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Galanin pathogenic mutations in temporal lobe epilepsy

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Abstract

Temporal lobe epilepsy (TLE) is a common epilepsy syndrome with a complex etiology. Despite evidence for the participation of genetic factors, the genetic basis of TLE remains largely unknown. A role for the galanin neuropeptide in the regulation of epileptic seizures has been established in animal models more than two decades ago. However, until now there was no report

of pathogenic mutations in *GAL*, the galanin-encoding gene, and therefore its role in human epilepsy was not established. Here, we studied a family with a pair of monozygotic twins affected by TLE and two unaffected siblings born to healthy parents. Exome sequencing revealed that both twins carried a novel *de novo* mutation (p.A39E) in the *GAL* gene. Functional analysis revealed that the p.A39E mutant showed antagonistic activity against galanin receptor 1 (GalR1)-mediated response, and decreased binding affinity and reduced agonist properties for GalR2. These findings suggest that the p.A39E mutant could impair galanin signaling in the hippocampus, leading to increased glutamatergic excitation and ultimately to TLE. In a cohort of 582 cases, we did not observe any pathogenic mutations indicating that mutations in *GAL* are a rare cause of TLE. The identification of a novel *de novo* mutation in a biologically-relevant candidate gene, coupled with functional evidence that the mutant protein disrupts galanin signaling, strongly supports *GAL* as the causal gene for the TLE in this family. Given the availability of galanin agonists which inhibit seizures, our findings could potentially have direct implications for the development of anti-epileptic treatment.

Introduction

Temporal lobe epilepsy (TLE) is the most common partial epilepsy in adults (1). Originally considered as an acquired condition, twin studies and the description of familial forms have demonstrated the importance of genetic factors in TLE (2,3). Up to date, two genes (*LGI1* and *DEPDC5*) have been found mutated in familial temporal lobe epilepsies; the *LGI1* gene (leucine-rich, glioma inactivated 1) has been found mutated in autosomal dominant lateral temporal lobe epilepsy (ADTLE), and these mutations lead to failure of glutamate re-uptake resulting in elevated glutamate concentration and increased activation of NMDA receptors in the pyramidal neurons, causing epileptic seizures (4). Recently, the *DEPDC5* gene (dishvelled, Egl-10 and pleckstrin domain-containing protein 5) has been found mutated in familial partial epilepsy with variable foci (FPEVF) (5). DEPDC5 is part of the GATOR complex and negatively regulates the mTOR pathway which controls numerous functions including cellular proliferation, protein synthesis, and transcription (6).

The galanin neuropeptide was discovered more than 30 years ago and described as being able to contract smooth muscle and cause hyperglycemia (7). Galanin is 30-amino-acid peptide produced from the cleavage of a 123-amino-acid protein precursor encoded by the galanin/GMAP prepropeptide gene (*GAL*; NM_015973.3). The *GAL* gene contains 5 coding exons among which exons 2 and 3 encode for galanin. The galanin neuropeptide acts as a cellular messenger within the central and peripheral nervous systems, modulating diverse physiological functions (8). In 1992, Mazarati and collaborators first demonstrated that galanin has anticonvulsivant activity in rodents (9). Over the past two decades, significant progress has been made in the understanding of the role of galanin as an endogenous inhibitor of epileptic activity as well as in deciphering the involvement of three G-protein-coupled galanin receptors (GalR1, 2 and 3). Galanin, which is highly expressed in the hippocampus, exerts an inhibitory effect on glutamatergic transmission through the activation of GalR1 and GalR2, ultimately inhibiting epileptic seizures (10). The anticonvulsivant effects of galanin have prompted the development of agonists as well as encapsulated galanin-producing cells in the prospect of designing galanin-based anti-epileptic strategy (11,12). Despite all the past knowledge and advanced understanding of the anticonvulsivant properties of galanin, there has not been yet any evidence for pathogenic mutations in the human galanin gene related to epilepsy.

Here, we report on the identification of a missense mutation (p.A39E) in exon 2 of the *GAL* gene, which encodes for galanin, in patients with TLE. We have shown that this galanin mutant affects galanin binding to receptors and downstream signaling and may ultimately lead to TLE. The results of our study further strengthen the role of galanin in epilepsy and could have direct implications for patient care.

Results

Exome sequencing data

Whole exome sequencing was carried out on the parents–child trio (individuals I-1, I-2 and II-3; Fig. 1). On average, we produced 262 (±9.3 SD) million reads per sample, 255 (±8.8 SD) million were properly paired and mapped uniquely to the reference genome (hg19), 233 (±8.2 SD) million reads remained after removal of duplicate reads, and among these, 165 (±5.3 SD) million were on target. This resulted in an average coverage of at least 8× for 97.43% (±0.04 SD) of the coding part of the RefSeq genes. An average of 27 412 (±210 SD) variants was detected per individual. Supplementary Material, Table S1 provides a summary of the exome sequencing results for each individual.

Identification of *de novo* and recessive variants

In the absence of positive family history of seizures or other neurological manifestations, we hypothesized that the affected monozygotic twins were sporadic cases and looked for pathogenic *de novo* mutations in one member of the twin pair (individual II-3). We identified 2 *de novo* single nucleotide variants (SNVs) (Table 1). All small insertions/deletions (indels) classified as *de novo* were rejected during visual inspection mainly due to miscalling of indels in homopolymer tracts or trinucleotide repeats (data not shown). The first *de novo* SNV resulted in an aspartic acid to glycine change at position 137 of the cysteine-rich secretory protein LCCL domain-containing 2 precursor (*CRISPLD2*) gene (c.A410G; p.D137G). The second variant caused an alanine to glutamic acid substitution at position 39 of the galanin/GMAP prepropeptide (*GAL*) gene (c.C116A; p.A39E). These two variants were novel, not present in dbSNPv138 nor in the Exome Aggregation Consortium (ExAC) database and were classified as damaging by SIFT, Polyphen2 and Mutation taster (Table 1). Up to date, CRISPLD2 has never been associated with seizures or epilepsy but with non-syndromic cleft lip with or without cleft palate in several studies (13–15) except for one (16). The unaffected sister (II-1) did not carry this variant. However, as none of the twins presented with this condition, this gene was not further considered.

