background predisposes to the development of TLE, while another genetic background is protective. As variants in the GAT genes could play such a role, we have investigated the frequency of single-nucleotide polymorphisms (SNPs) in genes encoding GAT-1 (*SLC6A1* gene, chromosome 3) and GAT-3 (*SLC6A11* gene, chromosome 3) in a group of drug-resistant TLE patients with and without a background of FS and TBI, and in healthy controls. Regarding GAT-1, the SNP with dbSNP identifier rs2697153 (A>G allele, 5′ flanking region, exon 1) has been associated with increased panic attacks in a group of anxiety disorder patients (Thoeringer *et al*., 2009). This SNP is located in the 5′ flanking region of *SLC6A1* within only 10 kb of exon 1, potentially including the putative promoter region, providing functional and structural relevance to this specific SNP (Thoeringer *et al*., 2009). Another study (Carvill *et al*., 2015) has identified six *SCL6A1* (GAT-1) gene mutations in seven individuals with myoclonic-atonic seizures. The alterations in these patients most probably led to loss of GAT-1 function with consequently decreased clearance of synaptic GABA, provoking both phasic and tonic inhibition, leading to enhanced spike-wave discharges and, hypothetically, seizures. Fifteen other SNPs in the *SLC6A1* gene were identified, but this study focused on non-clinical samples and therefore the detected variants could not be related to certain diseases (Hirunsatit *et al*., 2007). Johannesen *et al*. recently found four recurring missense variants, suggesting possible mutational "hot spots" within *SLC6A1*. They studied patients with myoclonic atonic epilepsy (MAE) syndrome and found that the clinical hallmark of these gene variants is cognitive impairment, expressed with different levels of intellectual disability. Most of these patients also had epilepsy since childhood. This study, together with the study of Carvill *et al*., suggests that *SLC6A1* mutation-positive patients have a combination of intellectual disability, language delay and epilepsy, most frequently associated with MAE syndrome (Johannesen *et al*., 2018).

Regarding GAT-3, the SNP with dbSNP identifier rs2272400 (GAT-3 c.1572 C>T, exon 12) has been associated with antiepileptic drug resistance in a Korean study (Kim *et al*., 2011). Structurally, the SNP rs2272400 is located in the coding region of GAT-3 on exon 12, however, this does not lead to amino acid changes. It has become increasingly clear that "silent" SNPs may influence promoter activity (and thereby gene expression) or may lead to synthesis of proteins with the same amino acid sequence, but different structural and functional properties (Kim *et al*., 2011). No other SNPs in the

SLC6A11 gene have thus far been identified. We therefore performed an association study for both SNPs in a cohort of drug-resistant TLE patients and healthy controls.

Materials and methods

Study population

All TLE patients (*n*=138) were diagnosed with drug-resistant TLE (Kwan *et al.*, 2010). They were subjected to resective brain surgery (anterior temporal lobectomy and amygdalahippocampectomy) after an extensive preoperative work-up including video-EEG, 3T-MRI and neuropsychological examination. Temporal lobe neocortical and hippocampal specimens were collected during surgery. Upon resection, specimens were immediately frozen on dry ice and stored at -80°C until molecular analysis. All TLE patients gave their written informed consent to use their resected tissue samples for medical research. The research was conducted in The Netherlands only. According to Dutch legislation, it is not obligatory to obtain permission from the local ethics committee for medical research with patient data and tissue samples. At the time of tissue collection for this study, ethical approval was deemed unnecessary not only because of this legislation but also because the tissue collection did not determine the course of surgery or treatment, either before or during follow-up, and the investigated samples would otherwise have been discarded. All tissue samples and patient data were anonymized for members of the experimental research team.

Based on routine histopathological evaluation, samples consisted of both HS and non-HS (tumour and vascular malformation) cases. HS samples were graded as mild HS (Grade 1-2) or severe HS (Grade 3-4) (Wyler *et al.*, 1992). Controls (*n*=94) consisted of buccal swabs or blood samples collected from a pool of anonymized healthy subjects without a (familial) history of neurological or psychiatric disease. The control group was matched with the study population for age, gender and ethnicity.

