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Novel α1 and γ2 $GABA_A$ receptor subunit mutations in families with idiopathic generalized epilepsy

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Abstract

Epilepsy is a heterogeneous neurological disease affecting approximately 50 million people worldwide. Genetic factors play an important role in both the onset and severity of the condition, with mutations in several ion-channel genes being implicated, including those encoding the $GABA_A$ receptor. Here, we evaluated the frequency of additional mutations in the $GABA_A$ receptor by direct sequencing of the complete open reading frame of the *GABRA1* and *GABRG2* genes from a cohort of French Canadian families with idiopathic generalized epilepsy (IGE). Using this approach, we have identified three novel mutations that were absent in over 400 control chromosomes. In *GABRA1*, two mutations were found, with the first being a 25-bp insertion that was associated with intron retention (i.e. K353delins18X) and the second corresponding to a single point mutation that replaced the aspartate 219 residue with an asparagine (i.e. D219N). Electrophysiological analysis revealed that K353delins18X and D219N altered $GABA_A$ receptor function by reducing the total surface expression of mature protein and/or by curtailing neurotransmitter effectiveness. Both defects would be expected to have a detrimental effect on inhibitory control of neuronal circuits. In contrast, the single point mutation identified in the *GABRG2* gene, namely P83S, was indistinguishable from the wildtype subunit in terms of surface expression and functionality. This finding was all the more intriguing as the mutation exhibited a high degree of penetrance in three generations of one French Canadian family. Further experimentation will be required to understand how this mutation contributes to the occurrence of IGE in these individuals.

Introduction

Epilepsy is one of the most common neurological conditions, with a prevalence of approximately 5–10 per 1000 in North America (Theodore *et al.*, 2006). For most patients (approximately 65%) with either generalized or partial epilepsies, the underlying cause is unknown and the condition is referred to as being 'idiopathic'. Overall, 30% of all individuals with epilepsy have idiopathic generalized epilepsy (IGE; McCorry *et al.*, 2006), a condition marked by generalized seizures and specific electroencephalographic abnormalities with no discernible defects in brain structure. In the past two decades, an increasing number of mutations in genes predisposing for various forms of IGE have been identified (Turnbull *et al.*, 2005; Macdonald *et al.*, 2010). Most of the mutations are found in genes encoding for voltage- or ligand-gated ion channels; they account for most familial forms of the disease (Scheffer & Berkovic, 2003; Hahn & Neubauer, 2009; Catterall *et al.*, 2010; Macdonald *et al.*, 2010; Zamponi *et al.*, 2010). This finding has led to the conclusion that IGE is primarily a 'channelopathy' (Kullmann & Waxman, 2010), although not all mutations encode for ion channels (e.g. Pal *et al.*, 2003).

γ-Amino-butyric acid $(GABA)_A$ receptors feature among several of the candidate ion channels thought to be involved in IGE (Galanopoulou, 2010; Kullmann & Waxman, 2010; Macdonald *et al.*, 2010). In keeping with this, signaling through $GABA_A$ receptors provides almost all inhibitory tone to the adult brain, balancing the tendency of excitatory neuronal circuits to induce convulsions (Ben Ari, 2006). To achieve this, $GABA_A$ receptors hyperpolarize neurons by fluxing chloride ions through a central anion-selective pore that is a pentamer assembled from 19 possible subunits, namely α1 – α6, β1 – β3, γ1 – γ3,

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δ, ε, π, θ and $\rho 1 - \rho 3$ (Olsen & Sieghart, 2008). Subunit number and organization around this central pore is ordered (Chang *et al.*, 1996) with the most commonly expressed $GABA_A$ receptor thought to consist of two each of α and β and a γ_2 (Sarto-Jackson & Sieghart, 2008). To date, most $GABA_A$ receptor mutations associated with IGE have been found in the γ_2 (*GABRG2*) subunit (Baulac *et al.*, 2001; Wallace *et al.*, 2001; Harkin *et al.*, 2002; Kananura *et al.*, 2002; Audenaert *et al.*, 2006; Sun *et al.*, 2008; Shi *et al.*, 2010) though mutations in the α_1 (*GABRA1*; Cossette *et al.*, 2002; Maljevic *et al.*, 2006), δ (*GABRD*; Dibbens *et al.*, 2004) and β_3 (*GABRB3*; Lachance-Touchette *et al.*, 2010; Tanaka *et al.*, 2008) subunits have also been described.

Here, we identify three novel mutations from a population of French Canadians with IGE. Two mutations found in the *GABRA1* gene were associated with disrupted plasma membrane delivery of the fully assembled $GABA_A$ receptor as well as deficits in its signaling properties. In contrast, a single point mutation found in the *GABRG2* gene had little effect on surface membrane expression or the functional properties of the mature receptor, a property not yet described for any other IGE-related $GABA_A$ receptor mutants. Future experiments will be needed to ascertain whether this mutation disrupts a more nuanced aspect of $GABA_A$ receptor biology.

Materials and methods

Subjects and healthy-control samples

Ninety-five individuals with IGE were selected from the Quebec population of Canada. The methods used for the clinical characterization of this cohort have been described previously (Kinirons *et al.*, 2008). The phenotypes in these probands include 21 cases with juvenile myoclonic epilepsy, 12 with childhood absence epilepsy, eight with generalized epilepsy with febrile seizures plus and 54 with IGE not otherwise specified. This cohort includes 46 familial cases (17 families with two affected individuals, nine with three affected individuals and 20 with four or more affected individuals) and 49 singletons. A summary of clinical manifestations of affected individuals with mutations in *GABRA1* or *GABRG2* are provided in Supporting Information Table S1. All the individuals, including 190 healthy ethnically-matched controls, gave informed consent, after which patient information and blood samples were collected. DNA was extracted from peripheral blood through the use of standard protocols.

