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**LATERAL TEMPORAL EPILEPSY ASSOCIATED
WITH LGI1 MUTATIONS:
A CLINICAL, GENETIC AND FUNCTIONAL STUDY**

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ABSTRACT

Introduction. Mutations in the leucine-rich glioma-inactivated 1 (*LG1*) gene or epitempin cause autosomal dominant lateral temporal epilepsy (ADLTE), an epileptic syndrome characterized by focal seizures with prominent auditory symptoms and benign clinical course. Lgi1 function is not completely defined and it seems to mediate proteins to proteins interactions in synapses. To date, 38 *LG1* mutations have been described and most of them inhibit protein secretion (loss-of-function). In the present study we aimed to better define the clinical phenotype of ADLTE associated with *LG1* mutations and to further investigate the pathogenic mechanisms underlying the syndrome. Particularly we evaluated the functional effect of some identified mutations.

Methods. Families were selected on the basis of an ADLTE diagnosis according to defined diagnostic criteria. Almost all the affected individuals were submitted to clinical assessment, video-electroencephalogram (EEG) monitoring and magnetic resonance imaging (MRI). A positron emission tomography (PET) and a psychiatric assessment by means of validated psychometric scales were obtained in some individuals. Genetic analysis included *LG1* sequencing and multiple ligation-dependant probe amplification (MLPA) assay in patients without point mutations. The expression of Lgi1 mutant proteins was evaluated in cultured cells by a secretion assay. The interaction of extracellular Lgi1 mutated proteins with ADAM 22/23 receptors (a Disintegrin and Metallopeptidase domain family) was investigated by means of both co-transfection and immunofluorescence and co-immuno-precipitation assays.

Results. Four families out of eight identified were included into the study. Three presented missense mutations and one a microdeletion. PET study demonstrated a mild hypermetabolism of the right temporal lobe in patients compared to controls at SPM analysis. Psychiatric assessment provided evidence for a psychiatric diagnosis in most of the patients and for higher level of impulsiveness in patients compared to a control group. Two mutations (R406C; T380A) were showed to not impair completely protein secretion. However they reduced Lgi1 binding to ADAM22/23 receptors.

Discussion and conclusions. We described four families with different *LG1* mutations. In our patients we noticed a prevalence of ictal symptoms different from

auditory auras higher than previously reported in other series. A psychiatric comorbidity was also present and seems to emerge as a new aspect in some ADLTE families. As to genetic aspects we found a microdeletion in one family, confirming the possibility of copy number variations (CNVs) as causative mutations. The functional study demonstrated a pathogenic mechanism different from inhibition of secretion for two mutations suggesting a possible role of the ADAM22/23 receptors in the pathogenesis of this condition.

Key-words: temporal epilepsy, genetic, epitempin

1. INTRODUCTION

1.1 Autosomal Dominant Lateral Temporal Epilepsy (ADLTE)

Autosomal dominant lateral temporal epilepsy (ADLTE), also known as autosomal dominant partial epilepsy with auditory features (ADPEAF), is a rare familial condition characterized by focal seizures with prominent auditory or aphasic symptoms, onset in adolescence or early adulthood, absence of brain structural abnormalities and overall benign outcome [1; 2]. The syndrome segregates with an autosomal dominant pattern with reduced penetrance [3]. Its prevalence is unknown but it may represent about 19% of genetic focal epilepsies [4]. Clinical diagnosis is based on the presence of at least two cases with non symptomatic focal or secondarily generalized seizures whose symptoms suggest a lateral temporal lobe origin. Focal seizures are characterized by auditory auras in about 2/3 of the cases [2]. Auditory symptoms are often described as simple sounds (such as humming, buzzing, ringing); less frequently as complex hallucinations (music, voices) or a distortion of sounds. Aphasic seizures may also sometimes be present in some kindred. Other less frequent auras include complex visual, psychic, autonomic, vertiginous and other sensory symptoms, usually in association with auditory phenomena. Secondary generalized tonic-clonic seizures are frequent particularly during sleep, sometimes causing elementary focal seizures be underestimated [2; 5; 6]. Standard MRI is normal by definition. Interictal electroencephalograms usually show mild lateral temporal abnormalities such as slow/sharp waves in about half of the patients, sometimes with left side prevalence [7; 8]. Genetic studies have revealed mutations in the leucine-rich, glioma inactivated gene (*LG11*) in about 30% of the families providing evidence for a genetic heterogeneity of this condition. Detailed analysis of families with and without *LG11* mutations failed to demonstrate significant phenotypic differences among them [5; 6]. Since its first description, 39 mutated families have been reported worldwide, most of which with unique *LG11* mutations [9]. Very recently a new gene, reelin (*RELN*), has been associated to ADLTE in seven families without *LG11* mutations. Dazzo et al reported *RELN* heterozygous mutations in 17.5% of ADLTE families [10]. Moreover

linkage to chromosome 19q13.11-q13.31 has been reported in a large Brazilian family with ADLTE and migraine [11].

Sporadic, non familial patients with auditory seizure have been reported and named IPEAF (Idiopathic Epilepsy with Auditory Features) [12]. Their clinical picture is almost identical to ADLTE except for their negative family history. De novo *LG11* mutations have been found in three sporadic cases [13; 14; 15].

1.2 LGI 1/Epitempin

The *LGI1* gene is located on chromosome 10q and consists of eight exons. It is mainly expressed in neurons, particularly in the neocortex and limbic regions [16; 17]. It encodes a protein of 557 aminoacids which is secreted into the extracellular compartment and shows no similarities with ion channels [18; 19]. The *Lgi1* protein's structure consists of a signal peptide and two distinct structural domains: the N terminal region contains four leucine-rich repeats (LRR) flanked by conserved cysteine clusters [20], whereas the C-terminal contains seven copies of a repeat named EPTP or epitempin [21], which form a beta-propeller domain [22]. Both domains have been showed to mediate protein to protein interactions [20; 22]. This gene and its product have been associated with different clinical phenotypes. It was first described in 1998 as a putative tumor suppression gene, involved in a chromosomal rearrangement in the T98G malignant glioma cell line. Subsequent studies failed to confirm its role in oncogenesis [23; 24]. In 2002, *LG11* heterozygous mutations were identified to cause ADLTE in several European and American families [16; 17]. To date, a total of 38 *LGI1* mutations have been described: 35 mutations segregate in 39 affected families, whereas three are *de novo* mutations identified in IPEAF patients [13; 15; 17]. Of these mutations, 24 allow single aminoacid substitutions, 13 result in protein truncation due to frameshift deletions or insertions and to non-sense or splice-site mutations. So nearly all *LG11* mutations described are point mutations, either nonsynonymous or splice-site mutations or short indels, distributed along the gene length [25; 26], and two microdeletions, one spanning the first four exons, have been reported [27; 28]. The overall penetrance of *LGI1* mutations is estimated to be around 66% [3] and the proportion of families with penetrance $\geq 75\%$ was similar among missense and

truncation mutations [26]. All but three *LG11* mutations so far reported have been demonstrated to inhibit protein secretion, suggesting a loss of function effect [25; 29; 30]. A three-dimensional *in silico* model of the two epitempin domains was built to predict possible structure/function relationship. It identifies a possible protein binding site in the beta propeller domain and another in the LRR domain. This functional model indicates that Lgi1 may mediate the interactions between proteins to different synaptic sites. It may also be used to predict the effects of identified mutations on protein folding or map them to functional surface regions [31].

Epitempin shows no homology with known ion channels. Its function and the mechanism by which it causes epilepsy remain still unclear. Three main functions have been proposed in the central nervous system:

- 1) Inhibition of inactivation of the presynaptic voltage-gated potassium channel Kv1.1 [31];
- 2) Potentiation of AMPA receptors-mediated synaptic transmission in the hippocampus through interaction with the transmembrane receptors ADAM22 and ADAM23 (a Disintegrin and Metalloproteinase domain family) [33; 34];
- 3) Postnatal maturation of glutamatergic synapses, regulation of spine density, and dendritic pruning [35].

Lgi1 is associated to a postsynaptic complex containing PSD95 and ADAM 22. It was shown to participate through this interaction in the regulation of synaptic strength at excitatory synapses [36]. Moreover it binds the presynaptic receptor ADAM23 to stimulate neurite outgrowth *in vitro* and dendritic arborisation *in vivo* and may act as a trans-synaptic protein connecting the pre-synaptic ADAM23 with the post-synaptic ADAM22 receptors [19; 37]. It has been shown that the ligand-receptor complexes between Lgi1 and ADAM22/23 regulate AMPA receptor-mediated synaptic transmission in the hippocampus, suggesting a possible molecular mechanism underlying ADLTE [19]. This mechanism is further supported by recent findings showing that Lgi1 antibodies neutralize the specific protein-protein interaction between Lgi1 and ADAM22/23 and this disruption reduces AMPA receptors in rat hippocampal neurons [34]. Remarkably, Lgi1-knockout (KO) mice present spontaneous seizures [19;

38; 39]. Homozygous Lgi1 KO mice have spontaneous seizures at postnatal day 10 and die before the end of the third postnatal week, while heterozygous Lgi1 +/- mice exhibit increased susceptibility to sound-induced or pentylenetetrazole-induced seizures [19; 37].

The pivotal role of LGI1 in epileptic disorders has been recently expanded by the finding of autoantibodies against Lgi1 in the sera of patients with limbic encephalitis (LE). LE is an autoimmune neurological disorder characterized by amnesia, confusion, seizures involving mostly the temporal lobes or faciobrachial dystonic motor seizures, and personality changes or psychosis [40; 41]. It belongs to a group of autoimmune synaptic encephalopathies, in which the patients develop antibodies against synaptic proteins, and usually is not associated with tumors [42]. Both temporal lobe and motor cortex seem to be sequentially involved in this disorder [43].