SNP genotyping

In both patients and controls, the alleles of the SNPs in the GAT-1 gene (*SLC6A1*), SNP rs2697153, and the GAT-3 gene (*SLC6A11*), SNP rs2272400, were analysed. Genomic DNA was extracted from 20-25 mg frozen neocortical tissue (patients) and blood samples/buccal swabs (controls) using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The DNA samples were subsequently submitted to PCR, followed by allele-specific restriction fragment length polymorphism (RFLP) analysis, as follows: PCR reactions for GAT-1 and GAT-3 were carried out in a volume of 50 μL containing 200 ng genomic DNA, 0.5 μM of each primer (GAT-1 forward and reverse primer: 5'TCAATTGGGCACGAGGGTAG3' and 5'CCTTAGGATGTCAAAGGGCCA3', respectively [Thoeringer et al., 2009]; GAT-3 forward and reverse primer: 5'GGATCACCTTCCGCCTTTCT3' and 5'CGTGGGTTGGAGGGTAGATG3', respectively [Dong-Uk et al., 2011]), 0.2 mM dNTPs (Invitrogen), 1 U fast start *Taq* polymerase (Roche Applied Science, The Netherlands), and 5 μL PCR buffer (10x) with 1.5 mM $MgCl_2$. Next, SNP-dependent fragments were generated by digestion of PCR products with allele-specific restriction endonucleases according to the manufacturer's recommendations (*supplementary table 1* for lengths of bp fragments). Finally, fragments were resolved by gel electrophoresis on a 3.5% agarose gel containing Gelstar and visualized by UV light using a Biorad Geldoc 2000 system with Quantity One software (*supplementary figure 1A, B*).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences 12.0 software. Differences in genotype between patient groups and controls were analysed by the two-tailed Fisher exact test and the Chi-Square-Test. A p value <0.05 was considered significant.

Results

Demographic characteristics of the patient cohort are presented in *table 1*.

The patient group included 138 neocortical samples, of which we were able to genotype 115 for GAT-1 (*tables 2, 4*) and 129 for GAT-3 (*tables 3, 5*).

The control group consisted of 94 samples, from which we were able to genotype 75 samples for GAT-1 (*table 2*) and 86 samples for GAT-3 (*table 3*).

Table 1. Descriptive statistics of the patient group.

	Patients
n	138
Sex (% male)	51% (*n*=70)
Age at onset, years (SD)	15.2 (11.5)
Age at surgery, years (SD)	37.9 (13.3)
Epilepsy duration, years (SD)	23.0 (13.0)

Table 2. Association between SNP rs2697153 (*SLC6A1* gene) and TLE.

GAT-1 SNP rs2697153	Genotype*			Allele		Carrier of G allele
	AA	GA	GG	G	A	
Control group *n*=94, Genotype obtained *n*=75	17 (23%)	42 (56%)	16 (21%)	74 (49%)	76 (51%)	58 (77%)
Epilepsy group *n*=138, Genotype obtained *n*=115	46 (40%)	55 (48%)	14 (12%)	83 (36%)	147 (64%)	69 (60%)

Fisher Exact Test *p = 0.027; Chi-Square-Test (df=2) p= 0.028

Table 3. Association between SNP rs2272400 (*SLC6A11* gene) and TLE.

GAT-3 SNP rs2272400	Genotype*			Allele		Carrier of T allele
	CC	CT	TT	C	T	
Control group *n*=94, Genotype obtained *n*=86	86 (100%)	0	0	172 (100%)	0	0
Epilepsy group *n*= 138, Genotype obtained *n*=129	124 (96%)	5 (4%)	0	253 (98%)	5 (2%)	5 (4%)

Two-tailed Fisher exact test *p = 0.16

Regarding the three GAT-1 genotypes, GA was most common (48% in epilepsy patients and 56% in controls), while the GG genotype was least common (12% in epilepsy patients and 21% in controls) (*table 2*). The AA genotype was significantly more common in the epilepsy group than in controls (40 vs 23%; *p*=0.03).

Regarding the three GAT-3 genotypes, CC was most common (96% in epilepsy patients and 100% in controls), while the TT genotype was not present at all. In the control group, the CT genotype was not present either, though it was found in 4% of epilepsy cases (*table 3*) (*p*=0.16). Furthermore, the C allele was present in 98% of epilepsy cases and in all controls, indicating that the T allele was only found in 2% of epilepsy cases and not at all in controls.

Within the patient group, data on FS history was available for 136 patients (*tables 4, 5*). Of these, 31/136 patients (23%) had a history of FS (FS^+). In this FS^+ group, we were able to determine the GAT-1 genotype in 25 and the GAT-3 genotype in 28 patients (*tables 4, 5*). In the FS^- group, we were able to determine the GAT-1 genotype in 87, and the GAT-3 genotype in 100 patients (*tables 4, 5*). The GAT-3 CT genotype was significantly more common in the FS^+ group (14%) than in the FS^- group (1%; *p*=0.01). Both the patient group and the control group were in Hardy-Weinberg equilibrium.