Screening for mutations and variation analysis

We detected a total of three novel variants in *GABRA1* and *GABRG2* genes by re-sequencing them in a cohort of 95 subjects (53 females and 42 males) with epilepsy (Table 1). The open reading frame of each gene codes for the $\alpha 1$ and $\gamma 2$ subunits of the $GABA_A$ receptor. The genomic organization of each gene was obtained from the UCSC Genome Browser (Hg18 build; http://genome.ucsc.edu/). All the coding regions of the genes were amplified by polymerase chain reaction (PCR, AppliedBiosystems9700; Applied Biosystems, Foster City, CA, USA). We designed primers from at least 50 bp of the exon–intron boundaries to examine the entire exonic sequences of the gene and splice sites. PCR primer pairs were designed using the software *Exon primer* from the UCSC Genome Browser. We used the sequence of reference NM_000806 and NM_000816 for the *GABRA1* and *GABRG2* genes respectively. The amplicons were analyzed on an ABI3730 automatic sequencer (Applied Biosystems) by using *Mutation Surveyor* software version 3.0 (SoftGenetics, State College, PA, USA). All mutations and variants were confirmed by

TABLE 1. Mutations identified in $GABA_A$ receptor genes

Gene	Nucleotide (genomic)	Nucleotide (mRNA)	Amino acid (protein)	Maf	Polyphen		SIFT		SNAP		Panther		Conservation (amino acid)
					PSIC score	Prediction	Score	Prediction	Score	Prediction	subP SEC	Pdeleterious	
GABRA1	g.chr5:161242237G>A	r.655G>A	p.D219N	0.01	0.787	Benign	0.33	Tolerated	89%	Neutral	−4.8313	0.86192	Highly conserved
GABRA1	g.chr5: 161256674_161256675ins25	r.1059_1060 ins1242	p.K353delins18X	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
GABRG2	g.chr5:161453551C>T	r.247C>T	p.P83S	0.01	1.982	Possibly damaging	0.00	Affects protein function	70%	Non-neutral	−5.5871	0.93003	Highly conserved

Maf, Minor allele frequency; PSIC, position-specific independent counts; subPSEC, substitution position-specific evolutionary conservation; Pdeleterious, probability of functional impairment.

resequencing. For the analyses of detected single-nucleotide polymorphisms (SNPs) in our cohort, we used the dbSNP Build 133 based on NCBI Human Genome (version 5.2) and The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff. The novel SNPs and known mutations were tested by sequencing the corresponding fragment in a control population of 190 individuals. These French Canadian controls underwent a systematic questionnaire in order to exclude symptoms compatible with seizures as well as any familial history of epilepsy. Transmission of any variant or mutation was tested in parents and relatives of the probands when available.

In silico predictions

The combination of four programs (*PolyPhen*, *SIFT*, *SNAP* and *PANTHER*) was used to predict the potential disruptiveness of an amino acid substitution on the structure and function of a human protein (Table 1). The genome information and homology searches were based on the UCSC Genome Browser database (NCBI36/hg18 assembly). Sequence cluster alignments were performed by using the Clustal W algorithm. All novel SNPs identified were also tested for their potential effects on splicing enhancer/inhibitor elements by using *ESEfinder* (Cartegni *et al.*, 2003) and *RESCUE-ESE* programs (data not shown; Fairbrother *et al.*, 2002).

Cloning and mutagenesis

For biochemical and functional analysis of each mutation we cloned human α1, β2 and γ2 subunits of the $GABA_A$ receptor into the pcDNA3.1(-) vector (Invitrogen, Carlsbad, CA, USA). As a positive control, we also cloned the A322D mutant of *GABRA1* gene which has been described previously (Cossette *et al.*, 2002; Krampfl *et al.*, 2005). The QuickChange site directed mutagenesis kit (Stratagene, Agilent Technologies, Cedar Creek, TX, USA) was used to introduce D219N and P83S mutations in the *GABRA1* and *GABRG2* clones respectively, as described previously (Cossette *et al.*, 2002; Krampfl *et al.*, 2005). The sequence of the mutant *GABRA1* (K353delins18X) subunit was obtained by PCR from the cDNA of the patient A-III-I and subcloned into the vector containing the wildtype *GABRA1* to replace the 3′ part of the gene as described below. For the immunofluorescence experiments, a myc tag was inserted by PCR at the N-terminus between amino acids four and five of both the α1 and the γ2 subunits. All constructs were sequenced to confirm the presence of the mutations and to exclude any other variants that may have been introduced during PCR amplification.

Reverse transcriptase PCR (RT-PCR)

To obtain the K353delins18X mutant, total cellular RNA from patient A-III-I was obtained from lymphoblastoid cell lines. RNA of the sample was reverse-transcribed to complementary DNA (cDNA) using the ThermoScript RT-PCR System kit (Invitrogen). PCR-amplification was performed on the cDNA using the Advantage kit (Applied Biosystems). The cDNA was amplified with *GABRA1*-specific primers flanking intron 11: *GABRA1*-5′ (exon 10): GAGCATCAGTGCCAGAAACTCCCTCCC, *GABRA1*-3′ (exon11): CGGGCTTGACCTCTTTAGGTTCTATGG and *GABRA1*-3′-UTR: CCCCCTCTTTTCTCTCTCATTCTGTCTCC. All primers were designed using NM_000806 as the reference sequence. PCR conditions were: 95 °C for 1 min, 40 cycles of 20 s at 95 °C, 30 s at 65 °C, 45 s at 98 °C, followed by 68 °C for 7 min. Reactions were cycled on an AppliedBiosystems9700 PCR instrument. The amplicons were analyzed on an ABI3730 automatic sequencer by using *Mutation Surveyor* software version 3.0.

Immunofluorescence

To assess the surface and cellular distribution of wildtype and mutant receptor subunits, myc-tagged wildtype and mutant α1 subunits were co-transfected with wildtype β2 and γ2. Myc-tagged wildtype and mutant γ2 subunits were also co-transfected with wildtype α1 and β2 in HEK 293 cells that had been plated on 22-mm glass coverslips coated with poly-L-lysine in DMEM containing 10% fetal bovine serum and incubated for 18 h. All transient transfections were performed at a ratio of 1 : 1 : 2 for α1 : β2 : γ2 (2 : 2 : 4 μg per dish) using Lipofectamine 2000. For the endoplasmic reticulum (ER) colocalization experiments, a plasmid for the expression of the dsRed2-ER marker (Clontech, Mountain View, CA, USA) was co-transfected in a ratio of 1 : 1 : 2 : 1 for α1 : β2 : γ2 : dsRed2-ER respectively. After 24 h, cells were washed and fixed in 4% formaldehyde in PBS. Cells were washed and blocked once with PBS–glycine, 20 mM, and once with PBS containing 10% goat serum, 0.5% BSA and 0.1% Triton X-100 (10 min). Cells were then incubated with anti-myc-tag rabbit monoclonal antibody (1 : 200 dilution in blocker; cat, no. 2278; Cell Signaling, Millipore, Billerica, MA, USA) alone or with pan-Cadherin mouse monoclonal antibody for membrane co-localization (ab6528, 1 : 250 dilution, 1 h at room temperature; Abcam, Cambridge, MA, USA). This step was followed by three washes with blocking solution. Cells were then incubated for 1 h at room temperature with Alexa Fluor 488 rabbit IgG antibody (Molecular Probes, Invitrogen) and/or Alexa Fluor 568 mouse IgG antibody (Molecular Probes, Invitrogen) at a 1 : 400 dilution in blocking solution. Cells were washed three times with blocking solution and mounted onto glass slides. Fluorescently labelled cells were imaged using a confocal microscope (Leica TCS SP5).