We also investigated the possibility of a recessive model and looked for the presence of homozygote or compound heterozygote mutations in the twins. We identified three genes (*BCR*, *MYO9A* and *SH3TC1*) with mutations compatible with an
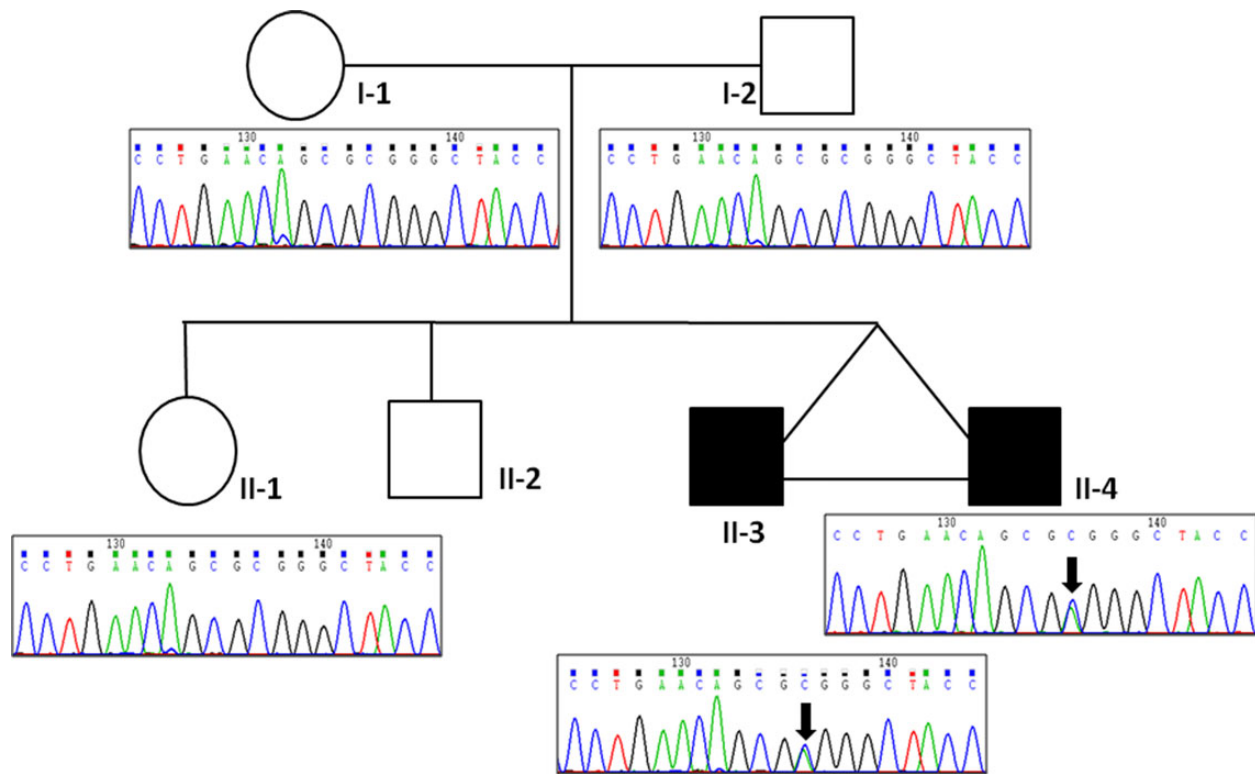
Figure 1. Pedigree structure of family EPI-ORA-AFF. Sanger sequencing traces showing the c.C116A (p.A39E) mutation in exon 2 of the *GAL* gene. The black arrows indicate the variant present in both affected twins (II-3 and II-4).

Table 1. List of validated *de novo* variants

Chr	Position	Gene (Acc. number)	Nucl. change	AA change	Cov.[a]	SIFT	PP2	MT	GERP
11	68'453'096	GAL (NM_015973.3)	c.C116A	p.A39E	370	0	0.93	0.98	2.45
16	84'883'042	CRISPLD2 (NM_031476.3)	c.A410G	p.D137G	140	0.02	0.99	0.99	5.03

SIFT, Sorting Intolerant from Tolerant algorithm; PP2, Polyphen2; MT, MutationTaster; GERP, Genomic Evolutionary Rate Profiling score.

Cov.[a]: Number of reads after the removal of duplicates that covers this position.

DP4#: Number of reads after the removal of duplicates that supports from left to right: forward reference allele—reverse reference allele—forward mutant allele—reverse mutant allele. Sum can be smaller than coverage because low-quality bases are not counted.

autosomal recessive mode of inheritance (Supplementary Material, Table S2). However, none of these genes has been linked to epilepsy. The *BCR* gene is the site of breakpoints used in the generation of the 2 alternative forms of the Philadelphia chromosome translocation found in chronic myeloid leukemia and acute lymphocytic leukemia (OMIM#151410). The *MYO9A* and *SH3TC1* genes have not been associated with disorders. These genes were not further considered.

The galanin neuropeptide was found to act as a potent anticonvulsant and regulate epileptic seizures in animal models (17, for review). However, until now its role in human epilepsy was not established. All family members were subsequently screened for the p.(A39E) mutation using Sanger sequencing. We confirmed the wild-type allele in both parents and the unaffected sister and the mutated allele in both affected twins (Fig. 1).