2. AIM OF THE STUDY

The present study aims to better define the clinical phenotype of ADLTE associated with LGI1 mutations and to further investigate the pathogenic mechanism underlying this epileptic condition. Therefore the aims of our research were:

- i) To identify ADLTE families among patients followed or coming to first evaluation for epileptic disorders at our Epilepsy Center;
- ii) To characterize phenotypically the probands and their relatives (electroencephalograms, MRI, PET) and to evaluate possible psychiatric comorbidities;
- iii) To ascertain the presence of LGI1 mutations in the ADLTE families identified;
- iv) To study the effect of the identified mutations on protein secretion and its interactions with ADAM 22/23 by means of *in vitro* functional tests.

Genetic and functional studies have been conducted in collaboration with the Laboratory directed by doctor Carlo Nobile at the Institute of Neuroscience-CNR-Section of Padua. PET study has been conducted in collaboration with doctor Sabina Pappatà at the Institute of Biostructure and Bioimaging-CNR-Section of Naples.

3.MATERIALS AND METHODS

3.1 Families

Probands were identified among outpatients attending the Epilepsy Tertiary Center at the University Federico II in Naples during a five years period. Families were classified as possible affected with ADLTE according to the following criteria: two or more affected members with a history of unprovoked seizures that in at least one of them are focal and characterized by auditory, aphasic, or visual symptoms highly suggestive of a lateral temporal onset. The absence of structural brain abnormalities and an autosomal pattern of inheritance were also requested. Each proband and almost all affected individuals were interviewed directly and examined by a skilled epileptologist. The clinical interview included personal and family history, as well as details concerning the following features: age at onset, description of ictal semeiology, verbatim of auras, seizure frequency and response to the treatment, possible risk factors. A physical and neurologic examination was also obtained. Seizure types were classified according to the Partial Seizure Symptom Definitions [44]. Video-EEG monitoring both in wakefulness and during afternoon nap and magnetic resonance imaging (MRI) were obtained in almost all affected members.

3.2 PET study

Interictal brain metabolism was studied in some affected subjects by means of 18-fluorodeoxyglucose positron emission tomography (18FDG-PET).

FDG-PET. Subjects fasted for at least 6 hours. Before radiopharmaceutical injection, blood glucose was checked and was <140 mg/dL in all cases. After a few minutes (min) rest in a silent and obscured room, patients and controls were injected with a dose of 185–250 MBq of 18F fluorodeoxyglucose in resting state and eyes closed conditions, and remained in a dimly lit room with minimal background noise for about 40 minutes. PET acquisition started between 45 and 60 minutes after injection and lasted 15 minutes. Brain images were acquired in 3-dimensional mode using a whole body PET/CT scanner (Discovery LS, GE Medical System) with an axial field of view of 15.2 cm, yielding 35 slices of 4.25 mm thickness and an axial and transaxial resolution

(full width at half maximum [FWHM]) of 4.7 and 4.8 mm. Images were reconstructed with iterative reconstruction (FORE-Iterative) and corrected for attenuation using the CT scan. FDG-PET images were converted to Analyze format and exported for data analysis using a voxel-based methods. Images were spatially normalized into the Montreal Neurological Institute (MNI) space using the PET imaging–derived template and the default spatial normalization settings (affine transformation with nonlinear components, voxel size of 2 x2 _x2 mm) of SPM2 (Wellcome Department of Cognitive Neurology, London, UK; <http://www.fil.ion.ucl.ac.uk/spm>).

Data analysis. Visual and statistical analyses were performed in patients with ADLTE. For statistical voxel-based analysis performed using SPM2, spatially normalized FDG-PET images were smoothed with a gaussian filter of 10 mm FWHM. A single-subject conditions (controls, ADLTE) and covariates model of SPM2 were applied, using global normalization. Differences among patients and controls (n=12; age range: 35-64 years) were set at a threshold of $p < 0.001$ uncorrected for peak height and $p < 0.05$ corrected at cluster level. Both the hypotheses patients<controls and patients>controls were explored.

3.3 Psychiatric assessment

A psychiatric interview and a complete assessment by means of validated psychometric scales (SCID I and II, TAS-20, BDI-II, STAY-Y, BIS-11) were obtained in family members who gave their consent.

Specifically trained psychiatric interviewers administered the research version of the Structured Clinical Interview for DSM-IV (SCID). The Structured Clinical Interviews for DSM-IV Axis I Disorders (SCID-I) [45] and Axis II Disorders (SCID-II) [46] are diagnostic semi-structured interviews used to determine both DSM-IV Axis I disorders (major mental disorders) and DSM-IV Axis II disorders (personality disorders). Structured psychiatric interviews are proven to specifically contribute to a better evaluation of the true prevalence of psychiatric comorbidities in temporal lobe epilepsy [47] and chronic epileptic patients [48]. SCID-II has been previously used in epileptic patients to define personality disorders [49]. The Toronto Alexithymia Scale (TAS-20) is a self-report 20 items commonly used to measure alexithymia [50]. It has been

validated in a sample from Italy [51] and it has also been used for psycho-pathological assessment of epileptic patients [52]. The Barratt Impulsiveness Scale (BIS-11) is a 30 items questionnaire designed to assess the personality/behavioral construct of impulsiveness and divided into three subscales [53]. It has an overall score ranging from 30 to 120 points and its values show a normal distribution in the general population [54]. A validated Italian version of BIS-11 is available [55]. Moreover it was used to investigate the impulsive dimension among temporal lobe epileptic patients [56]. According to the literature, we decided to classify patients as high impulsive for BIS total score ≥ 72 and mild impulsive for values between 63 and 71. However no standardized cut-off score has been previously stated for this test. So the overall scores in these patients were compared to a control group matching sex and age distribution (psychiatric diagnosis or drug abuse in anamnesis were considered exclusion criteria). Data were described using means and standard deviation (SD) and the scores compared by *t-test*. The software Stata/IC 11.1 was used for the analysis. The Beck Depression Inventory (BDI-II) is a 21-question multiple-choice self-report inventory to explore main symptoms of depression and is also considered a useful screening tool in epileptic patients [57]. The State-Trait Anxiety Inventory (STAI) is a commonly used measure of trait and state anxiety and has already been used in temporal lobe epileptic patients to assess levels of anxiety [58; 59].

3.3 Genetic analysis

Blood samples were collected from the proband and other family members after signing the informed consent. DNA was extracted by a standard phenol method and LGI1 exon analysis was performed by Sanger sequencing. Exons were PCR amplified (conditions described in Michelucci et al 2003) and sequenced by the Big Dye Terminator Cycle Sequencing kit (ABI PRISM, Applied Biosystems) and an ABI3730 automated sequencer. Predictions of pathogenicity of LGI1 point mutations were made with Polymorphism Phenotyping 2 (PolyPhen-2; <http://genetics.bwh.harvard.edu/>) and Sorting Intolerant from Tolerant (SIFT; <http://sift.jcvi.org/>) programs. In probands with no LGI1 point mutations identified by standard methods, the number of copies of each LGI1 exon was analyzed by multiplex ligation-dependent analysis (MLPA). It was

performed using the commercial kit SALSA MLPA P408 ADLTE-LGI1 probemix (MRC-Holland, Amsterdam, The Netherlands) and following the manufacturer's instructions. MLPA data were analyzed with the Coffalyser NET software (MRC-Holland). Ratios of 1.0 were regarded as normal copy number, and ratios below 0.7 or above 1.3 were considered as deletion or duplication, respectively. Validation of the results were performed by real time quantitative (q)PCR. Type-it CNV SYBR Green PCR kit (Qiagen, Hilden, Germany) was used for relative quantification of exon copy number. qPCR was performed on 7500 real time PCR system (Applied Biosystems-Life Technologies). qPCR reactions were carried out with 10 ng of template DNA, 17.5 μ M of each primer, 2 \times Type-it SYBR Green PCR Master Mix in a final reaction volume of 25 μ l. Standard curves were created by four serial dilutions from 50 ng to 1.8 ng of C1 calibrator genomic DNA and run in triplicate.

3.4 Functional studies

3.4.1 Secretion assay

Human embryonic kidney 293T (HEK 293T) cells were transfected with expression constructs containing the wild type and mutated LGI1 cDNAs using Lipofectamine 2000 (Life Technology, Grand Island, NY, USA), following the manufacturer instructions. Twenty-four hours after transfection, cells were washed twice and then refed with serum-free medium Opti-MEM (Life Technology, Grand Island, NY, USA). After 16-20 hours, cells were lysed in Triton lysis buffer (25mM Tris pH 7.4, 150 mM NaCl, 1% (vol/vol) Triton, 10% (vol/vol) Glycerol, 1mM EDTA) supplemented with proteases and phosphatase inhibitors. The medium was collected and centrifuged to pellet cell debris, and the supernatant was concentrated about 20x using Centricon YM30 concentrators (Merk-Millipore, Billerica, MA, USA). Cell lysates and concentrated media were separated on 5-15% SDS-polyacrylamide gels and then electroblotted onto nitrocellulose membrane. They were incubated with primary antibodies in tris buffered saline (TBS) containing 2% (vol/vol) skimmed milk for 2 hours. The proteins immunostained with anti-Lgi1 antibody were detected with a horseradish

peroxidase-labelled secondary antibody and enhanced chemiluminescence (ECL) reagent and visualized by autoradiography.