Affinity purification of surface receptors

HEK 293 cells were plated in 10-cm dishes coated with poly-L-lysine in DMEM containing 10% fetal bovine serum and incubated for 18 h. Cells were transiently transfected with the cDNA encoding for wildtype or mutant α1 and gamma2, together with the wildtype beta2 subunit using a ratio of 1 : 1 : 2 for α1 : β2 : γ2 (12 : 12 : 24 μg). Twenty-four hours post-transfection, intact cells were washed twice with ice-cold PBS and incubated with the membrane-impermeable biotinylation reagent Sulfo-NHS SS-Biotin (0.5 mg/mL in PBS; Pierce, Rockford, IL, USA) for 30 min at 4 °C to label surface membrane proteins. To quench the reaction, cells were incubated with 10 mM glycine in ice-cold PBS twice for 5 min at 4 °C. Sulfhydryl groups were blocked by incubating the cells with 5 nM *N*-ethylmaleimide (NEM) in PBS for 15 min at room temperature. Cells were solubilized overnight at 4 °C in lysis buffer (Triton X-100, 1%; Tris–HCl, 50 mM; NaCl, 150 mM; and EDTA, 5 mM; pH 7.5) supplemented with protease inhibitors (Complete Mini; Roche, Mississauga, ON, Canada) and 5 mM NEM. Lysates were cleared by centrifugation (16 000 ***g***, 10 min at 4 °C). An aliquot of each lysate was saved. Biotinylated surface proteins were affinity-purified from the remaining cell lysates by incubating for 1 h at 4 °C with 100 μL of immobilized neutravidin-conjugated agarose bead slurry (Pierce). The samples were then subjected to centrifugation (16 000 ***g***, 10 min, at 4 °C), an aliquot of the supernatant was kept and the beads were washed six times with buffer (Triton X-100, 0.5%; Tris–HCl, 50 mM; NaCl, 150 mM; and EDTA, 5 mM; pH 7.5). Surface proteins were eluted from beads by incubating for 30 min at room temperature with 200 μL

of LSB/Urea buffer [2× Laemmli sample buffer (LSB) with 100 mM DTT and 6 M urea; pH 6.8]. Protein concentration of the lysates and the supernatants was determined using the Bradford method. Equal amounts of lysate and supertatant proteins were prepared by adding 2× LSB/urea buffer and loaded into an SDS-polyacrylamide gel (SDS-PAGE), as well as 30 μL of affinity-purified proteins.

Western blotting

Protein samples were run on 10% SDS-PAGE and transferred to PVDF membranes. Blots were incubated for 1 h at room temperature in blocking buffer containing 5% non-fat milk in TTBS (NaCl, 500 nM; Tris base, 20 mM; and Tween-20, 0.1%) followed by a 2-h incubation at room temperature with anti-α1 (1 : 2000; Chemicon MAB339; Chemicon, Millipore, Billerica, MA, USA) antibodies in blocking buffer. The blots were then washed (3 × 20 min) with TTBS and incubated for 1 h at room temperature in horseradish peroxidase-conjugated donkey anti-mouse IgG or horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA) at a 1 : 5000 dilution in blocking buffer. The blots were then washed (3 × 20 min in TTBS) and revealed by chemiluminescence with Western Lightning Plus-ECL (PerkinElmer, Waltham, MA, USA). GAPDH was used as a control to demonstrate that equal amounts of protein were loaded (mouse monoclonal anti-GAPDH; Chemicon MAB374, 1 : 2000 dilution). Similarly, pan-cadherin protein expression was used to normalize the loading of membrane proteins (mouse monoclonal pan-cadherin; Abcam ab6528, 1 : 5000 dilution).

Cell culture and transfection

Techniques used to culture and transfect mammalian cells have been described in detail elsewhere (Bowie, 2002). Briefly, tsA201 cells (provided by R. Horn, Jefferson Medical College, PA, USA) were maintained at a 70–80% confluency in minimal essential medium with Earle's salts, 2 mM glutamine and 10% fetal bovine serum supplemented with penicillin (100 units/mL) and streptomycin (100 μg/mL). After plating at low density (2×10^4 cells/mL) on 35-mm plastic dishes coated with poly-D-lysine, cells were transfected with the cDNAs encoding the human $GABA_A$ receptor subunits in a 1 : 1 : 2 ratio (α1 : β2 : γ2), using the calcium phosphate technique as previously described (Bowie, 2002). For analysis of the mutant subunits, the cDNAs encoding for the α1 (A322D), α1 (K353delins18X), α1 (D219N) and γ2 (P83S) replaced the corresponding wildtype subunits. The cDNA for enhanced green fluorescent protein was routinely co-transfected (0.1 μg per dish) to identify transfected cells.