The 30-amino-acid galanin is located at position 33 to 62 of the 123-amino-acid galanin/GMAP prepropeptide which also comprises a 5′-hydrophobic signal peptide and a 3′-galanin message-associated peptide (GMAP). These peptides are released upon cleavage at two Lysine-Arginine (KR) dibasic sites located

on either side of galanin (18). The p.(A39E) mutation affects the alanine at position 39 of the GAL prepropeptide (galanin precursor), which corresponds to the 7th residue of the 30-amino-acid mature galanin peptide. The first 15 residues of galanin are highly conserved throughout evolution and crucial for its biological activity (Fig. 2). The last 15 residues, which are less conserved, lack receptor affinity and are believed to protect the C-terminal half from proteolysis (19–21).

Screening of the *GAL* gene for mutations in patients with focal epilepsy (FE)

To further appreciate the contribution of galanin mutations in FE in humans, we performed mutation analysis of coding sequences and splice site junctions of exons 2 and 3 of the *GAL* gene, which encode for the galanin neuropeptide, in 530 individuals with TLE and 52 patients with various types of focal epilepsies. We also analyzed exon 1 to seek for potential nonsense and frameshift mutations. We did not find any potential pathogenic mutations suggesting that *GAL* mutations are a rare cause of TLE.

Human	GWTLNS**A**GYLLGPHAVGNHRSFSDKNGLTS
Gibbon	GWTLNS**A**GYLLGPHAVGNHRSFSDKNGLTS
Baboon	GWTLNS**A**GYLLGPHAVGNHRSFSDKNGLTS
Marmoset	GWTLNS**A**GYLLGPHAVGNHRSFSDKLGLTS
Gorilla	GWTLNS**A**GYLLGPHAVGNHRSFSDKNGLTS
Squirrel_monkey	GWTLNS**A**GYLLGPHAIDNHRSFSDKHGLTG
Crab-eating_macaque	GWTLNS**A**GYLLGPHAVGNHRSFSDKNGLTS
Lesser_Egyptian_jerboa	GWTLNS**A**GYLLGPHAIDNHRSFHEKHGLAG
Chinese_tree_shrew	GWTLNS**A**GYLLGPHAVDNHRSLHEKHGLAG
Mouse	GWTLNS**A**GYLLGPHAIDNHRSFSDKHGLTG
Rat	GWTLNS**A**GYLLGPHAVGNHRSFSDKNGLTS
Chinese_hamster	GWTLNS**A**GYLLGPHAIDNHRSFSDKHGLTG
Cape_golden_mole	GWTMNS**A**GYLLGPDAMDKHRPLQEKQDLAG
Guinea_pig	GWTLNS**A**GYLLGPYAIDNHRSFSDKHGLAG
Chinchilla	GWTLNS**A**GYLLGPHAVDNHRSFSDKHGLAG
Prairie_vole	GWTLNS**A**GYLLGPHAIDNHRSFNDKHGLTG
Pika	GWTLNS**A**GYLLGPHAIDNHRSFNDKHGLAG
Squirrel	GWTLNS**A**GYLLGPHAIDNHRSFPDQSSLAG
Pig	GWTLNS**A**GYLLGPHAIDTHRSLSDKHGLAG
White_rhinoceros	GWTLNS**A**GYLLGPHAIDNHRSFHDKHGLAG
Alpaca	GWTLNS**A**GYLLGPHAIDNHRSFQDKHGLAG
Bactrian_camel	GWTLNS**A**GYLLGPHAIDNHRSFQDKHGLAG
Dolphin	GWTLNS**A**GYLLGPHAIDNHRSFNDKHGLTG
Killer_whale	GWTLNS**A**GYLLGPHAIDNHRSFHDKHGFTG
Cat	GWTLNS**A**GYLLGPHAIDNHRSFQEKPGLTG
Dog	GWTLNS**A**GYLLGPHAIDNHRSFHEKPGLTGG
Cow	GWTLNS**A**GYLLGPHALDSHRSFQDKHGLAG
Ferret	GWTLNS**A**GYLLGPHAIDNHRSLHEKPGLAG
Pacific_walrus	GWTLNS**A**GYLLGPHAIDNHRSFQEKPGLTG
Sheep	GWTLNS**A**GYLLGPHAVHNHRSFNDKHGFSG
Domestic_goat	GWTLNS**A**GYLLGPHAIDNHRSFHDKHGLAG
Weddell_seal	GWTLNS**A**GYLLGPHAIDNHRSFHEKPGLTG
David's_myotis_bat	GWTLNS**V**GYLLGPKAVDNLRSFHDKHGLAG
Microbat	GWTLNS**A**GYLLGPNAVDNHRSFHDKHGLAG
Big_brown_bat	GWTLNS**A**GYLLGPHAVDNHRSFHDKHSLAG
Tibetan_antelope	GWTLNS**A**GYLLGPHAIDNHRSLHDKHGLAG
Horse	GWTLNS**A**GYLLGPHAIDNHRSFHDKHGLAG
Shrew	GWTLNS**A**GYLLGPHAVDNHRSFNDKHGLAG
Elephant	GWTLNS**A**GYLLGPHAIDNHRSFHEKHGLAG
Cape_elephant_shrew	GWTLNS**A**GYLLGPHAIDNHRSFHEKHGLAG
Wallaby	GWTLNS**A**GYLLGPHAVDNHRSLNDKHGLAG
Opossum	GWTLNS**A**GYLLGPHAVDNHRSFNDKHGLAG
Armadillo	GWTLNS**A**GYLLGPHAVDNHRSFHDKHGLAG
Aardvark	GWTLNS**A**GYLLGPHAVDNHRSFQERHGLTG
Tasmanian_devil	GWTLNS**A**GYLLGPHAVGNHRSFSDKLGLTS
Zebra_finch	GWTLNS**A**GYLLGPHAIDNHRSFHDKYGLAG
Tibetan_ground_jay	GWTLNS**A**GYLLGPHAVGNHRSFNDKNGLTS
Turkey	GWTLNS**A**GYLLGPHAVDNHRSFNDKHGFTG
Spiny_softshell_turtle	GWTLNS**A**GYLLGPHAVDNHRSFNDKHGLAG
Green_seaturtle	GWTLNS**A**GYLLGPHAVDNHRSFNDKHGLAG
Medaka	GWTLNS**A**GYLLGPHAIDNHGSFHDKHGFTG
Lamprey	GWTLNS**A**GYLLGPNAVDNHRSFNDKHGLAG
Spotted_gar	GWTLNS**A**GYLLGPHAVDNHRSLSDKHGLAG

Figure 2. Multiple species alignment of the predicted amino-acid sequences of the galanin peptide and flanking cleavage sites produced using the 100-way multiZ new (hg19) option in Galaxy (https://usegalaxy.org/). The flanking dibasic Arg-lys (KR) cleavage sites are depicted in green. Red arrows point to the residue found mutated in TLE patients (p.A39E).