Within the epilepsy group, data was available regarding TBI history in 133 patients (*tables 6, 7*). Of these, 13 had a history of TBI (10%), of whom the GAT-1 genotype was determined in 11 patients, and GAT-3 genotype in 13. Of the 120 patients without a history of TBI, the GAT-1 genotype was determined in 88 patients, and GAT-3 genotype in 111. The GAT-1 GG genotype was present in 18% of the TBI^+ group and in 10% of the TBI^- group. The GAT-3 CT genotype was not present in the TBI^+ group at all, but was present in 4% of the TBI^- group. The GAT-1 and GAT-3 genotypes did not significantly differ between TBI^+ and TBI^- patients (*tables 6, 7*).

Table 4. Distribution of GAT-1 SNPs in TLE patients with and without a history of FS.

GAT-1 SNP rs2697153	AA	GA	GG
FS^+ (total *n*=31) (*n*=25, obtained genotype) (*n*=6, unknown genotype)	8 (32%)	14 (56%)	3 (12%)
FS^- (total *n*=105) (*n*=87, obtained genotype) (*n*=18, unknown genotype)	38 (44%)	39 (45%)	10 (11%)

FS history, FS+ or FS-, was known in 136 cases, and unknown or not available in two cases. Two-tailed Fisher exact test p = 0.555; Chi-Square test p=0.56.

Table 5. Distribution of GAT-3 SNPs in TLE patients with and without a history of FS.

GAT-3 SNP rs2272400	CC	CT*	TT
FS$^+$ (total *n*=31) (*n*=28, obtained genotype) (*n*=3, unknown genotype)	24 (86%)	4 (14%)	0
FS$^-$ (total *n*=105) (*n*=100, obtained genotype) (*n*=5, unknown genotype)	99 (99%)	1 (1%)	0

FS history, FS+ or FS-, was known in 136 cases, and unknown or not available in two cases. Two-tailed Fisher exact test *p = 0.008.

Table 6. Distribution of GAT-1 SNPs in TLE patients with and without a history of TBI.

GAT-1SNP rs2697153	AA	GA	GG
TBI$^+$ (total *n*=13) (*n*=11, obtained genotype) (*n*=2, unknown genotype)	2 (18%)	7 (64%)	2 (18%)
TBI$^-$ (total *n*=120) (*n*=88, obtained genotype) (*n*=32, unknown genotype)	37 (42%)	42 (48%)	9 (10%)

TBI history, TBI+ or TBI-, was known in 133 cases, and unknown or not available in five cases. Two-tailed Fisher exact test p = 0.266.

Table 7. Distribution of GAT-3 SNPs in TLE patients with and without a history of TBI.

GAT-3 SNP rs2272400	CC	CT	TT
TBI$^+$ (total *n*=13) (*n*=13, obtained genotype) (*n*=0, unknown genotype)	13 (100%)	0	0
TBI$^-$ (total *n*=120) (*n*=111, obtained genotype) (*n*=9, unknown genotype)	107 (96%)	4 (4%)	0

TBI history, TBI+ or TBI-, was known in 133 cases, and unknown or not available in five cases. Two-tailed Fisher exact test p = 0.99.

Discussion

In this association study, we analysed the rs2697153 SNP in the GAT-1 gene (*SLC6A1*) and the rs2272400 SNP in the GAT-3 gene (*SLC6A11*) in a well-circumscribed cohort of patients with drug-resistant TLE and matched healthy controls. The SNPs were selected based on relevant recent literature (Thoeringer *et al*., 2009; Kim *et al*., 2011; Carvill *et al*., 2015; Johannesen *et al*., 2018), however, the clinical significance of both SNPs is not yet reported in major databases (dbSNP, gnomAD and ExAC).

We observed that the AA GAT-1 genotype was significantly more frequent in patients than in controls. The obtained frequencies in the control group for both SNPs were comparable with data from the above-mentioned major databases (dbSNP, gnomAD) corresponding to the European population (dbSNP: rs2697153 G=0.4085, A=0.5915 and rs2272400 C=0.99257, T=0.00743).