Electrophysiological solutions and techniques

Experiments were performed 24–48 h after transfection. GABA (Sigma, Oakville, ON, Canada) was dissolved in external solution containing (in mM): NaCl, 150; HEPES, 5; $MgCl_2$, 2; and $CaCl_2$, 1; with 2% phenol red. Internal pipette solution contained (in mM): KCl, 150; EGTA, 5; $MgCl_2$, 1; and MgATP, 2. The pH and osmotic pressure of internal and external solutions were adjusted to 7.3–7.4 and 310 mOsm respectively. For Zn^{2+}-block experiments, $ZnCl_2$ (Sigma) was diluted to a 10-μM final concentration in control and agonist external recording solutions from 10-mM frozen stocks. Diazepam was diluted to a 1-μM final concentration in control and agonist external recording solutions from 1-mM frozen stocks. All recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Inc., CA, USA) using thin-walled borosilicate glass pipettes (2–5 MΩ) coated with dental wax to reduce electrical noise. Control and agonist solutions were rapidly applied to outside-out patches excised from transfected tsA201 cells using a piezoelectric stack (Physik Instrumente, Germany). Solution exchange (10–90% rise-time, 150–250 μs) was routinely determined at the end of each experiment by measuring the liquid junction current (or exchange current) between the control and external solution containing an additional 10 mM NaCl. Current records were filtered at 5 kHz and digitized at 25 kHz, and series resistances (3–10 MΩ) compensated by 95%. All recordings were performed at a holding potential of −60 mV. Data acquisition was performed using pClamp9 software (Molecular Devices) and analysis and statistics were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). All datasets reported in this study exhibited a normal distribution as determined by the Shapiro–Wilk normality test with *P*-values > 0.05. Figure illustrations were made using Origin 7.0 (OriginLab, Northampton, MA, USA). All experiments were carried out at room temperature (22–23 °C). For whole-cell recordings, control and agonist solutions were locally applied to the recorded cell.

Results

Identification of mutations in the GABRA1 and GABRG2 genes

We have identified three novel genetic mutations in the alpha1 (α1) and gamma2 (γ2) subunits of the $GABA_A$ receptor (Fig. 1). The amino acid numbering for these mutations refers to the immature protein including the signal peptide. Screening the *GABRA1* gene identified two variants, the first of which was an insertion of 25 nucleotides in the intron close to the splice acceptor site of exon 11 (Fig. 2A). The exact sequence of the insertion has been obtained by subcloning the PCR fragment of the mutated allele in a TOPO-cloning vector (Invitrogen). This 25-bp insertion in the genomic sequence of *GABRA1* (g.chr5:161256674_161256675ins25) changes the position of the putative branch site to a distance of 63 bp from exon 11. The maximal distance between the splice acceptor and the branch sites is usually between 20 and 50 bp (Keren *et al.*, 2010). We therefore hypothesized that this mutation prevents cleavage at the 3′ end of intron 10 (Fig. 2A). Consistent with this hypothesis, RT-PCR of the A-III-I individual has shown a 1242-bp insertion which corresponds to the retention of intron 10 (r.1059_1060ins1242). This splice mutation, which results in a complex rearrangement of the *GABRA1* transcript, predicts a deletion of the fourth transmembrane domain in the mature α_1 protein, as well as the insertion of 18 amino acids and a premature stop codon (p.K353delins18X; Fig. 2B). A total of three affected members from this family carry this K353delins18X mutation in *GABRA1*, as well as an obligated carrier (Fig. 1A). The second α1 variation was due to a missense mutation in the aspartate 219 residue to an asparagine (i.e. D219N) which was detected in four out of five affected individuals with either IGE or febrile seizures in pedigree B (Fig. 1B). Sequence analysis indicated that carriers are heterozygous with respect to a G>A substitution, which is predicted to change an aspartic acid to an asparagine at position 219 of the α1 $GABA_A$ receptor subunit. The K353delins18X and D219N mutations were not detected in 190 healthy controls (102 females and 88 males), nor were additional variations in *GABRA1* detected.

We also detected a missense mutation in the γ2 $GABA_A$ receptor subunit which changed the proline 83 residue of the immature protein to a serine (i.e. P83S; Fig. 1C), in a large family exhibiting febrile seizures and IGE over three generations. This P83S mutation was found in all affected individuals ($n = 9$) of this multiplex family (Fig. 1C). The heterozygous mutation Pro83Ser is found near a region which affects benzodiazepine binding on the extracellular ligand-

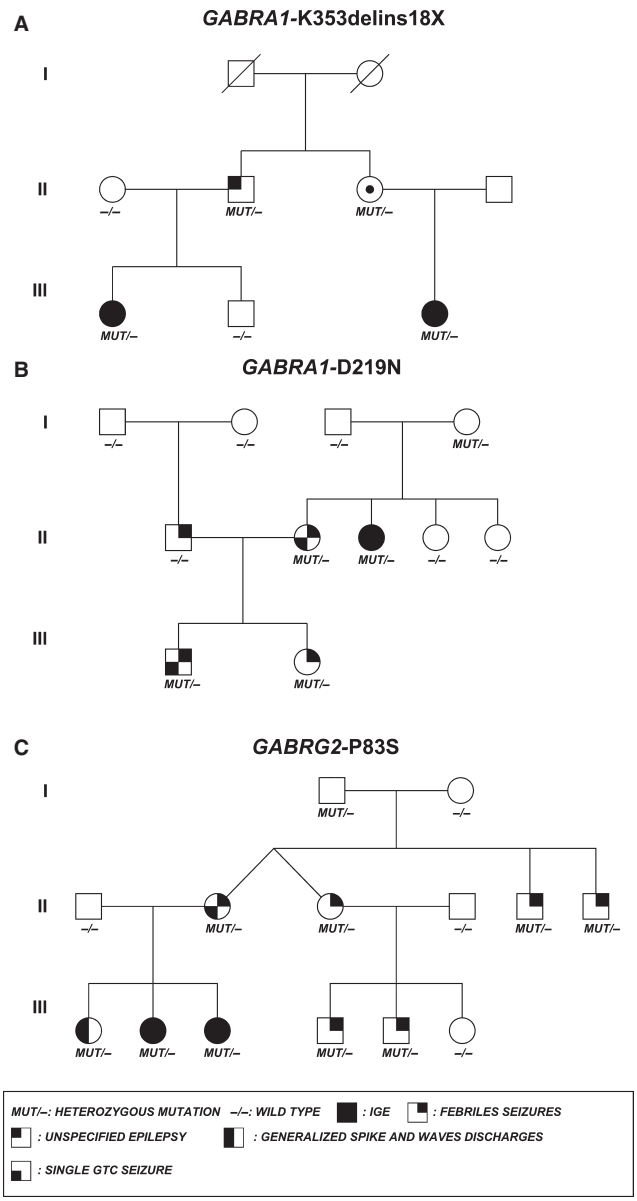


FIG. 1. Segregation profiles of the novel $GABA_A$ receptor mutants in French Canadian families affected with IGE and related phenotypes. (A and B) The (A) K353delins18X and (B) D219N mutations in *GABRA1* were found in four individuals of the same family over two generations. (C) The P83S mutation in *GABRG2* was found in nine individuals of the same family over two generations. Blackened symbols indicate affected individuals.

binding domain (Goldschen-Ohm *et al.*, 2010). Importantly, this mutation was not identified in the 190 French Canadian control subjects. In addition, no other additional mutations in *GABRG2* gene were detected in this cohort of IGE patients.