Functional analysis of the p.(A39E) mutation

Galanin-receptor affinity analysis

Displacement studies of ^{125}I-galanin with hGal(WT) or hGal(A39E) peptides, were performed on cell membranes from human Bowes melanoma cells endogenously expressing human GalR1 (hGalR1), CHO cells stably transfected with human GalR2 (hGalR2) and finally Flp-In T-REx 293 cells with inducible expression of human GalR3 (hGalR3). When compared to that of hGal(WT), hGal(A39E) peptide showed similar binding affinities to hGalR1 (t-test two-tailed P-value = 0.25) and hGalR3 (t-test two-tailed; P-value = 0.10) but significantly decreased affinities to

hGalR2 (t-test two-tailed P-value = 0.03; Fig. 3 and Table 2). hGal (A39E) peptide also showed preferred binding to hGalR1 with a 8-fold selectivity when compared to hGalR2 and 259-fold selectivity when compared to hGalR3 (Table 2).

Galanin signaling analysis

Bowes cells expressing hGalR1 were pre-incubated with increasing concentrations of hGal(A39E) peptide (from 0.1 nM to 10 μM) for 1 h before being stimulated with 100 nM hGal(WT). Cell index was measured before and after the addition of the peptides. hGal(WT) peptide showed a half maximal effective

concentration (EC$_{50}$) value of 22.4 ± 2.56 nм (Fig. 4). hGal(A39E) peptide exhibited a dose-dependent inhibition of the hGal(WT) response, with an half maximal inhibitory concentration (IC$_{50}$) value of 486.5 ± 12.46 nм, suggesting that hGal(A39E) acts as an antagonist of galanin GalR1-mediated signaling (Fig. 4).

Signal transduction was examined by assessing the ability to stimulate inositol phosphate (IP) production in CHO cells expressing hGalR2. Both hGal(WT) and hGal(A39E) peptides stimulated IP production (Fig. 5). hGal(WT) stimulated IP production with a EC$_{50}$ value of 171 ± 9.37 nм, whereas hGal(A39E) showed a statistically significant higher EC$_{50}$ value of 1243 ± 103 nм (t-test two-tailed, P-value = 0.0005) indicating that this mutant is a 7-fold less potent activator of hGalR2 (Fig. 5). The additive effect of different concentrations of hGal(A39E) on IP production obtained with 100 nм hGal(WT) was also assessed. hGal(A39E) showed a statistically significant additive effect starting from concentration of 1 μм (one-way ANOVA, P-value < 0.001; Fig. 6). Indeed, an increase in IP production was observed when CHO cells were co-stimulated with hGal(WT) and hGal(A39E) compared to stimulation with hGal(WT) alone suggesting that hGal(A39E) acts as a
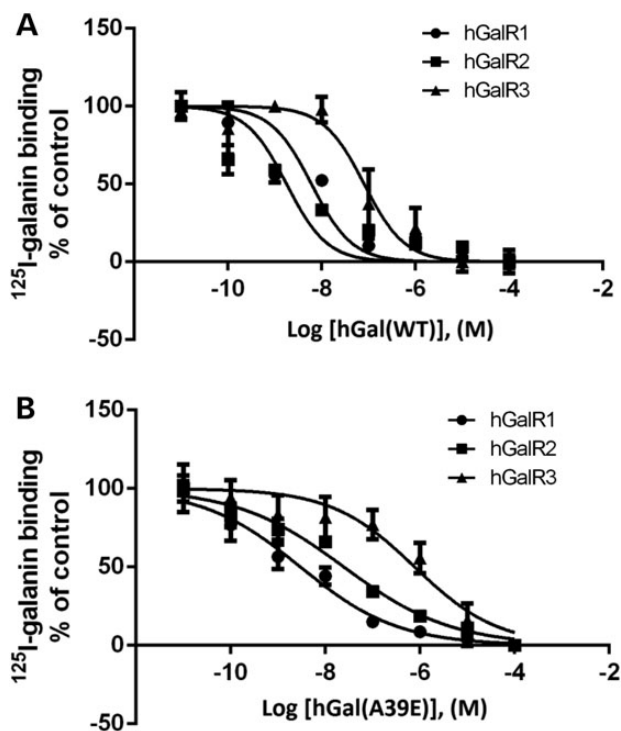

Figure 3. Galanin-receptors binding studies: Displacement of porcine-[125I]-galanin from membranes by peptide hGal(WT) (**A**) or hGal(A39E) (**B**). Membranes were from human Bowes melanoma cells expressing GalR1 (closed circle), CHO cells expressing GalR2 (closed square) and Flp-InT-REx 293 cells expressing GalR3 (closed triangle). The data are from three representative experiments performed in duplicates, presented as mean ± SEM.

hGalR2 agonist (Fig. 6). hGal(A39E) at concentration below than 1 μм did not show significant synergistic effect on the IP production induced by 100 nм hGAL(WT) (Fig. 6).

Discussion

Here, we report on the identification of a *de novo* p.(A39E) mutation in the galanin/GMAP prepropeptide (*GAL*) gene in a family with TLE. The *GAL* gene encodes for the galanin neuropeptide which possesses anticonvulsant activities in animal models. Functional analysis of the mutant galanin peptide showed that it impairs galanin function in a dominant negative manner.