3.4.2 Co-immunofluorescence assay

The assay was performed to evaluate if the mutations R407C and T380A affect the interactions of extracellular Lgi1 with ADAM 22/23 receptors. COS7 cells were co-transfected with wild-type or mutant *LGI1*-Flag and HA-tagged *ADAM22* or *ADAM23*cDNAs overexpressed (3 ug of total DNA). Thirty-six hours after transfection, cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 10 min and blocked with PBS containing 2 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich) for 40 min. Fixed cells were stained with anti-Flag antibody, followed by Cy3-conjugated secondary antibody. Then, the cells were permeabilized with 0.1% Triton X-100 for 10 min, blocked with PBS containing 2mg/ml BSA and stained with anti-HA monoclonal antibody, followed by Alexa-Fluor 488-conjugated secondary antibody. All the antibodies were diluted in 1% BSA/PBS; this was followed by washes with PBS. Finally, they were fixed with mounting medium containing DAPI (Vectashield; Vector Laboratories). Two coverslips were made for every transfection experiment, and twenty random fields were taken (magnification 400X). For every field the number of co-immunostained cells (Lgi1 and ADAM 22 or ADAM 23) was counted and the percentage of cells with both signals on the cell surface was estimated. In total, three independent experiments were performed. For cell counting, slides were analyzed using a Leica-DM 5000B Epifluorescence microscope. Confocal images were acquired with a Radiance 2000 confocal microscope (BioRad). The GraphPad software program (<http://www.graphpad.com/quickcalcs/>) was used to calculate Chi-square and the two-tailed P-value to compare the frequency of membrane-bound Lgi1 proteins between cells carrying the wild type Lgi1 protein and cells expressing mutated Lgi1 proteins. The same procedures were performed using COS7 cells co-transfected with wild-type *LGI1*-Flag and HA-tagged *ADAM22* in presence of human serum (HS) of a patient diagnosed as affected with limbic encephalitis. The number of co-immunostained cells (Lgi1 and ADAM 22) was counted and the percentage of cells with both signals on the

cell surface was estimated in the presence respectively of 2.5%, 5%, 7.5 % and 10% amount of human serum.

3.4.3 Co-immunoprecipitation assay

In order to confirm the findings of the COS7 cell co-transfection and immunofluorescence experiments a co-immunoprecipitation assay was performed. Lgi1-Flag wild-type or clinical mutants were co-transfected with HA-ADAM22 or HA-ADAM23 into HEK293T cells seeded on a six wells plate. Thirty-six hours after transfection, cells were lysed in 200 μ l of lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium β -glycerophosphate, 5 mM sodium pyrophosphate, 0.27 M sucrose and 1% (w/v) Tween 20 in the presence of a protease inhibitor cocktail (Sigma Aldrich) and incubated on ice for 20 minutes. Lysates were subsequently clarified by centrifugation at 14000 g for 20 minutes and 0.5 mg of total proteins in 500 μ l of volume were incubated with 0.5 μ g of mouse monoclonal anti-HA antibody (Roche). After 2 hours of incubation at 4°C with gentle rocking, 20 μ l of Protein G sepharose (GE Healthcare Life Sciences) were supplemented and incubated 1 hour at 4°C. After 5 washes in washing buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium β -glycerophosphate, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1% (w/v) tween 20 and 250 mM NaCl), proteins were eluted in 2x sample buffer (Invitrogen), separated in NuPAGE 4-12% (Life Technology) and electro-blotted on polyvinylidene fluoride membrane (PVDF) (Merk-Millipore). PVDF membranes were saturated with 10% skimmed milk in TBS supplemented with 0.05% tween 20 and proteins detected using primary anti-HA and anti-Lgi1 antibodies and secondary antibodies conjugated with horseradish peroxidase.

4. RESULTS

4.1 Clinical findings

According to the previously stated diagnostic criteria, we identified eight ADLTE families among patients followed or coming to first evaluation for epileptic disorders at the Epilepsy Centre of the University of Naples Federico II during the years 2010 to 2015. Five families presented mutations in *LG11*; two were negative and one proband refused consent to genetic analysis. One of the mutated families was lost to follow up and clinical and genetic data were previously described by our group [27]. We have described some clinical and genetic aspects for families I and II in two papers [28; 29]. Finally four families were included into the study. The families' trees are represented in figures from 1 to 4 A. The clinical, electrophysiological and neuroimaging data are summarized in table 1.

Overall there were 12 subjects (1 deceased) with epilepsy belonging to four families. The age of seizure onset ranged from 9 to 65 years, with a mean of 26.4 years. In most cases the diagnosis began in the adolescence or in the young childhood. Auditory auras isolated or in association were reported in 5/11 patients (45%). One patient reported seizures induced by external sounds (alarms, fireworks). Non auditory auras occurring in isolation and consisting of visual hallucinations, aphasia, déjà-vu and vertigo were found in 5/11 patients (45%). The auditory symptoms were reported as elementary or unformed sounds (whistles, buzzing) in four patients. Almost all the patients (9/11) presented rare generalized tonic clonic seizures (GTCS) during sleep; one patient presented generalized seizures alone. Clinical course is benign in almost all the patients and particularly in some of them who were seizures free after drug discontinuation (V:2 family I, II:5 family III) or never needed antiepileptic treatment. The EEGs showed mild abnormalities over the temporal regions in 5/11 patients, involving the left side in 4 of them (80%) [figure 5]. In patient V:2 (family I) an ictal EEG was obtained and showed focal fast rhythmic activities over the left anterior fronto-temporal areas during a brief episode characterized by vertigo, déjà-vu, fear and pallor. Brain MRI was available for all the patients and showed no abnormalities. Diagnosis was uncertain for the patient II:3 in family IV, since she did

not accept clinical evaluation. The subject III:1 in family IV presented febrile seizures in the childhood but he had no mutation in LGI1. Common migraine was reported by one patient in family IV.

4.2 PET findings

¹⁸F-FDG-PET scans showed mild asymmetric metabolism in the lateral temporal or parietal cortex ($p < 0.05$) in 3/4 patients on a visual evaluation (figure 6 A). A voxel-based statistical analysis performed with SPM showed a relative metabolic reduction at the level of the right superior frontal gyrus (Brodmann's area (BA) 6) (24x-2y-70z) and left middle frontal gyrus (BA6) (-30x 8y 56z) [figure 6B]. No significant hypometabolism was evident in other regions including the temporal cortex. In contrast the patients group showed a relative increase of glucose metabolism in the right middle temporal gyrus (BA21) (50x-28y-10z) as compared to controls (figure 6B).

4.3 Psychiatric findings

The psychiatric interview and the administration of the previously described scales were completed in 7/11 patients from three different families, who gave their consent. Analysis of the data showed a high prevalence of psychiatric disturbances. All the patients but one presented at least one major psychiatric disturbance or a personality disorder diagnosed according to DSM IV. Borderline personality disorder was the most frequent finding (4/7). An eating disorder not otherwise specified was found in all the family III members. Moreover 5/7 patients showed high or mild elevation at the scale assessing impulsivity. BIS-11 scores were significantly higher in ADLTE patients than in healthy controls ($p = 0.021$, *t-test*) (table 2, figure 7). We also found high levels of anxiety (evaluated by State-Trait Anxiety Inventory) in 5/7 patients. Depression symptoms instead were quite infrequent. Only two patients showed alexithymia. Detailed results of psychiatric evaluations are summarized in table 3.

4.4 Genetic results

Sequencing of *LG11* exons revealed mutations in the proband of families I, III and IV. In **family I** a heterozygous c.1219C>T mutation in exon 8 (figure 1B), causing an arginine to cysteine substitution at position 407 of the protein sequence (Arg407Cys), was found in the proband, in patients IV:4 and III:4 and in one asymptomatic individual (IV:9). The mutation was not found in other seven family members (figure 1) and in 225 unrelated healthy controls of Italian ancestry (50 from the same geographic region of the study family). The Arg407 residue is evolutionarily conserved in many species; its substitution with cysteine is predicted to have deleterious effects (Poly Phen score 2.031). In **family III** the sequencing of *LG11* revealed a novel heterozygous c.1138A>G mutation, giving rise to an arginine to threonine substitution at position 380 (T380A), in the proband and in three other affected subjects (figure 3B). The threonine 380 residue is highly conserved in many species and the mutation occurs in the fourth repeat of the EPTP domain. It was not found in 225 unrelated healthy controls and is predicted as “potentially damaging” by PoliPhen2 (score 0.997). Both mutations were predicted to exert a dangerous effect on protein structure by an *in silico* model of *LG11* functional domains. They seem to modify a side chain of the protein *Lgi1* that could be crucial to form a circular conserved region which has been hypothesized to be the main interaction interface of *Lgi* family proteins [32]. In **family IV** the sequencing of *LG11* discovered a heterozygous three base-pair deletion, c.377-379delACA, within exon 4, in the proband (III:2) and in her father. No mutation was identified in her brother (III:1), affected with febrile seizures during the childhood. The mutation results in the in frame deletion of an asparagine residue, p. Asn126del, located in the second LRR. The same mutation was reported in a French family and showed to inhibit protein secretion [60]. In **family II** MLPA identified a novel microdeletion spanning exon 2 in the proband and in his affected father (figure 2 B). The microdeletion was validated by real time qPCR. Deletion of *LG11* exon 2 does not alter the translation frame and, therefore, results in a protein lacking the first LRR repeat. Although it is less conserved than LRR repeats 2—4, the first LRR repeat of the *Lgi1* protein is important for the stability and interaction properties of the LRR domain, and, therefore, its complete deletion likely results in a functionally defective protein [25; 31].

4.5 Functional results

Previous 3D modelling of the Lgi1 protein predicted that some ADLTE-causing, non synonymous, mutations in the C-terminal EPTP domain have no apparent effect in protein folding but are crucial for proteins interactions mediated by EPTP domain [31]. In agreement with this hypothesis, we evaluated the effects of the identified LGI1 mutations, R407C and T380A, both affecting residues at the C-terminal EPTP domains, on the secretion of Lgi1 by means of a secretion test assay. Both mutant proteins as well as the wild-type protein were detected in the media of transfected cells and, though in variable amounts, in the cell lysates (figure 8). However we consistently observed in several experiments a lower amount of the Lgi1 T380A mutant protein in the medium than in the cell lysate, suggesting that the secretion of this mutant protein was only partially hampered. Thus, according to this qualitative secretion assay, the T380A and R407C mutations do not inhibit completely secretion of the Lgi1 protein.