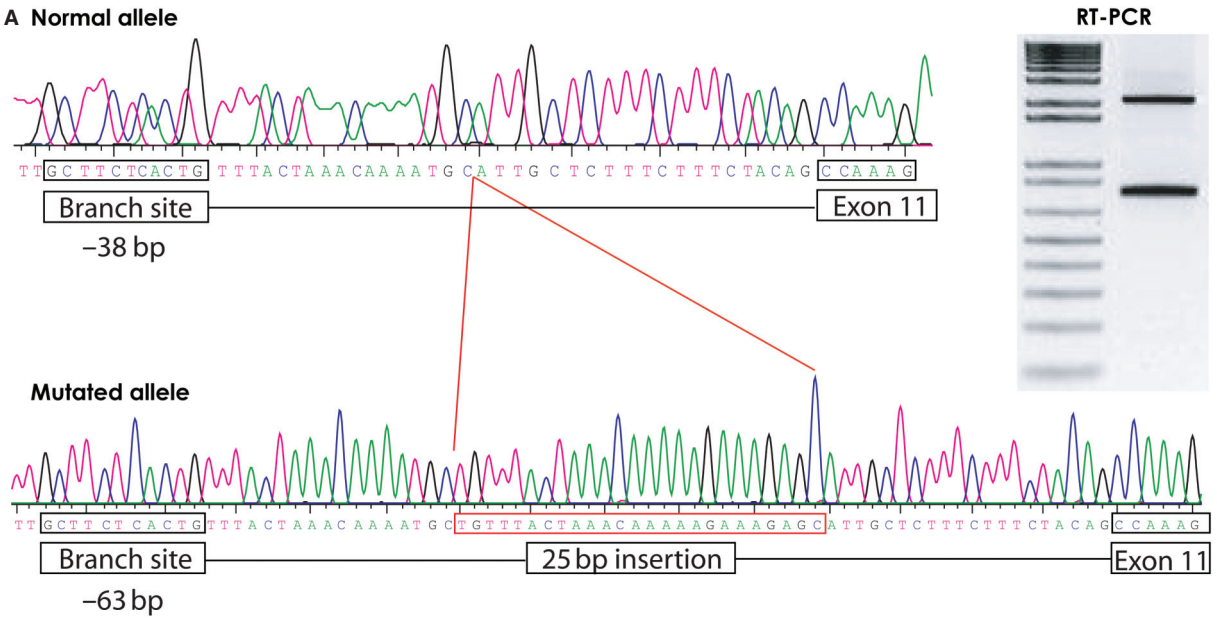
In silico analysis, using a combination of four different programs (*PolyPhen*, *SIFT*, *SNAP*, *PANTHER*), predicts that the P83S mutation should have a damaging effect on protein structure or function whereas the D219N in α1 is predicted to have a benign outcome (Table 1).

Subcellular distribution of mutant and wildtype $GABA_A$ receptors

Prior work has shown that other $GABA_A$ receptor mutations associated with IGE can affect the cellular distribution of the mature protein (Macdonald *et al.*, 2010). In many cases, the mutant receptor is held up in the ER and/or exhibits reduced surface expression (Macdonald *et al.*, 2010). To explore both of these issues, we examined the subcellular distribution of mutant α1 subunits that had been co-expressed with wildtype β2 and γ2 subunits (Figs 3 and 4). As controls, we compared the staining pattern of the wildtype α1 subunit which traffics normally and the α1 A322D mutant which accumulates in the ER and exhibits reduced plasma-membrane surface expression (Cossette *et al.*, 2002; Krampfl *et al.*, 2005; Maljevic *et al.*, 2006). As antibody recognition for the myc-tagged γ2 $GABA_A$ receptor subunit was variable, we focused only on examining its functional properties using electrophysiology (see below). Myc-tagged constructs of wildtype and mutant α1 subunits were expressed in HEK293 cells and visualized with a fluorescent-tagged myc antibody (Figs 3 and 4). ER localization was then estimated by overlaying the α1 subunit staining pattern with that of dsRed2-ER as a selective marker of this organelle (Fig. 3). Surface expression levels were determined in a similar manner using red-fluorescent pan-cadherin as a marker of the plasma membrane (Fig. 4).

As expected, there was little overlap in the staining pattern for the wildtype α1 subunit and that of the ER (Fig. 3, left column) whereas the distribution of the α1 A322D mutant co-localized almost exclusively with dsRed2-ER (Fig. 3, middle column) in agreement with prior work showing that this subunit is retained within the cell during protein synthesis and assembly. The staining pattern for the α1 K353delins18X also closely matched that of dsRed2-ER (Fig. 3, middle) whereas there was only partial, though substantial, overlap in the distribution of the α1 D219N mutant (Fig. 3, right column), indicating that some of this latter mutant protein may be trafficked to the surface of the plasma membrane. In support of this, the pattern of expression of both pan-cadherin and α1 D219N mutant were similar and comparable to the wildtype α1 subunit (Fig. 4A, left and right columns). In contrast, the staining pattern of pan-cadherin did not match that of α1 K353delins18X or α1 A322D subunits (Fig. 3, middle columns), further confirming that these mutations disrupt plasma membrane surface expression.