A role of galanin in epilepsy was first demonstrated 22 years ago by Mazarati and collaborators who showed that galanin decreased the severity of picrotoxin-kindled convulsions in rats and that seizures-induced galanin depletion may contribute to the maintenance of seizure activity, whereas the increase of galanin concentration may favor the cessation of seizures (9,22). In addition, transgenic mice that overexpress galanin showed increased resistance to seizure induction, whereas mice deficient for galanin showed higher seizure susceptibility (23). Galanin elicits a range of biological effects by interactions with three G-protein-coupled receptors (GalR). Both GalR1 and GalR2, which are expressed in the hippocampus, mediate anticonvulsivant effect of galanin (24). GalR3, which is mainly expressed in the preoptic/hypothalamic area, has not been reported to play a role in epilepsy (25). The hippocampal formation, which is involved in seizures underlying TLE, receives galaninergic and excitatory glutamatergic innervations. Galanin fibers suppress this excitatory action through the opening of potassium channel which triggers membrane hyperpolarization and eventually inhibits glutamate release from presynaptic terminals (26). Our data on the function of the p.A39E mutant are consistent with this model; indeed, the p.A39E mutant, which has been shown to reduce galanin signaling, is predicted to increase neuronal excitability in the hippocampus, ultimately leading to TLE. The discovery of galanin mutations suggests that genetic variation in galanin may be important in TLE.

Analysis of *GAL* for mutations in 530 individuals with TLE and 52 patients with various types of focal epilepsies did not reveal additional pathogenic mutations indicating that galanin mutations are not important contributors to the genetics of TLE. However, mutation screening of other genes involved in galanin signaling, for instance the galanin receptors, would be necessary to comprehensively assess the contribution of galaninergic system to the genetics of TLE. Indeed, GalR1 has been shown to exacerbate hippocampal neuronal loss after status epilepticus (27) and CYM2503, a GalR2-positive allosteric modulator, has been shown to exhibit anticonvulsant effects in animal models (11).

Seventy two missense and loss-of-function mutations in the galanin/GMAP prepropeptide (*GAL*) gene have been reported by ExAC (http://exac.broadinstitute.org/). Among these mutations, only ten were predicted to affect galanin synthesis and/or

Table 2. Ki values of hGal(WT) and hGal(A39E) towards galanin receptors 1, 2 and 3 (GalR1, 2 and 3)

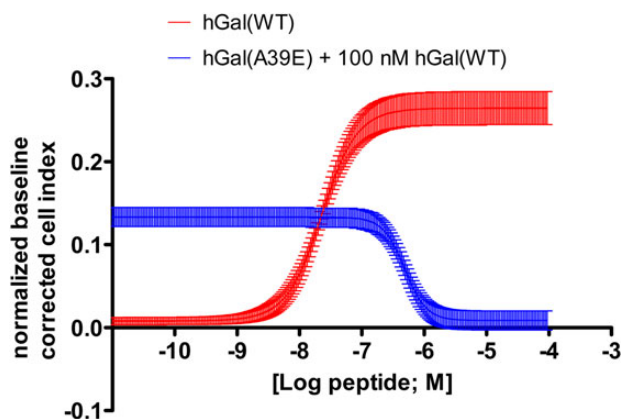| Ki (nM; mean ± SEM)					
Ligand	GalR1	GalR2	GalR3	Ki GalR2/ Ki GalR1	Ki GalR3/ Ki GalR1
hGal(WT)	6.071 ± 2.134	1.789 ± 0.684	80.90 ± 27.08	0.29	13.32
hGal(A39E)	3.001 ± 0.848	24.90 ± 7.01	780 ± 337	8.29	259.91
P-value (t-test)	0.252	0.03	0.1		

Figure 4. Inhibition of hGal(WT) stimulation by hGal(A39E) peptide. Serum-starved Bowes cells expressing GalR1 were pre-incubated for 1 h with hGal (A39E) peptide (0.1nM to 10 μM). Subsequently, cells were stimulated with 100 nM hGal(WT). Data are presented as mean ± SEM. Cell index values were normalized with respect to the cell index at the time of ligand addition and baseline-corrected by subtracting the cell index obtained with PBS treatment.
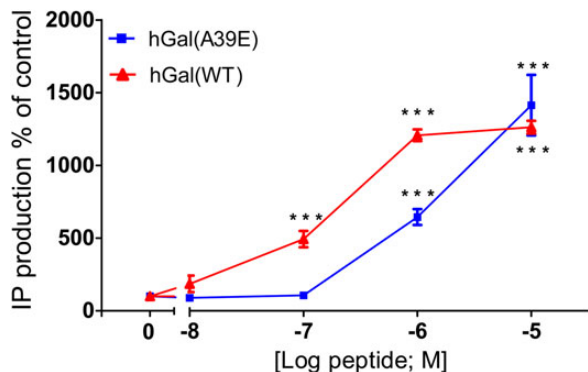

Figure 5. Inositol phosphate production in CHO cells expressing human GalR2. CHO cells were pre-incubated with 1 μCi [3H]-myo-inositol for 24 h before being stimulated with peptides hGal(WT) or hGal(A39E) at increasing concentrations. Data are presented as percent of control (no peptide) and mean ± SEM of three separate experiments. Significant level compared to controls: ***$P < 0.001$; one-way ANOVA, Tukey HSD post hoc comparison test.