To investigate whether these mutations affect the interaction of extracellular Lgi1 protein with ADAM22/23 receptors, we overexpressed the wild type and mutant *LG11*-Flag cDNAs together with HA-fused *ADAM22* or *ADAM23* constructs in COS7 cells and anti-HA antibodies and a double-immunofluorescence analysis was carried out using confocal imaging. As shown in figure 9 and table 4, we observed that the wild-type Lgi1 protein, when co-expressed with either ADAM22 or ADAM23, mostly co-localized with either receptor on the cell surface, whereas mutant Lgi1 proteins failed to interact or poorly interacted with ADAM22/23 receptors. Overall, the percentage of Lgi1 mutant proteins bound to either ADAM receptor on the cell surface (0-33%) was significantly lower than that of wild-type Lgi1 (76-88%). In order to demonstrate that the binding of the two proteins happens in the extracellular space, the percentage of Lgi1 wild-type protein bound to ADAM22 on cell surface was also evaluated in the presence of different concentrations of human serum of a patient with limbic encephalitis containing antibodies against Lgi1. These antibodies were demonstrated to bind Lgi1 on the cell surface [42]. As shown in table 5, the increasing of human serum caused a significant reduction of Lgi1 associated to ADAM22 since the serum antibodies competitively prevent the binding.

To confirm co-immunofluorescence findings, a co-immunoprecipitation assay from HEK293T cells overexpressing HA-tagged ADAM22 or HA-ADAM23 and Lgi1-Flag was performed. As shown in Fig. 8, HA-ADAM22, which is present in both immature (110 kDa) and mature (90 kDa) forms as previously observed, efficiently co-precipitated wild-type Lgi1. Instead, both Lgi1 described mutations affected Lgi1-ADAM22 interactions: the T380A mutation completely disrupted the interaction, whereas R407C mutation reduced the affinity for ADAM22. We also assessed the effect of these mutations on Lgi1-ADAM23 interaction using co-immunoprecipitation. The experiment showed that only wild-type Lgi1 was efficiently immunoprecipitated by ADAM23, consistent with the results obtained with ADAM22, while pathological Lgi1 mutations displayed reduced affinity for ADAM23. However, the transfection efficiency of HA-ADAM23 in the presence of LGI1 was low.

5. DISCUSSION

5.1 Clinical and instrumental findings

We identified eight families diagnosed as possible affected with ADLTE according to the diagnostic criteria described in the methods section. These criteria are quite different from those usually reported in the literature, where auditory or aphasic auras are considered mandatory for the diagnosis [6]. We decided for less strict criteria based on the hypothesis that ADLTE associated to *LG11* mutations might not be limited to auditory auras but includes a subset of ictal symptoms probably originating from the temporal lateral cortex. Mutations in *LG11* were identified in five families and four were included into the study. Thus we found a proportion of ADLTE mutated families higher than reported in the literature (around 30%). This may be merely a causality or related to the families geographic origin. We might also suppose that the prevalence of *LG11* mutations is underestimated if we consider families with only auditory features. Age at onset and clinical course are similar to those described in other series. We also observed some patients with very mild and underdiagnosed clinical manifestations and no need for antiepileptic treatment. In our families we found a higher prevalence of ictal symptoms different from auditory auras, such as visual or psychic. Families with aura features different from auditory symptoms have been described, including a kindred in which the majority of the patients had only visual auras [61]. So our observation supports the notion that, in addition to auditory symptoms, which are prominent in most families, other type of auras may be found in ADLTE, sometimes occurring in isolation. Tonic clinic generalized seizures occurred in the majority of cases as expected in epilepsies with lateral temporal origin [62]. In agreement with what was previously suggested [7; 8] we found a left predominance of the EEG abnormalities. Other ADLTE series did not confirm this finding.

PET is a very sensitive technique for lateralization and general localization of the epileptogenic zone in pharmaco-resistant epilepsy even when MRI and EEGs are not contributive [63; 64]. To date, only two PET studies have been reported in two patients from different Japanese families [65; 66]. They found a decreased glucose metabolism in the left temporal area in two patients who also showed a small volume of the left

superior temporal gyrus at 1.5 tesla MR brain imaging. An interictal SPECT in one patient was reported by Poza et al and showed a hypoperfusion of the left temporal lobe [61]. PET study was performed only in a small subgroup of patients. We found mild asymmetric metabolism in the lateral temporal or parietal cortex (sn<dx) at visual evaluation in 3/4 patients and no abnormalities in the patient who was seizure free from almost 10 years. A SPM voxel-based statistical analysis comparing ADLTE subjects and controls demonstrated a significant relative decrease of glucose metabolism in the left superior frontal gyrus and right middle frontal gyrus but failed to detect significant hypometabolism in the left temporal cortex despite the slight asymmetry observed in the PET-FDG images of 3 patients. The small number of patients could in part explain this last finding. It is interesting to note, however, that we found a significant relative metabolic increase in the right temporal cortex. We might argue that a very mild congenital dysfunction of the left temporal cortex, resulting only in very mild hypometabolism, might in turn induce a compensative hyperfunction of the right side as previously reported in mesial temporal lobe epilepsy [67]. In this respect some authors reported possible dysfunction of the left temporal cortex explored by long-latency auditory evoked potentials or neuropsychological tests specific for auditory processing [7; 8]. No certain clinic correlation may be proposed for the relative hypometabolism in the rostral premotor regions (BA6). Interestingly an involvement of motor cortex has recently been demonstrated in limbic encephalitis associated with anti-Lgi1 antibodies [43]. These regions might be implicated in motor but also in non motor mental-operation tasks [68]. Further neuropsychological and PET-FDG studies in a larger number of patients are required to confirm our preliminary results. Overall the evidence of only mild PET alterations in our patients seems to be in agreement with the clinical benign course of the syndrome and do not support the hypotheses of underlying focal developmental abnormalities of potential epileptogenic significance. Tessa et al previously reported a cluster of increased functional anisotropy at voxel based analysis of diffusion tensor imaging in the left middle temporal gyrus of 8 ADLTE patients [69].

Finally, the psychiatric evaluation demonstrated a high prevalence of psychiatric disturbances diagnosed by means of validated psychometric scales. Psychiatric comorbidities are often reported in epileptic patients [47, 49] but they have rarely been

found in ADLTE families. Heiman and colleagues recently evaluated depression risk in *LG11* mutation carriers and found that only the ones who were clinically affected with epilepsy showed an increased risk [70]. However depression was not a major finding in our patients. A high risk prevalence of hyperactive behavior has been recently reported in a Dutch family [71]. Psychiatric symptoms, such as explosive violent behaviors and panic-attack-like symptoms, have been described in a large Japanese ADLTE kindred carrying the *LG11* c.1418C>T (S473L) mutation [65]. We also noticed depression and anxiety symptoms in some members of a family we previously described [27]. These observations lead to the suggestion that psychiatric symptoms may be part of the constellation of *LG11*-related clinical manifestations. Three of the families described in this study exhibit psychiatric symptoms, lending support to this hypothesis. Psychiatric aspects look quite homogeneous and typical in the different families. Both DSM IV diagnosis (eating and bipolar disorders, borderline personality disorder) and high scores at the BIS-11 refer to the impulse control spectrum. A role of *Lgi1* dysfunction in these disorders is also supported by the observation of psychiatric manifestations in limbic encephalitis. The effect of glutamatergic transmission might be a final common pathway [72].

5.2 Genetic and functional studies

Sequencing of *LG11* exons revealed three missense mutations in families I, III and IV. The first two mutations were predicted to possibly impair protein to protein interactions by an *in silico* *Lgi1* model [31]. The mutation identified in family IV, a three base-pair deletion, c.377-379delACA, and its effect on protein secretion had been previously described in a French family [60]. However despite the French family, in our patients we do not find visual symptoms and only one of them suffered from migraine without aura. This is against the authors' hypothesis of a possible link between migraine-like episodes and ADLTE in some families. In family II MLPA identified a microdeletion spanning exon 2 in the proband and in his affected father. MLPA is the method of choice for screening large cohorts of patients for microrearrangements in specific genes. Two recent studies utilized this method to screen ADLTE and sporadic