To examine more subtle defects in the targeting of the mutant subunits to the plasma membrane, we used cell surface biotinylation and Western blotting to quantify and compare surface expression of the mutants to wildtype subunits. To do this, HEK293 cells transfected with wildtype and mutant $GABA_A$ receptors were surface-biotinylated with the membrane-impermeable reagent Sulfo-NHS-SS-Biotin and biotinylated surface expressed proteins recovered from the soluble lysate fraction (i.e. L) by affinity purification (i.e. M; Fig. 4B). The unbound or nonbiotinylated fraction present in the supernatant (i.e. S) was also analysed as it represents the proportion of receptor subunits that are retained intracellularly. As expected of controls, the wildtype α1 subunit was found in both the membrane (M) and supernatant (S) fractions whereas the α1 A322D mutant was largely restricted to the supernatant (Fig. 4B, left and middle columns). Likewise, the K353delins18X mutant was also absent from the membrane fraction and, although it was detected in the supernatant, the band migrated at a lower molecular weight (Fig. 4B, middle column). This finding is consistent with the prediction that this mutation introduces a premature stop codon which truncates the fourth transmembrane domain of the mature α_1 protein. Although we were not able to detect the α1 A322D mutant and K353delins18X in the membrane fraction, it does not exclude the possibility that a small number of receptors were expressed at the plasma membrane. In contrast, the α1 D219N mutant was detected in both the membrane and supernatant fractions (Fig. 4B, right column), in agreement with the staining studies. Compared to the wildtype α1 subunit, there was a 50% decrease in the ratio of surface to total expression of the α1 D219N mutant ($P = 0.0013$, Student's *t*-test).



B WT α_1 subunit protein sequence length = 456

MRKSPGLSDCLWAWILLLSTLTGRSYGQPSLQDELKDNTTVFTRILDRLLDGYDNRLRPGLGERVTEVKTD
IFVTSFGPVSDHDMEYTIDVFFRQSWKDERLKFKGPMTVLRLNNLMASKIWTPDTFFHNGKKSVAHNMTMP
NKLLRITEDGTLLYTMRLTVRAECPMHLEDFPMDAHACPLKFGSYAYTRAEVVYEWTREPARSVVVAEDGS
RLNQYDLLGQTVDSGIVQSSTGEYVVMTTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFWLNRESVPARTV
FGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAVCYAFVFSALIEFATVNYFTKRGYAWDGKSVVPEKPK
KVKDPLIKKNNTYAPTATSYTPNLARGDPGLATIAKSATIEPKEVKPETKPPEPKKTFNSVSKIDRLSRIA
FPLLFGIFNLVYWATYLNREPQLKAPTPHQ

K353delins18X α_1 subunit protein sequence length = 371

MRKSPGLSDCLWAWILLLSTLTGRSYGQPSLQDELKDNTTVFTRILDRLLDGYDNRLRPGLGERVTEVKTD
IFVTSFGPVSDHDMEYTIDVFFRQSWKDERLKFKGPMTVLRLNNLMASKIWTPDTFFHNGKKSVAHNMTMP
NKLLRITEDGTLLYTMRLTVRAECPMHLEDFPMDAHACPLKFGSYAYTRAEVVYEWTREPARSVVVAEDGS
RLNQYDLLGQTVDSGIVQSSTGEYVVMTTHFHLKRKIGYFVIQTYLPCIMTVILSQVSFWLNRESVPARTV
FGVTTVLTMTTLSISARNSLPKVAYATAMDWFIAVCYAFVFSALIEFATVNYFTKRGYAWDGKSVVPEK**VN**
ALMVTVVHQYYVSLNLX

FIG. 2. Sequencing of the K353delins18X mutation in *GABRA1*. (A) The normal and mutant alleles were amplified by PCR, sub-cloned in a TOPO-cloning vector (Invitrogen) and sequenced separately. The mutation consists of a 25-bp insertion located between the splice acceptor and branching sites of exon 11 of the *GABRA1* gene. RT-PCR shows an abnormal lower band; sequencing of this band confirmed the retention of the intron leading to a premature stop codon. (B) The amino acid sequence of the wildtype (top) and mutant (bottom) $GABA_A$ receptor. The mutation results in the translation of 18 intronic amino acids and a premature stop codon (red) and the deletion of 103 amino acids.

Functional analysis of mutant α1 and γ2 $GABA_A$ receptor subunits

To measure the functionality of surface receptors, we performed electrophysiological recordings on outside-out patches excised from tsA201 cells transiently transfected with wildtype and mutant $GABA_A$ receptor subunits (Fig. 5). Following activation, $GABA_A$ receptors exhibit rapid desensitization kinetics with a time course of tens of milliseconds (Jones & Westbrook, 1996). Consequently, the advantage of performing experiments on excised membrane patches is that it permits rapid exchange of agonist solution (within 400–500 μs) prior to the onset of desensitization and therefore peak response amplitude and decay kinetics are determined with accuracy.

Typical membrane currents elicited by 10 mM GABA on wildtype or mutant $GABA_A$ receptors are shown in Fig. 5A. Note that peak responses of wildtype $\alpha_1\beta_2\gamma_2$ receptors were considerably larger than $\alpha_1\beta_2$ assemblies (Fig. 5A, upper row), an observation expected from earlier work highlighting the importance of subunit composition to agonist responsiveness (e.g. Verdoorn *et al.*, 1990; Boileau *et al.*, 2003). Likewise, patches containing α1(A322D)β2γ2 receptors elicited much weaker responses to GABA (Fig. 5A, middle row), which is consistent with their reduced surface expression (Fig. 4) and lower agonist sensitivity (Krampfl *et al.*, 2005). We failed to detect responses in any excised patches from cells transfected with the α1 K353delins18X subunit (Fig. 5A, middle row). The lack of responsiveness in excised patches is not due to detection failure as cells did not respond to GABA even in recordings performed in the whole-cell configuration (Fig. 6). Taken together with our staining and biochemical experiments, we conclude that the K353delins18X mutation completely eliminates surface expression of mature $GABA_A$ receptors, an observation supported by the fact that the α-subunit is required for the formation of functional receptors (Mizielinska *et al.*, 2006).