function including six loss-of-function mutations and four missense mutations (Supplementary Material, Table S3). For instance, the p.Trp25leufsTer47 frameshift mutation is predicted to disrupt the synthesis of the galanin peptide which is located at position 33 to 62 of the 123-amino-acid galanin/GMAP prepropeptide. The other variants in the galanin/GMAP prepropeptide were missense mutations located in exons which do not encode for galanin and were therefore unlikely to affect galanin function. The pathogenic significance of these 10 variants is currently unknown as (i) there is no experimental data on their potential effect on galanin function and (ii) the phenotypic status of the carriers is not available. Even if considering that they are all pathogenic, their cumulative allele frequency was calculated at 0.0246% which is well-below the prevalence of TLE estimated at 0.17% (28). Our functional analysis of the hGal(A39E) mutant favored dominant negative effect over haploinsufficiency as a potential mechanism for causation. Among the ten variants predicted to affect galanin, the missense mutations are most likely to act in a dominant negative manner. Their cumulative frequency was calculated at 0.0075% which is compatible with their involvement as a very rare cause of TLE.
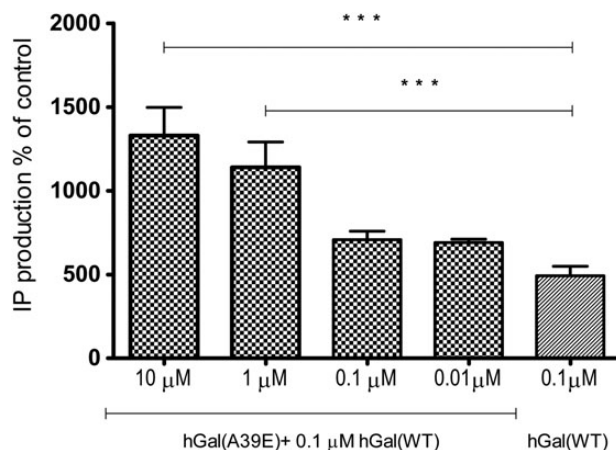

Figure 6. Inositol phosphate production in CHO cells expressing human GalR2. CHO cells were pre-incubated with 1 μCi [3H]-myo-inositol for 24 h before being stimulated with increasing concentrations of hGal(A39E) in the presence of 100 nM of hGal(WT). Data are presented as percent of control (no peptide) and mean ± SEM of three separate experiments. ***$P < 0.001$; one-way ANOVA, Tukey HSD post hoc comparison test.

The anticonvulsivant effects of galanin have prompted the development of various approaches targeting the galanin system for the treatment of epilepsy. For example, Lu *et al.* (11) have developed a GalR2-positive allosteric modulator, which potentiates the galanin action. Rodents with induced seizures that had received this compound showed increased seizures threshold and reduced seizures duration. The p.A39E mutant, which showed antagonist effects on GalR1 and reduced agonist effects on GalR2, is likely to have direct implications for the development of galanin-based anti-epileptic molecules.

Materials and Methods

Monozygotic twins with TLE

Monozygotic twins affected by TLE, born to healthy and non-consanguineous parents, were evaluated by one of the authors (AC) at the Neurology Department of Oran University Hospital (Algeria) (Fig. 1). Clinical data for the other members of the family (with the exception of II-2) were obtained in a field trip during which history and examination were performed by the same neurologist. All members of this family underwent an electroencephalography (EEG). All family members gave their informed consent for this study.

Based on detailed clinical seizure description in the twins, the diagnosis of TLE was established. Patient II-3, now 30 years old, denied both febrile seizures (FS) and central nervous system (CNS) infections. He has had a normal development. He started having isolated auras characterized by abdominal discomfort, incoherent speech, palpitations and blurred vision for one or two minutes at the age of 13 years. He did not come to medical attention until he had his first secondarily generalized seizure one year later. His EEG activity showed burst of theta oscillations in the temporal lobe. He had complex partial seizures at 19 years of age. Brain MRI did not show hippocampal sclerosis or any other anomalies. He is treated with carbamazepine (600 mg/day) and is seizure-free.

Patient II-4 is treated for TLE since the age of 13 years. His seizure description was suggestive of mesial temporal origin (auditory hallucinations, slow ideation, forced thoughts and

déjà-vu). The complex partial seizures included loss of awareness and staring. On occasion, they evolved into generalized tonic-clonic seizures. EEG showed a temporal focus and photosensitivity. Brain MRI analysis did not show hippocampal sclerosis or other anomalies. He became seizure-free with Levetiracetam (1 g/day).

Samples for screening of galanin mutations

Patients with partial epilepsy

Hundred and eighty-one unrelated individuals of European ancestry were admitted to the Epilepsy Unit at Montpellier University Hospital (France) with a diagnosis of FE according to ILAE Classification of 1989 (29). These patients suffered from a severe form of epilepsy with poor seizure control, although they were treated by anti-epileptic drugs (AEDs). Diagnosis was based on patient history, clinical examination, interictal and ictal EEG analysis carried out with monitoring video-EEG, and magnetic resonance imaging. The study group consisted of patients who suffered from non-lesional and lesional FE (including vascular malformation, cortical dysplasia, and nervous system tumor). These FE patients showed the following distribution of epilepsy syndromes: 129 patients with TLE (71.4%), 25 patients with frontal epilepsy (13.7%), 12 patients with frontotemporal epilepsy (6.6%), 4 patients with temporo-occipital epilepsy (2.2%), 3 patients with mesial-parietal epilepsy (1.6%), 2 patients with parietal epilepsy (1.1%), 2 patients with parieto-occipital epilepsy (1.1%), 1 patient with multifocal epilepsy (0.5%), 1 patient with temporo-parietal epilepsy (0.5%) and 2 patients with undetermined FE (1.1%). This study was approved by the ethics committee of the University Hospitals of Montpellier.

Thirty-seven unrelated patients were recruited in Lyon (*n* = 14) (GL, PR), Strasbourg (*n* = 18) (GR, EH) and Clermont Ferrand (*n* = 5) (DSR) through a multicenter research project (Projet Hospitalier de Recherche Clinique 2007-A00481-52 from the French ministry of health, coordinator Dr Philippe Ryvlin) that was approved by the local Ethics Committee (CPP SUD-EST IV). All these patients had a diagnosis of drug-resistant TLE based on patient history, clinical examination, interictal and ictal EEG analysis carried out with monitoring video-EEG, and magnetic resonance imaging.