LTE patients, and no microdeletions or microduplications were found in the *LG1* gene [73; 74]. Although these studies were performed on limited series of patients, it was concluded that *LG1* microdeletions are not a frequent cause of ADLTE. In our opinion, the use of MLPA as a diagnostic tool to detect causative *LG1* microdeletions should be considered, since we found two microdeletions in two out of 12 families from our Epilepsy Center that were screened at the Biology Laboratory of Padua [27; 28]. The overall ratio (5:1) between point mutations and gene rearrangements observed in the Italian cohort of ADLTE kindreds [6, this study] is in line with that found in other genes for Mendelian disorders [75]. Thus, MLPA analysis of *LG1* exons should be included in a molecular diagnostic protocol to detect possible disease-causing copy number variations in ADLTE families without sequence-based mutations. As to the functional study we have shown that the two identified mutations, R407C and T380A, affecting amino acids of the C-terminal EPTP domain, allow secretion of the mutant protein, and that these mutations reduce affinity of Lgi1 for ADAM22 and ADAM23 neuronal receptors. Since the first demonstration in 2005 [18], inhibition of secretion has been considered the sole mechanism by which *LG1* mutations cause loss of function. The present work shows that other pathogenic mechanisms should be considered. In fact these mutations seem to allow the protein fold to be maintained while disrupt Lgi1 interactions with other proteins. Such a decoupling is a strong indication for the functional relevance of the conserved surface residues forming a ring on the EPTP top surface. Indeed, it suggests the possibility that these residues are directly responsible for the Lgi1-ADAM22/23 interactions. The electrostatic surface between EPTP and ADAM22 is largely complementary, causing an attraction reinforced by steric complementarity. Any perturbation on the conserved EPTP top surface residues, or mutation of the ADAM22 D509 residue [33] would therefore weaken the interaction or even abolish it entirely. For mutations inhibiting secretion haploinsufficiency may also result from intracellular degradation of misfolded mutant proteins through protein quality-control mechanisms [76]. This hypothesis also provides a possible explanation for the variable amount of mutant proteins we observed in cell culture media. Particularly, the lower amount of the Lgi1-T380A protein in the medium compared to that seen in cell lysate might be due to a partial effect of this mutation on protein

folding. So a proportion of these proteins may be degraded and the other one secreted. Several studies have shown that the secreted Lgi1 protein binds to ADAM22 and ADAM23 receptors on the surface of neuronal cells and that these protein complexes exert various functions during neuronal maturation and synaptic transmission [33; 37]. Although their functions in the CNS are not clearly understood, the involvement of both *ADAM22* and *ADAM23* in epilepsy is suggested by studies of knock-out mice, showing that lack of expression of either of these genes results in spontaneous seizures [77]. Therefore, it has been suggested that the ligand-receptor complexes between Lgi1 and ADAM22/ADAM23 may be part of the molecular substrate underlying ADLTE [19]. So these results confirm the specificity of the binding of Lgi1 to ADAM22 and ADAM23, provide a pathogenic mechanism for *LG11* mutations different from the loss of the ability to be secreted, and strongly suggest a role for both ADAM22 and ADAM23 in the molecular mechanisms underlying ADLTE. Genetic disorders that are caused by defects in a ligand for a particular receptor are frequently mirrored by disorders in which there is a dysfunction of that receptor. However, a causative role for ADAM22 and ADAM23 receptors in ADLTE was widely evaluated in genetic studies of families without *LG11* mutations. The direct sequencing of *ADAM22* exons did not demonstrate any disease-causing mutations and linkage analysis with microsatellite markers within or near the *ADAM23* gene failed to reveal any significant linkage peak [78; 79]. The absence of causative mutations in *ADAM22* and *ADAM23*, however, does not exclude their involvement in the molecular pathway underlying ADLTE, and many explanations are possible. *ADAM22/23* genes may have important regulatory roles in CNS development and, therefore, a reduction of their expression levels due to inherited mutations could be detrimental for brain development in humans. It is also possible that mutations in these genes have not been detected in the limited set of ADLTE families tested because they occur at low frequency, a hypothesis supported by recent studies suggesting a relatively high genetic heterogeneity in ADLTE families free of *LG11* mutations [6]. Finally, *LG11*-associated ADLTE may reflect a partial loss of function of the Lgi1 ligand at both receptor proteins ADAM22 and ADAM23, whereas heterozygous mutations in only one of these receptors may not be sufficient to cause the syndrome. The importance of the ligand-receptor interaction between Lgi1 and

ADAM22/23 in epileptic disorders is also supported by the observations in limbic encephalitis. Lgi1 autoantibodies present in the patients' sera specifically impair this binding [34]. Reduced binding of Lgi1 to ADAM22/23 may therefore be a pathogenic mechanism for both genetically inherited and acquired epilepsy disorders. Our data are also partially confirmed by Yokoi et al in a recent study [30]. They identified two more mutations (S473L and R474Q) not impairing protein secretion among eight mutations still uncharacterized described in the literature. Then they examined the biochemical and histochemical behavior of Lgi1 in the brain tissue of mutant mice carrying respectively a secretion competent (S473L) and a secretion defective protein (E383A). They demonstrated in a tissue specific model that secretion defective proteins are misfolded and enter the ER-associated degradation pathway whereas secretion competent ones present a weaker binding to ADAM22. Binding to ADAM23 seemed not to be compromised in disagreement with our findings.

6. CONCLUSIONS

ADLTE is an epileptic condition characterized by a wide genetic and phenotypic heterogeneity. In the first observations families were collected according to the presence of partial seizures with precocious auditory symptoms. So the phenotype was as homogenous as possible in order to enhance the possibilities of success of the linkage analysis studies for gene detection. The identification of mutations in the LGI1 gene in a subset of families allowed the observation of ictal symptoms other than auditory auras in affected subjects. This finding is particularly evident in our families, where visual and aphasic auras or vertigo are more frequent than usually reported. In our families we also found a high prevalence of psychiatric comorbidities, particularly impulse control disorders, not previously described elsewhere in a systematic manner. This observation further widens the clinical spectrum of epilepsy associated with LGI1 mutations. It also provide useful tools in the management of the patients, that often show poor compliance to antiepileptic treatments and may often relapse despite an overall benign condition. PET study provided possible interesting functional aspects of the syndrome even if only in a small subgroup of patients. Finally in this work, genetic and functional studies in two families provided evidence for different pathogenic mechanisms and new insights into Lgi1 functions. LGI1 is unique among epilepsy related genes in encoding a secreted protein, whose physiologic functions and role in the epileptogenesis are still poor understood. An essential role for Lgi1-ADAM receptors interactions was demonstrated since some mutations disrupting this binding can cause ADLTE without impairing completely the secretion. The same interaction is disrupted in acquired limbic encephalitis. However a clinical, neuroimaging and genetic study of further families is desirable to better define some emerging aspects of this condition.

7. FIGURES AND TABLES

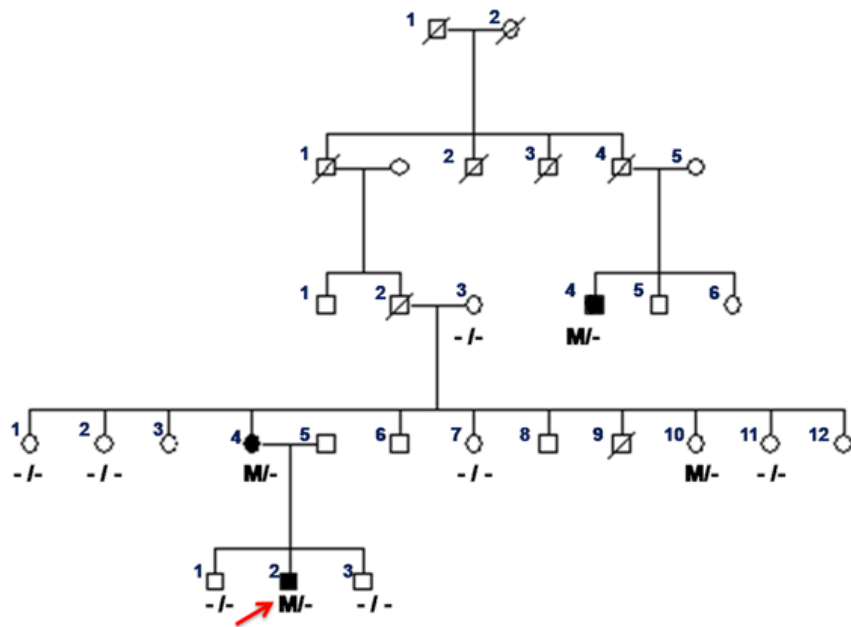
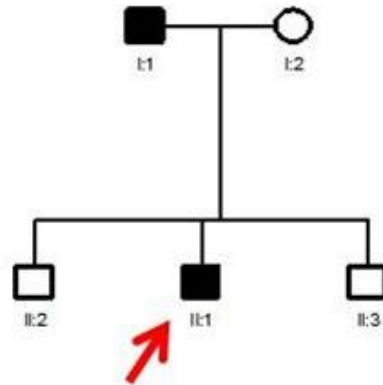


Figure 1. Pedigree of the family I. Individuals carrying one mutant and one normal allele are denoted by M/-, whereas those with no mutations by -/-.

A)



B)

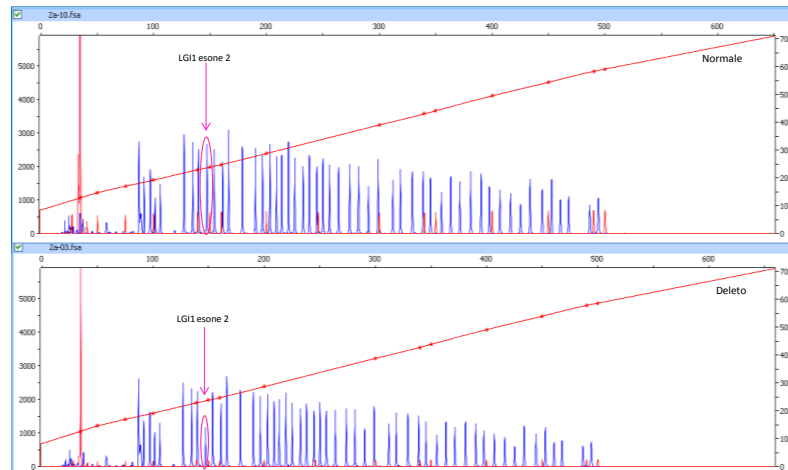
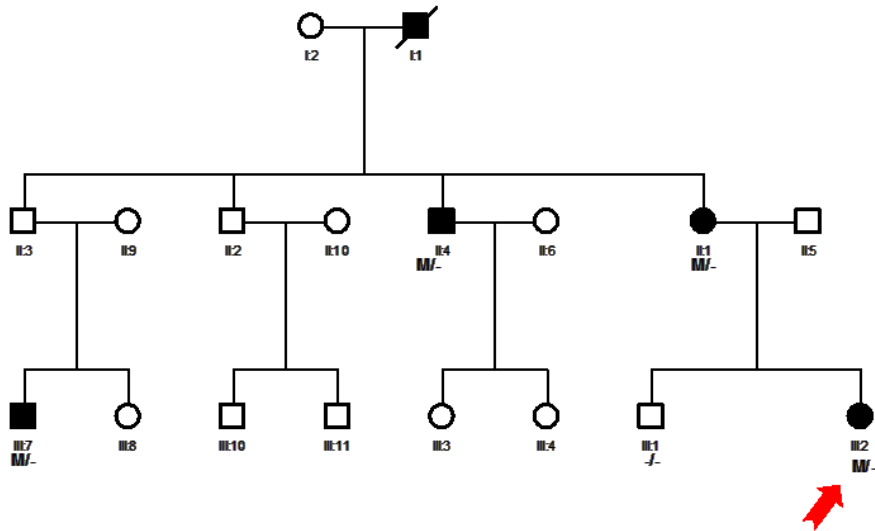


Figure 2. A) Pedigree of the family II. B) Original sequence and MLPA tracings used to detect disease alleles. Mutated exon is indicated by arrows.