In contrast, we routinely recorded GABA-mediated responses from the cells expressing the α1 D219N and the γ2 P83S subunit mutations (Fig. 5A, lower row). The level of surface expression of α1 D219N-containing receptors, as estimated by peak response amplitude, was modest compared to wildtype $\alpha_1\beta_2\gamma_2$ receptors (Fig. 5B). Although reduced functionality may reflect changes in unitary conductance or

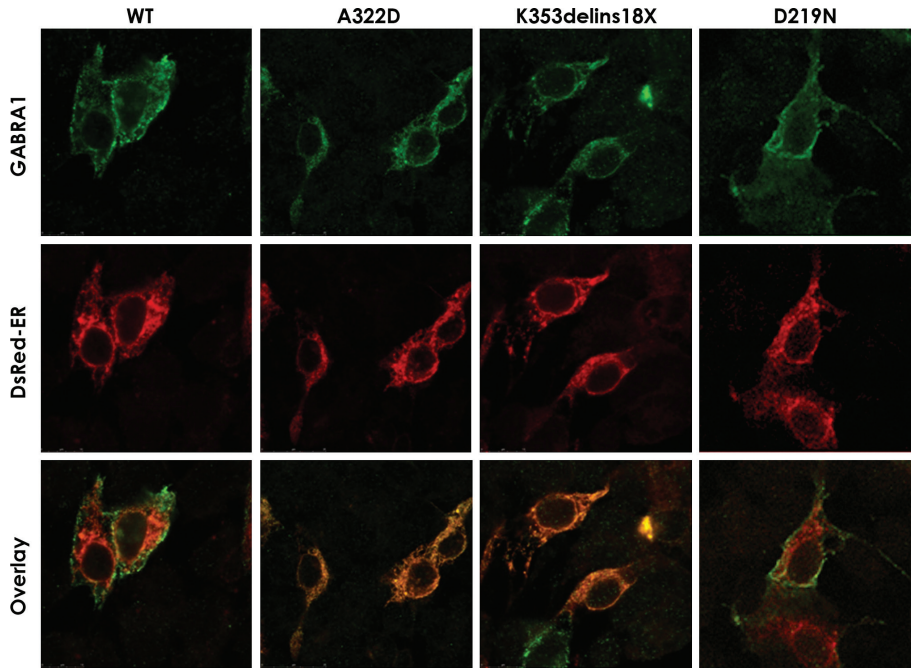


FIG. 3. Mutant α1 subunits co-localized with the ER. Immunostaining of the wildtype *GABRA1* subunit did not co-localize with the ER-marker DsRed-ER (left column). In contrast, the A322D and K353delins18X *GABRA1* mutants co-localized with the ER (middle columns), suggesting that the mutant subunits were retained in the ER and therefore did not reach the cell membrane. Staining for the D219N *GABRA1* mutant was partially co-localized with the ER.

agonist affinity/efficacy, much of it most probably reflects a genuine deficit in the delivery of mature $GABA_A$ receptors to the cell's surface, in agreement with immunohistochemical staining and Western blot analysis (Fig. 4A and B). Interestingly, peak membrane currents elicited by GABA at receptors containing the γ2 P83S subunit were indistinguishable from wildtype $\alpha_1\beta_2\gamma_2$ receptors (Fig. 5A and B). As peak responses of γ2 P83S-containing receptors were substantially larger than wildtype $\alpha_1\beta_2$ receptors (Fig. 5B), we tentatively concluded that this mutation does not affect the incorporation of γ2 subunits into mature $GABA_A$ receptors (see below). Finally, although the degree of equilibrium desensitization was similar for wildtype $\alpha_1\beta_2\gamma_2$ receptors compared to those containing the α1 D219N or γ2 P83S subunits (Fig. 5B, Table 2), we did observe a statistically significant difference between wildtype $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors ($n = 22$ and 4 patches for $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ respectively, $P = 0.0013$, Student's *t*-test; Fig. 5B) which has been reported by others (Boileau *et al.*, 2003).

α1 D219N mutation altered $GABA_A$ receptor gating kinetics

Structural modeling of the pentameric $GABA_A$ receptor (Ernst *et al.*, 2005) places Asp219 at the interface of the α1 and β2 subunits (Fig. 7A) based on homology with the Torpedo nicotinic acetylcholine receptor (Unwin, 2005) (Fig. 7B). Given that this interface is important for agonist binding and gating (Olsen & Sieghart, 2009), we examined whether replacement of the positively-charged Asp residue with a neutral Asn affects re-equilibration rates following agonist application (i.e. macroscopic desensitization) or removal (i.e. off-kinetics; Fig. 7C and D). To do this, each experimental trace was fitted with two or three exponential components to estimate desensitization and off-kinetics (Fig. 7 and Table 2). For comparison, we also fitted agonist responses mediated by wildtype $\alpha_1\beta_2\gamma_2$ receptors and receptors containing the γ2 P83S subunit.

Typical responses to GABA (1–10 mM) in patches containing wildtype or mutant receptors are shown as overlays in Fig. 7C and D. In all cases, rates into desensitization were fit with either two or three time constants whereas most off-kinetic relaxations were well described by bi-exponential functions (Table 2). As fit values of the fastest ($\tau \sim$1.3–2.8 ms) and slowest ($\tau \sim$93–287 ms) desensitization time constants were almost identical in all recordings, the occurrence of an intermediate time constant ($\tau \sim$14–21 ms) determined whether a third exponential component was included in the fit (Table 2). The biological basis of this intermediate component is not clear; however, prior work on native and recombinant $GABA_A$ receptors has noted variability in desensitization kinetics (e.g. Celentano & Wong, 1994). As we were able to exclude the possibility of variable proportions of $\alpha_1\beta_2$ and $\alpha_1\beta_2\gamma_2$ receptors in excised patches (see below), the distinct kinetic components of desensitization presumably correspond to an intrinsic property of the channel, perhaps modal gating (Lema & Auerbach, 2006).

Desensitization rates and off-kinetics of $GABA_A$ receptors containing the α1(D219N) subunit were appreciably faster than wildtype receptors and those containing the γ2 P83S subunit (Fig. 7C and D and Table 2). In contrast with the off-kinetics in wildtype patches, most of the off-kinetics for the α1 D219N-containing patches (7/9 patches) were fitted with three exponentials. Interestingly, the slowest component of desensitization had a larger contribution that in wildtype receptors (Table 2).