A potential role for GAT-1 in epilepsy is supported by Carvill *et al*. (2015), who found six mutations in the GAT-1 gene of myoclonic-atonic seizure patients. These mutations probably cause a decrease or loss of GABA transport activity (Ben-Yona and Kanner, 2013). Decreased GABA transport results in increased extracellular GABA levels and can paradoxically, as described in GAT-1 knockout mice (Jensen *et al*., 2003), provoke seizures and hypersynchronous epileptiform neuronal activity. Elevated synaptic GABA concentrations can overstimulate extra synaptic $GABA_A$ and $GABA_B$ receptors and enhance phasic and tonic inhibition, which consequently can be associated with spontaneous spike-wave discharges (Hosford, Wang and Cao, 1997). Cope *et al*. have shown that spontaneous discharges, typical of absence seizures, have been recorded in GAT-1 knockout mice and that $GABA_A$-mediated tonic inhibition was increased (Cope *et al*., 2009). Other studies have shown that prolonged $GABA_B$ receptor activation stimulates low-voltage activated Ca^{2+} channels, which can cause recurrent excitation within the thalamocortical system through excessive Na^+ spikes, associated with epilepsy (Han, Cortez and Snead, 2012).

A second finding of this study was that the CT genotype and T allele of GAT-3 SNP rs2272400 were more common in TLE patients than controls. The TLE group was subdivided into groups with and without epilepsy risk factors, FS and TBI. This sub-analysis revealed that the FS$^+$ group contained more CT-genotyped patients (14%) than the FS$^-$ group (1%; $p<0.05$). This GAT-3 SNP is a so-called synonymous SNP on exon 12, indicating that there is no amino acid change in the GAT-3 protein. It remains to be elucidated whether

this SNP can result in alternative mRNA or a change in turnover or capacity of GAT-3 protein, or is merely an indicator of a co-segregating functional SNP, as described for other silent SNPs (Komar, 2007; Shastry, 2009).

Regarding the function of GAT-3, astrocytic GAT-3 is capable of both uptake of GABA from the synaptic cleft (Wu, Wang and Richerson, 2003; Schousboe *et al*., 2004) and transport reversal, *i.e*. release of GABA into the synaptic cleft. These mechanisms may change under pathological conditions (Raiteri *et al*., 2002). Dysfunction of GAT-3 can be due to reduced transporter reversal, resulting in reduced extracellular GABA levels, thereby contributing to a prolongation of the ictal state (Kinney, 2005). The significance and possible pathological role of the GAT-3 T allele in TLE with a history of FS has not been revealed yet. A possible hypothesis is that (a rapid increase of) high temperature induces a progressive dysfunction of the already altered GABA transporter, leading to further lowering of the synaptic GABA level, lower inhibitory tonus, and consequently a lower seizure threshold. A similar mechanism has already been described for FS and $GABA_A$ receptor subunit mutations (Kang, Shen and Macdonald, 2006). Thus, the exact pathophysiological mechanism of this SNP in TLE and FS remains to be clarified. Though this study demonstrates a significant association between both SNPs and TLE, a limitation is due to the fact that not all ethnic groups were represented in this study. Therefore, we suggest validating this study in another cohort, preferably including different genetic backgrounds.

Conclusion

The results of this study demonstrate that the AA genotype of the GAT-1 transporter SNP (rs2697153) is significantly more common in epilepsy patients than in controls. The second finding is that the CT genotype, and as a consequence, the T allele, of the GAT-3 transporter SNP (rs2272400, GAT-3 c.1572 C>T) are significantly more frequent in TLE patients, especially the FS^+ group, compared with controls. These data suggest that the susceptibility to develop TLE is associated with SNPs in genes encoding GAT-1 and -3. In fact, GAT-3 c1572T may be a contributing factor in patients with TLE and FS. The exact pathophysiological mechanism remains to be elucidated. Generalizability of our findings would require validation in cohorts with different ethnic backgrounds, and the establishment of an international biobank containing genetic material from more epilepsy patients would be very helpful to achieve this. □

Highlights

- Susceptibility to develop TLE is associated with SNPs in GAT-1 and -3 genes
- AA genotype of GAT-1 SNP rs2697153 is significantly more frequent in TLE patients
- CT genotype of GAT-3 SNP rs2272400 is significantly more frequent in TLE patients
- GAT-3 SNP rs2272400 may be a contributing factor in patients with TLE and febrile seizures

Supplementary data.
Supplementary materials are available on the www.epilepticdisorders.com website.

Disclosures.
None of the authors have any conflict of interest to declare.

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