A)



B)

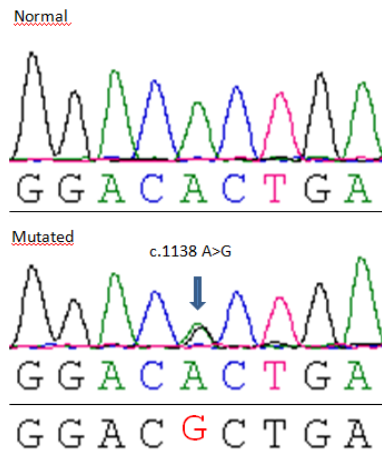
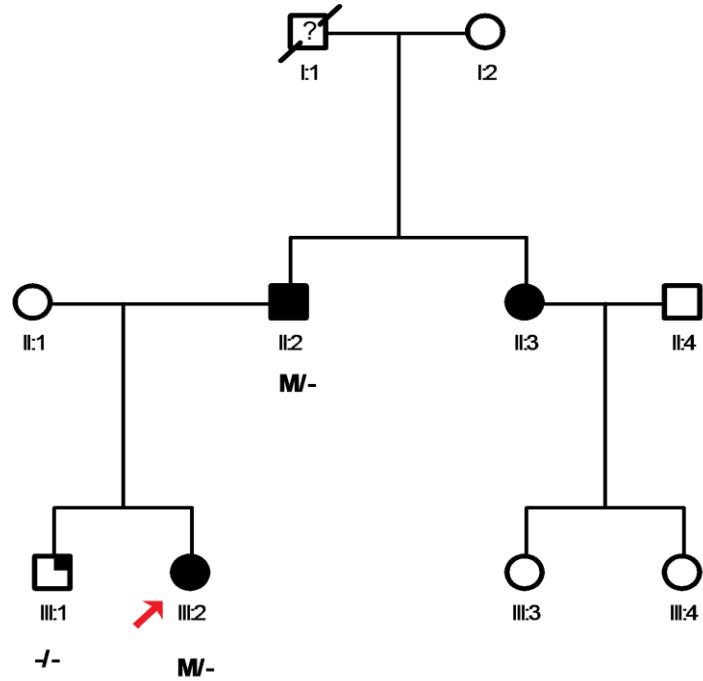


Figure 3. A) Pedigree of the family III. Individuals carrying one mutant and one normal allele are denoted by M/-, whereas those with no mutations by -/-. B) Chromatogram used to detect the disease allele (variant allele denoted by an arrow).

A)



B)

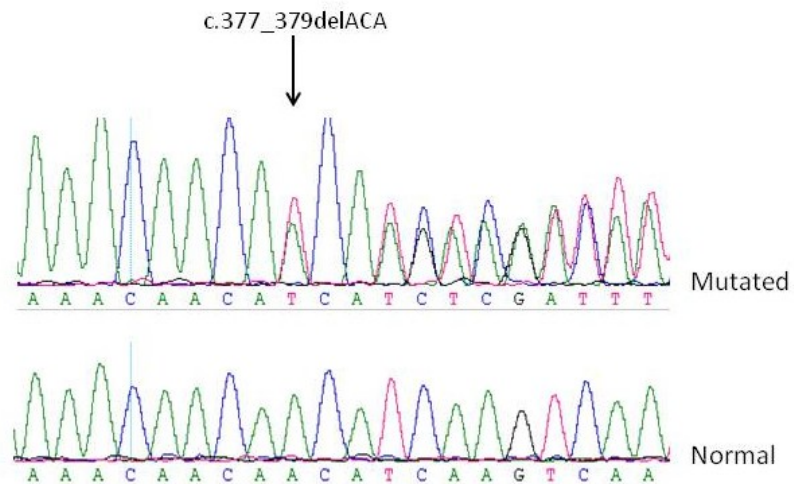


Figure 4 **A)** Pedigree of the family IV. Individuals carrying one mutant and one normal allele are denoted by M/-, whereas those with no mutations by -/-. **B)** Chromatogram used to detect the disease allele (variant allele denoted by an arrow).

TABLE I. Clinical and instrumental findings in mutated families.

Family ID	Patient ID/sex/age (y)	Seizure semeiology	Seizure onset (y)	Tonic-clonic seizures (age, y)	Interictal EEG	MRI	PET	Out-come	AED	LGI1 mutation
I	V:2/M/30	déjà-vù, vertigo, fear	12	yes (20)	rare left temporal sharp waves	N	ND	SF	CBZ *	R407C
	IV:4/F/55	déjà-vù	30	no	N	N	ND	SF	none	R407C
	III:4/M/82	staring	65	no	rare right temporal delta waves	N	ND	SF	none	R407C
II	I:1/M/57	visual (bright balls); auditory (whistles, noises that cover surrounding voices).	9	yes	normal	N	ND	SF	CBZ	del exon2
	II:1/M/23	visual (brightballs)	15	yes	normal	N	ND	SF	VPA	del exon2
III	I:2/M/dead (69)	NA	33	yes	ND	ND	ND		PB	ND
	II:5/M/51	whistles in both ears, brief confusion	20	yes (23)	N	N	ND	SF	none	T380A
	II:7/F/49	whistles in right ear, abdominal pain, Wernicke aphasia, loss of contact	41	yes (43)	left temporal sharp waves	N	Asymmetric metabolism in lateral temporal cortex (sn<dx)	monthly SPS and CPS	LEV, OXC	T380A
	III:1/M/32	vertigo, buzzing in both ears, visual disturbance (blurring or foggy)	26	no	left temporal sharp waves during sleep	N	Asymmetric metabolism in parietal mesio-temporal-cortex (sn<dx)	SF	CBZ	T380A
	III:8/F/24	GTCS during sleep	17	yes (17)	normal	N	Asymmetric metabolism lateral temporal cortex (sn<dx)	rare GTCS	LEV	T380A

IV	II:1/M/58	aphasia	12	yes (12)	N	N	N	seizure free	CBZ	c377-379delACA
	III:2/F/22**	auditory (deafness); aphasia	11	yes (11)	Left temporal slow waves	N	ND	Rare CPS	CBZ	c377-379delACA

EEG electroencephalogram; **MRI** magnetic resonance imaging; **AED** antiepileptic drugs; **GTCS** generalized tonic clonic seizures; **LEV**levetiracetam; **PB** Phenobarbital; **CBZ** carbamazepine; **VPA** valproate; **SF** seizure free; **ND** not done; **N** normal

*The patient spontaneously stopped CBZ two years ago. No more seizures were reported.

**The patient also complained migraine attacks.

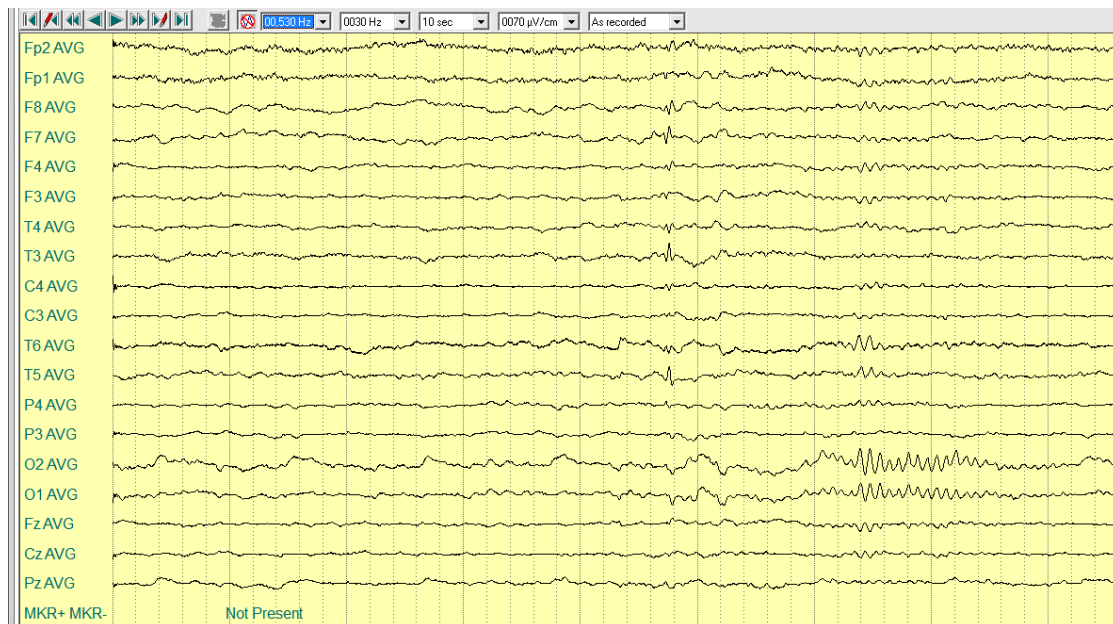


Figure 5. Interictal EEG of the patient V:2 (family I), that shows isolated low amplitude spikes over the left fronto-temporal region.