Hundred and thirty-one unrelated adult individuals of European ancestry were recruited at the Swiss Epilepsy Center in Zurich (Switzerland). All Individuals were diagnosed with TLE, two of which had a bilateral temporal focus. Within this cohort, severity of phenotypes, subgroups of TLE, familiarity as well as response to AEDs was diverse. The study was approved by the ethics committee of the Kanton Zurich.

Hundred and fifty-nine patients with sporadic lateral temporal epilepsy (LTE) were collected as part of a collaborative study supported by the Genetics Commission of the Italian League Against Epilepsy (LICE). Diagnosis of LTE was made according to a clinical history of at least two lifetime seizures with auditory symptoms such as ringing, humming, sounds, voices, music, or sudden hearing loss. Sporadic LTE patients were selected according to the following inclusion criteria: (i) absence of first and second-degree relatives (siblings, children, parents, grandparents, aunts and uncles) with epileptic seizures; (ii) absence of neuroradiological abnormalities including mesial temporal sclerosis; (iii) absence of mutations in the *LGI1* gene. Exclusion criteria were an insufficient or doubtful family history and lack of neuroimaging data. All patients underwent repeated EEG recordings according to the 10–20 International System during wakefulness and, when possible, during diurnal sleep induced by sleep deprivation. Each patient underwent high resolution MRI scan of the brain. This study was approved by LICE and local ethics committees.

Sixty-four unrelated patients were recruited at the Department of Neurology and Epileptology (Tübingen) and Department of Neuropediatrics (Kiel). Thirty-two children were diagnosed with focal epilepsies (FE), 18 children with TLE, 10 of which had abnormal MRI scan (mesial temporal sclerosis (*n* = 1), cortical dysplasia (*n* = 5), 1 with heterotopia (*n* = 1), arachnoïdal cysts (*n* = 2), proencephalic cyst temporal (*n* = 1)). Among these 18 TLE patients, 11 had normal intelligence, 6 had intellectual disability and 1 was not assessed. Six patients were diagnosed with frontal lobe epilepsy (FLE), two of which had abnormal MRI scan (cortical dysplasia frontal lobe (*n* = 1), unspecific gliosis (*n* = 1). Among these six FLE patients, one had behavior problems, one had mild intellectual disability and four had normal intelligence. Eight children with undetermined focal seizures (unclassified focal seizures/secondary generalized seizures), two of which had abnormal MRI scan [subcortical heterotopias suspected with hippocampus malrotation (*n* = 1), unspecific anomalies (*n* = 1)]. Among these eight patients, one had behavior problems, one had mild intellectual disability and six had normal intelligence.

Fourty-nine unrelated Caucasian individuals (mainly of German origin) with mesial TLE were recruited as a part of the genetic and pharmacogenetic studies which were approved by the local Ethics Committee (Department of Neurology and Epileptology University of Tübingen). All reported patients gave written informed consent previous to the study inclusion. The diagnosis of mesial TLE occurred according to patient history, clinical examination, EEG analysis and magnetic resonance imaging. A hippocampal sclerosis was detected in almost all cases (*n* = 48; 97%). Most of the patients (*n* = 40, 81%) suffered from refractory epilepsy, only 14% became seizure-free (*n* = 5 without surgery and *n* = 2 after surgery). In two cases, the outcome was unknown. Before study inclusion, the patients had an average of 5.4 (*n* = 49) trials of anti-epileptic medication and 6.6 (*n* = 42) seizures per month on average.

Exome capture and sequencing

Exome capture from the parents and one of the twins of family EPI-ORA-AFF (Fig. 1, individuals I-1, I-2 and II-3) was carried out on 2 μg of genomic DNA extracted from blood using the SureSelect Human ALL Exon v5 kit (Agilent Technologies). High-throughput sequencing was performed on a HiSeq2000. Demultiplexed fastq files were obtained for each sample using the Illumina CASAVA v1.8.2 software and processed by our 'in-house' pipeline running on the Vital-IT Center for high-performance computing of the Swiss Institute of Bioinformatics (SIB; (http://www.vital-it.ch). Specifically, Burrows–Wheeler Aligner (BWA) was used to align the sequencing reads to the human reference genome NCBI build (GRCh37/hg19). SAMtools was used to remove duplicate reads. SNVs and small insertions and deletions (INDELs) were called using bcftools and Pindel 0.2.4, respectively. The minimum number of reads required for allele calling was set at eight. SNVs and INDELS variants were then functionally annotated using the ANNOVAR package.

Identification of *de novo* and recessive variants

Potential pathogenic variants under *de novo* or recessive models were identified using VariantMaster (30). Variants detected in the proband with Samtools and PINDEL quality scores ≥150 and ≥600, respectively were retained for subsequent analysis. These

variants were further filtered so as to exclude variants found outside of exons or splice sites (±2) and variants with a minimum allele frequency greater than 0.02 in dbSNPv138 (Database of Single Nucleotide Polymorphism) and/or the Exome Variant Server (EVS) database. VariantMaster used the raw data (BAM files) to robustly estimate the probability of the remaining variants to be present in the parents and siblings. Variants were classified as *de novo* only if both parents were found to be homozygous for the reference allele. Homozygote or compound heterozygote variants in the proband that were predicted as damaging by at least two of the three prediction algorithms used (SIFT, Polyphen2 and Mutation Taster) were classified as recessive only if both parents were carriers and the genotype of the unaffected sister was different from that of the proband. All variants classified as *de novo* or recessive by VariantMaster, were subsequently visually inspected using the SAMtools text alignment viewer. The remaining candidate variants were then validated using Sanger sequencing on an ABI 3730xl DNA Analyzer (Applied Biosystems).