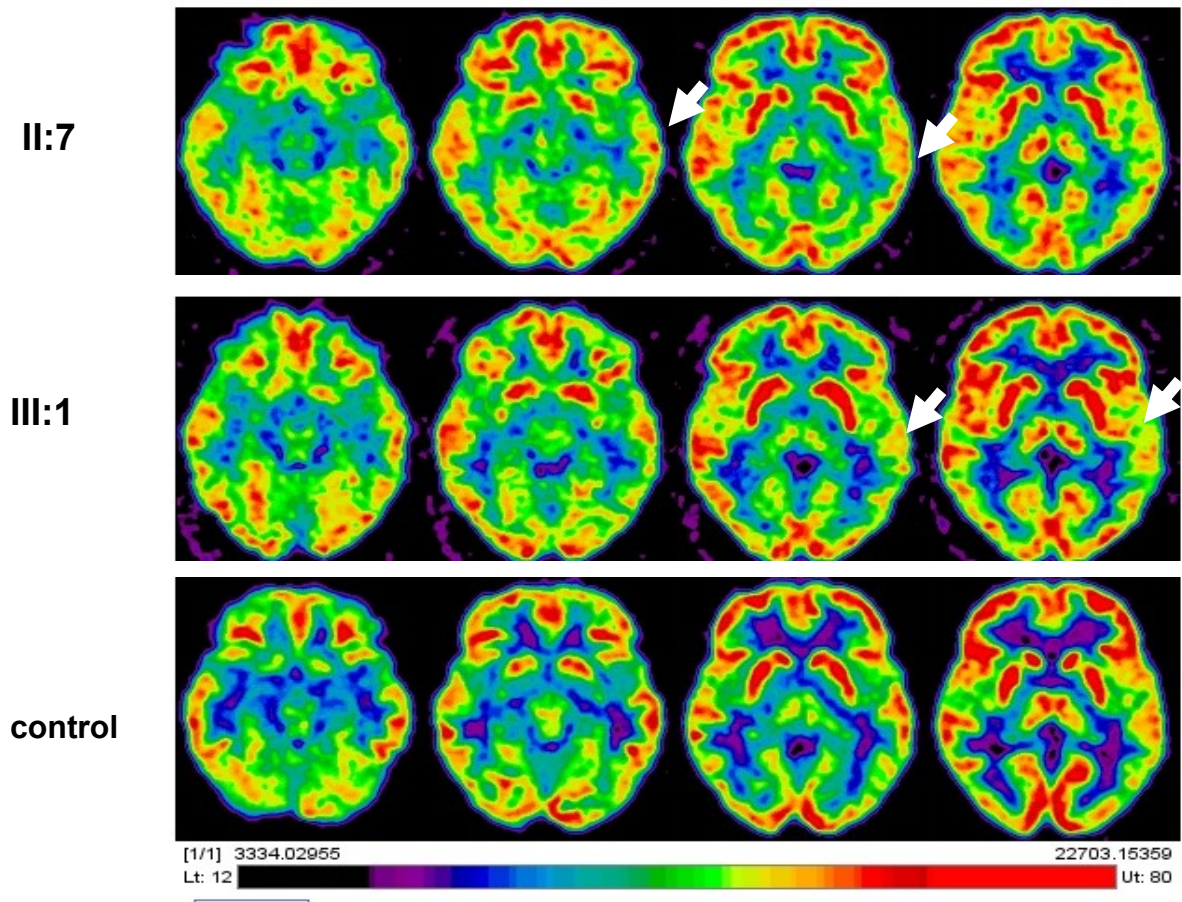
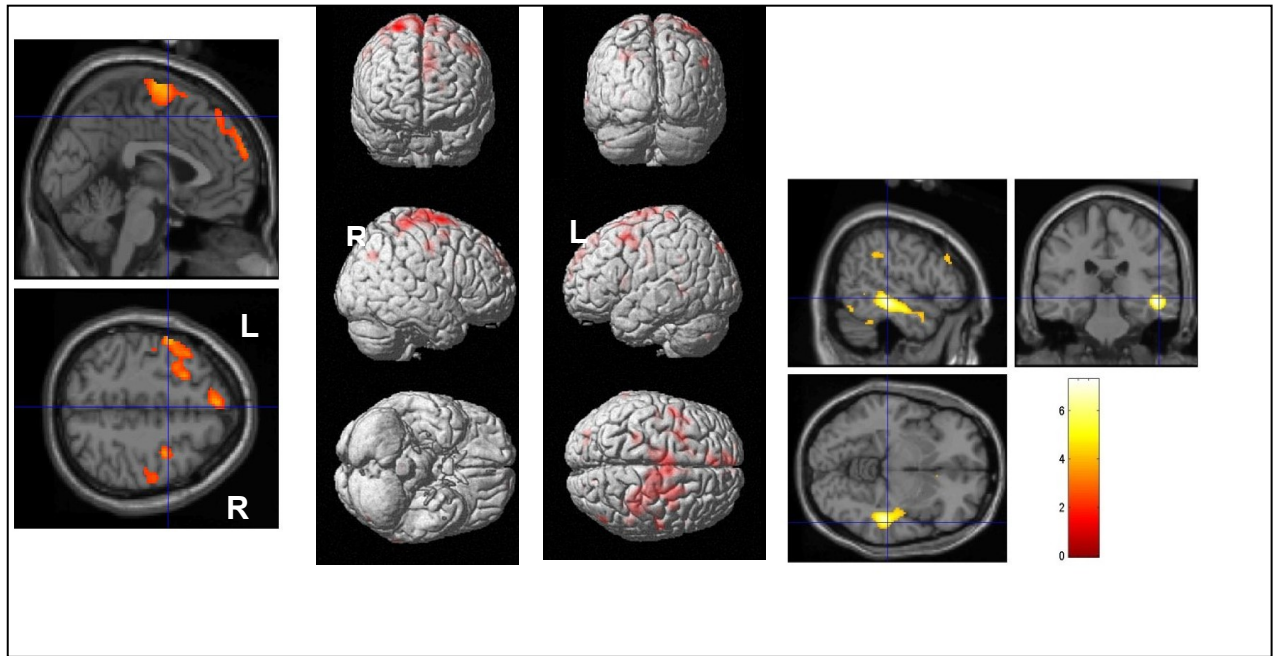


Figure 6. A) Axial FDG-PET images in two ADLTE patients carrying T380A mutation, that show a mild asymmetric FDG metabolism in lateral temporal cortex (sn<dx).



6 B) SPM voxel-based statistical analysis performed with SPM showing a relative metabolic reduction at the level of the right superior frontal gyrus and left middle frontal gyrus (BA6) (B left, middle) and a relative metabolic increase in the right middle temporal gyrus (BA21) (B right).

Table 2. Summary of the demographic characteristics and BIS-11 scores in ADLTE patients and healthy controls

Characteristics	ADLTE (n=7)	Healthy controls (n=7)	Significance of difference (test)
Age in years, median(IQR)	32 (22-58)	34 (21-60)	/
Gender, number of females (%)	3 (43)	3 (43)	/
BIS-11,mean (SD)	70 (9.4)	56.4 (9.7)	P<0.021 (t-test)

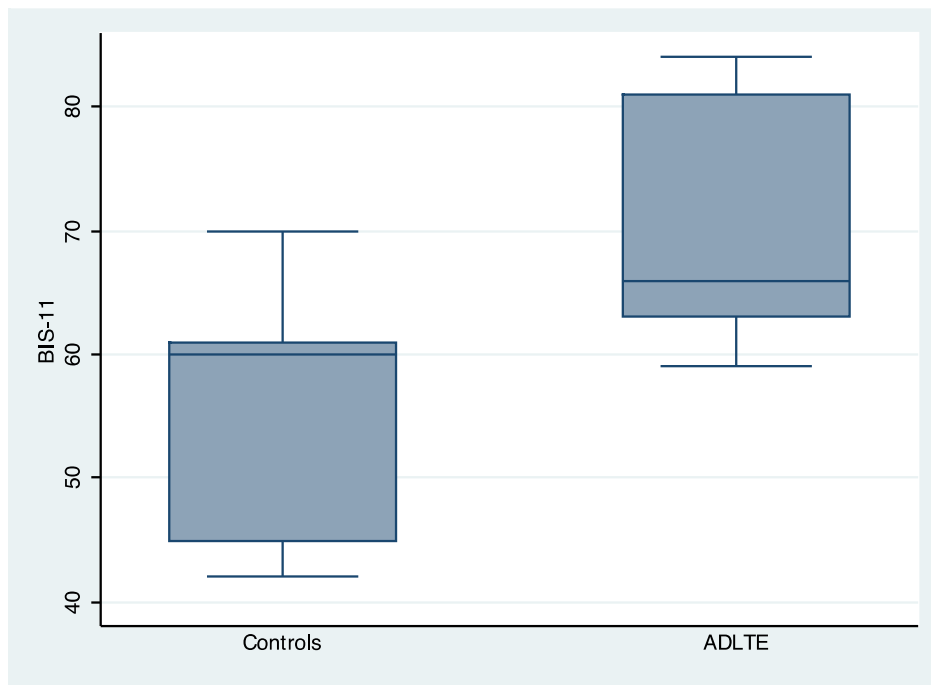


Figure 7. Graph box showing BIS-11 score in the two groups.

Table 3. Psychiatric assessment

Family	Patient ID/sex/age (y)	Psychiatric assessment					
		SCID I	SCID II	TAS-20	BIS-11	BDI-II	STAI-Y
I	V:2/M/30	BD-NOS	borderline personality disorder	normal	high impulsivity	euthymia	normal
	IV:4/F/55	major depressive episode; agoraphobia without panic disorder	normal	alexithymia	normal	depressive state	mild anxiety
II	II:7/F/49	ED-NOS; panic attack disorder; dysthymic disorder	borderline personality disorder	normal	high impulsivity	depressive state	high anxiety
	III:1/M/32	ED-NOS	normal	alexithymia	Mild impulsivity	euthymia	mild anxiety
	III:8/F/24	ED-NOS	borderline personality disorder	normal	high impulsivity	euthymia	mild anxiety
IV	II:1/M/58	normal	normal	normal	normal	euthymia	normal
	III:2/F/22	depressive disorder	borderline personality disorder	normal	mild impulsivity	depressive state	mild anxiety

SCID-I = Structured Clinical Interview for DSM-IV Axis I Disorders disorders; **SCID-II**= Structured Clinical Interview for DSM-IV Axis II disorders; **TAS-20**=Toronto Alexithymia Scale; **BIS-11**= Barratt Impulsiveness Scale 11; **BDI-II**= Beck Depression Inventory II; **STAI-Y**= State-Trait Anxiety Inventory Form Y; **ED-NOS**= Eating disorder not otherwise specified; **BD-NOS**= Bipolar Disorder not otherwise specified.

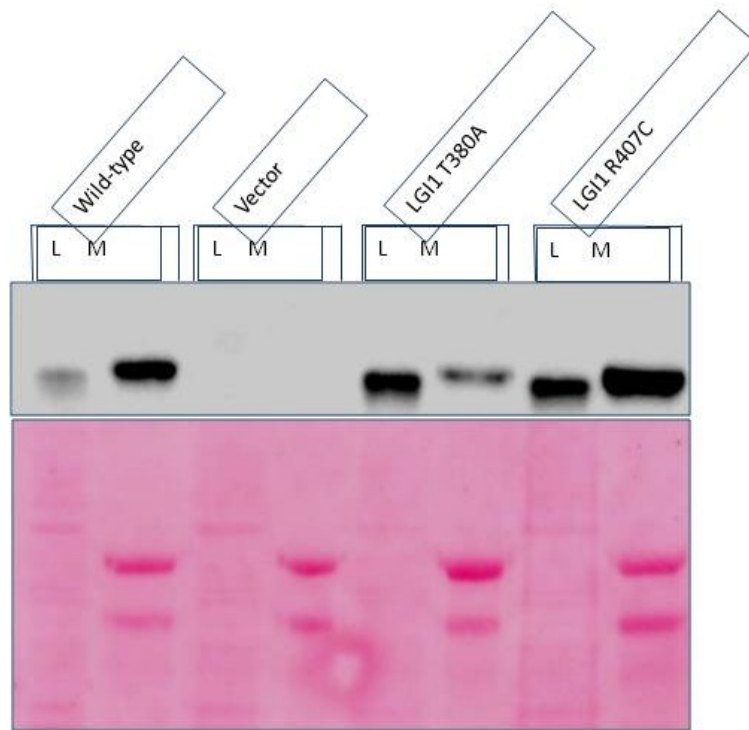
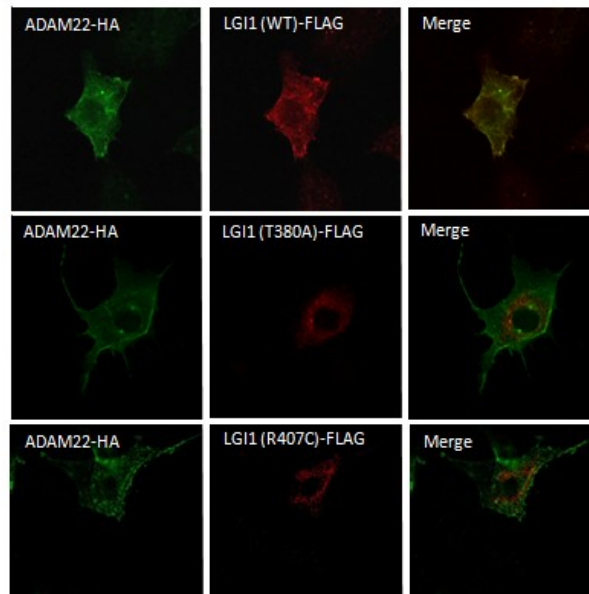


Figure 8. Western blot analysis of transfected HEK293 cells. Cell lysates (L) and concentrated (20x) media (M) of HEK293 cells transfected with LGI1 wild type, LGI1 1138A>G (p.T380A), LGI1 1219 C>T (R407C) expression constructs or empty vector were analyzed by western blot using an anti-LGI1 antibody (the image is shown with the permission of Dr. Nobile).

A)



B)

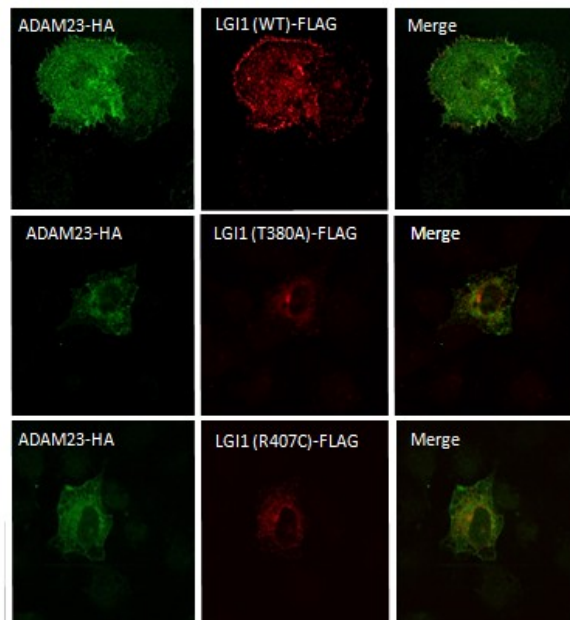


Figure 9 Interaction between secreted Lgi1 and ADAM22 (A) or ADAM23 (B) on the cell surface. COS7 cells were transiently co-transfected with wild-type or mutated *LGI1*-Flag and HA-tagged *ADAM22* or *ADAM23* expression constructs. Thirty-six hours after transfection, the Lgi1 proteins were labelled with anti-Flag antibody (red), then cells were permeabilized, and ADAM proteins were stained with anti-Ha antibody (green). Each panel displays representative confocal microscopy images, where wild-type Lgi1 co-localized with ADAM receptors on the cell surface, whereas mutant Lgi1 proteins failed to interact with ADAM receptors. Scale bars, 10 μ m (the images are shown with the permission of Dr. Nobile).

Table 4. Percentage of Lgi1 (wild-type or mutated) proteins bound to either ADAM receptor on cell surface

ADAM22	Total cells	Positive	Negative
WT	105	87 (83%)	28 (17%)
c.1138A>G (T380A)	81	0 (0%)	81 (100%)
c.1219C>T (R407C)	90	32 (35%)	58 (65%)

ADAM23	Total cells	Positive	Negative
WT	107	94 (88%)	13 (12%)
c.1138A>G (T380A)	78	0 (0%)	78 (100%)
c.1219C>T (R407C)	72	21 (29%)	51 (71%)

Table 5. Percentage of Lgi1 wild-type protein bound to ADAM22 on cell surface in the presence of different concentrations of human serum containing limbic encephalitis antibodies against Lgi1.

		% Human Serum			
		0	2,5	5	10
Lgi1 WT + ADAM22	Total Cells	115	273	254	236
	Membrane-bound Lgi1	87	102	93	47
	Not membrane-bound Lgi1	28	171	161	189

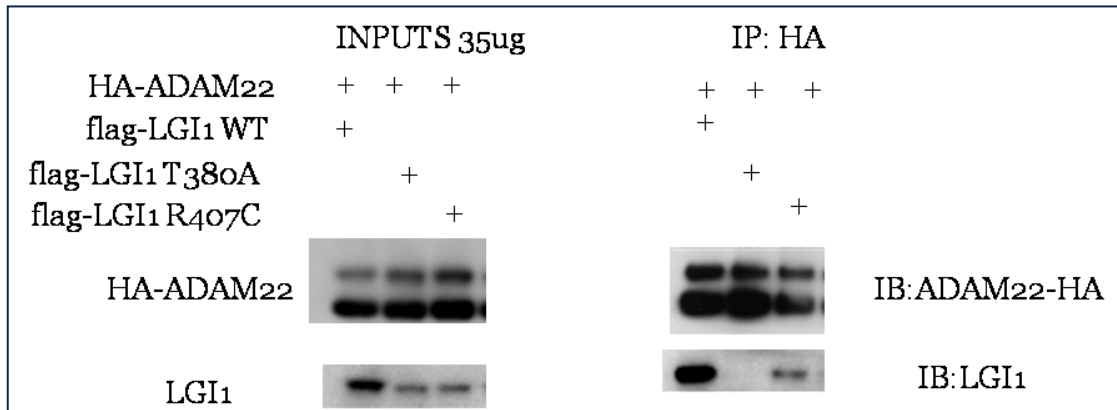


Figure 10. Wild-type or mutated Lgi 1 co-immunoprecipitated with ADAM22. The interaction between LGI1 and ADAM22 is affected by T380A and R407C mutations. FLAG-fused wt or mutated Lgi1 proteins were immunoprecipitated from equal amounts of HEK293T cells. Immunoprecipitates were blotted with either anti-HA (ADAM22) antibody or with anti-LGI1 antibody (these images are shown with the permission of Dr. Nobile).

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