Peptide synthesis

Peptides (Human galanin1–30 wild type [hGal(WT)]:GWTLNS**A**GYLLGPHAVGNHRSFSDKNGLTS and Human galanin A39E mutant [hGal(A39E)]: GWTLNS**E**GYLLGPHAVGNHRSFSDKNGLTS) were synthesized at 0.1 mmol scale on an automated microwave peptide synthesizer (Biotage® Initiator + Alstra™) using Fmoc (fluorenylmethyloxycarbonyl) solid-phase peptide synthesis strategy with HMPB-ChemMatrix 0.4 mmol/g resin as solid phase to obtain peptide acid. The first amino acid was attached to resin using symmetrical anhydride. All other coupling reactions were carried out using OxymaPure/DIC in DMF with DIEA as an activator base. The final cleavage was performed using standard protocol (95% TFA/2.5% TIS/2.5% H_2O). Peptides were purified by reversed-phase HPLC using BioBasic C-8 column (Thermo Scientific, Sweden) and a gradient of 20–80% acetonitrile/water containing 0.1% TFA. The identity of peptides was analyzed by MALDI-TOF mass spectrometry Voyager-DE STR (Applied Biosystems) in positive linear mode using α-cyano-4-hydroxycinnamic acid as matrix.

Galanin-receptor binding assays

Cells for ^{125}I-galanin-receptor displacement studies were seeded in 150-mm dishes and cultured 3–4 days until confluent. Cell dishes were washed and scraped into phosphate-buffered saline (PBS) and centrifuged twice at 4°C, 3000 g for 5 min. The pellet was re-suspended in assay buffer (20 mM HEPES, 5 mM $MgCl_2$, pH 7.4) supplemented with EDTA (5 mM EDTA) and incubated on ice for 45 min before centrifugation at 4°C, 8500 g for 15 min. After washing, the pellet was re-suspended in assay buffer supplemented with 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA) to a protein concentration of 1 mg/ml. Protein concentration was determined according to Lowry (BioRad, Stockholm, Sweden). Displacement studies on cell membranes were performed in a final volume of 200 μl, containing 0.15 nM porcine-[^{125}I]-galanin (2200 Ci/mmol; Perkin–Elmer Life Science, Boston, MA, USA), 30 μg cell membrane and various concentrations of peptide (10^{-5}–10^{-9} M). Peptide solutions were made in assay buffer supplemented with 0.3% bovine serum albumin (BSA). Samples were shaken at 37°C for 30 min and filtered through a MultiScreen-FB filter plate (Millipore, Billerica, MA, USA) pre-soaked in 0.3% polyethylenimine solution (Sigma–Aldrich). The filters were washed thrice with assay buffer and the retained radioactivity was determined in a β-counter (Tri-Carb Liquid Scintillation Analyzer, model 2500 TR; Packard Instrument Company, Meriden, CT, USA) using OptiPhase Supermix Cocktail (Perkin–Elmer Life Science, Boston, MA, USA) as scintillation fluid. IC_{50} values for the peptides were calculated using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) and converted into K_i values using the equation of Cheng–Prusoff ([31]).

xCELLigence cellular impedance assay

The impedance-based readout used by the xCELLigence System (Roche Applied Science) is based on the principle that the adhesion of cultured cells directly onto microelectrodes induces changes in the local ionic environment at the electrode/solution interface, conferring an increase in electrode impedance. As a result, any changes in cell physiological properties that modulate the physical contact between cell and electrode will be reflected by changes in the measured impedance, defined by the cell index variable. xCELLigence System can be used for functional screening of G-protein-coupled receptors (GPCRs) activity, based on its ability to modulate the actin cytoskeleton and cell adhesion ([32–34]). Bowes cells expressing GalR1 were seeded at 20 000 cells per well on 96-well E-Plates (ACEA Biosciences Inc.) and placed on the Real-time xCELLigence Cell Analyzer (Roche Applied Science) platform at 37°C and 5% CO2. After 24 h incubation, growth media was changed for assay buffer (HBSS containing 0.1% BSA). Three hours later, hGal(A39E) peptide was added (0.1 nM to 10 μM), after 1 h incubation, hGal(WT) was added at 100 nM. The xCELLigence System was employed to measure changes in cellular impedance following ligand stimulation. Resulting dose–response relationships were plotted using mean values from four replicates. Cell index values were normalized by dividing the cell index at the time of ligand addition and baseline-corrected by subtracting the cell index obtained with PBS treatment. Data analysis was done with built-in xCELLigence System software and presented with GraphPad Prism.

Inositol phosphate accumulation assay

CHO cells stably expressing human GalR2 were seeded in 12-well plates and cultured until confluent. Then cells were incubated 24 h with 1 μCi [3H]-myo-inositol in M-199 medium containing 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. CHO GalR2 cells were washed twice with HEPES Krebs Ringer (HKR) buffer (5 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05 mM MgCl2, 1.8 mM CaCl2, 1 g/l glucose, pH7.4) followed by 10 min pre-incubation in 800 μl HKR buffer with 10 mM LiCl at 37°C. hGal(A39E) peptide (10^{-5}–10^{-8} M) with or without hGal(WT) (10^{-7} M) was added to a final volume of 1000 μl and incubated for 60 min at 37°C. The reaction was terminated by addition of 200 μl ice cold 20% perchloric acid followed for 10 min followed by the addition of 1.5 M KOH/75 mM HEPES (pH7). Anion exchange chromatography was performed over 1 cm 50:50 Dowex (AG 1-X8 Resin, 200–400 mesh formate; BioRad, Hercules, CA, USA). The columns were washed with 5 ml distilled water before the IPs were eluted with 5 ml of 0.1 M formic acid/1.2 M ammonium formate. Radioactivity of the eluate was determined using scintillation counting in a β-counter (Tri-Carb Liquid Scintillation Analyzer, model 2500 TR, Packard Instrument Company, Meriden, CT, USA) using Utima Flo AF (Perkin–Elmer Life Science, Boston, MA, USA) as scintillation fluid. Each sample was normalized against the total count obtained before anion exchange chromatography.

Supplementary Material

Supplementary Material is available at *HMG* online.

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