γ2 P83S mutation did not alter Zn^{2+} or benzodiazepine sensitivity

The expression levels (Fig. 5) and kinetic properties (Fig. 7) of $GABA_A$ receptors containing the γ2 P83S subunit were indistinguishable from wildtype $\alpha_1\beta_2\gamma_2$ receptors. To examine whether replacement of Pro83 with Ser affects the allosteric regulation of $GABA_A$

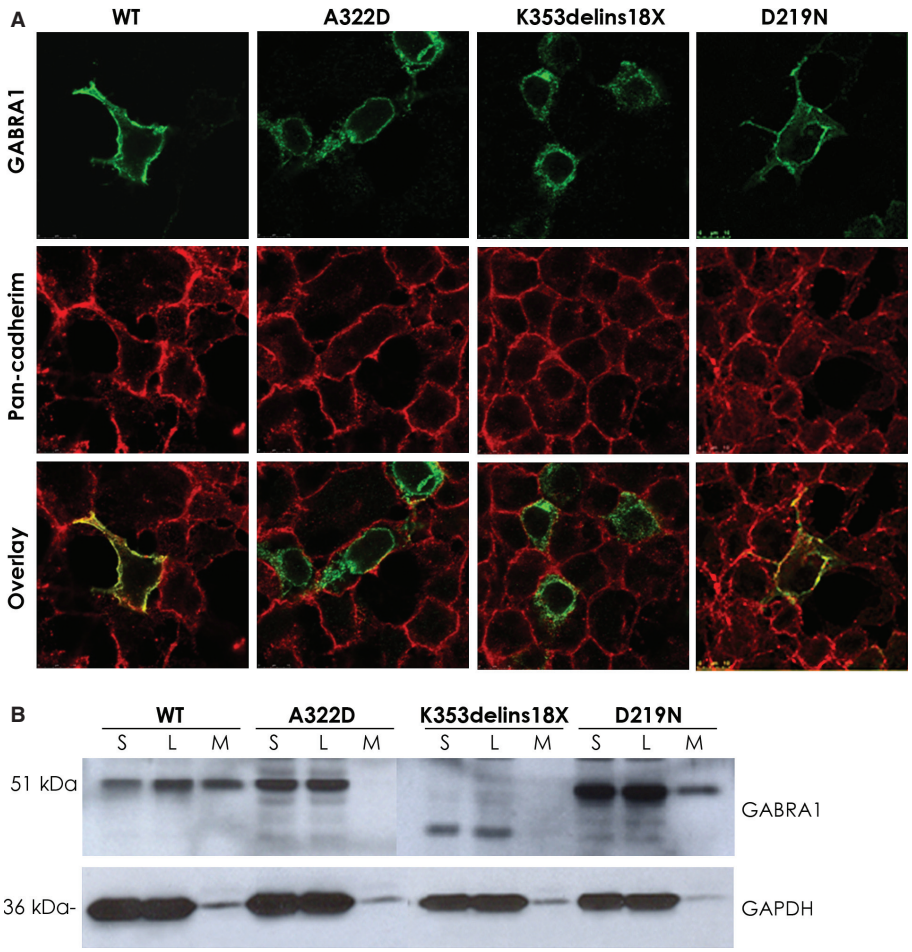


FIG. 4. Surface expression of the mutant α1 subunits. (A) In contrast to the wildtype α1 subunit (left column), immunostaining of A322D and K353delins18X *GABRA1* mutants did not co-localize with the marker for the cell membrane (pan-cadherin; red) when co-expressed with wildtype β2 and γ2 subunits. Staining for the D219N *GABRA1* mutant was also partially co-localized with pan-cadherin, suggesting that there is a reduction in surface expression of this subunit. (B) Western blot of total (L; lysate), biotinylated (M; membrane fraction) and non-biotinylated (S; supernatant) α1 proteins shows a reduction of approxiamtely 50% in the cell surface expression of the α1 D219N subunit. The absence of expression of the A322D and K353delins18X mutations at the cell surface is also shown. Consistent with the introduction of a premature stop codon, the K353delins18X subunit was detected at a lower molecular weight. GAPDH was used to normalize the supernatant and lysate proteins.

receptors, we examined their responsiveness to two known modulators, Zn^{2+} and the benzodiazepine diazepam, both of which are known to be dependent on the γ_2 subunit. For example, co-assembly with the γ2 subunit greatly diminishes sensitivity to Zn^{2+} block (Draguhn *et al.*, 1990) by disrupting some of its binding sites (Hosie *et al.*, 2003). Likewise, benzodiazepine potentiation of GABA-mediated responses is also γ2 subunit-dependent (Pritchett *et al.*, 1989) as it establishes a binding site at the interface with the α-subunit (Ernst *et al.*, 2005).

To examine Zn^{2+} sensitivity, block of 1 mM GABA-evoked responses by 10 μM $ZnCl_2$ was monitored in patches containing wildtype α1β2 or α1β2γ2 receptors which were compared with responses elicited by α1β2γ2(P83S) receptors (Fig. 8A). On average, Zn^{2+} reversibly antagonized α1β2 receptors by 86 ± 1% (n = 4 patches) whereas block of responses mediated by α1β2γ2 and α1β2γ2(P83S) receptors was much smaller (wildtype, 13 ± 4%, n = 6 patches; and P83S mutant, 13 ± 2%, n = 10 patches; $P < 0.0001$, one-tailed Student's t-test; Fig. 8B). Likewise, diazepam sensitivity was similar in α1β2γ2 and α1β2γ2(P83S) receptors (Fig. 9B and C). For example, although responses mediated by α1β2 receptors in 1 μM diazepam were unchanged (n = 3 patches), responses mediated by α1β2γ2 and α1β2γ2(P83S) receptors were potentiated by 20 ± 2 and 22 ± 2% respectively (n = 5 patches each, $P < 0.0001$, Student's paired t-test; Fig. 9B and C). The decrease in α1β2 receptors responses is most probably a consequence of time-dependent response rundown which probably leads to an underestimation of the potentiation of α1β2γ2 and α1β2γ2(P83S) receptors by diazepam. The summary plot in Fig. 9D reveals that there is a relationship between Zn^{2+} and diazepam sensitivity for the different $GABA_A$ receptors we have tested. α1β2 receptors were sensitive to Zn^{2+} block but insensitive to diazepam potentiation (blue squares). In contrast, wildtype α1β2γ2 receptors and those containing the mutant γ2 subunit (black circles and red triangles respectively) were resistant to block by Zn^{2+} but potentiated by diazepam. Taken together, these findings demonstrate that γ2 P83S mutation does not affect $GABA_A$ receptor sensitivity to Zn^{2+} or diazepam.

Discussion

Here we report the identification of three novel mutations in two $GABA_A$ receptor subunits found in patients with IGE and febrile seizures. The clinical manifestations observed in family C with the P83S mutation in *GABRG2* consist of a combination of febrile and absence seizures. This observation is consistent with the phenotype

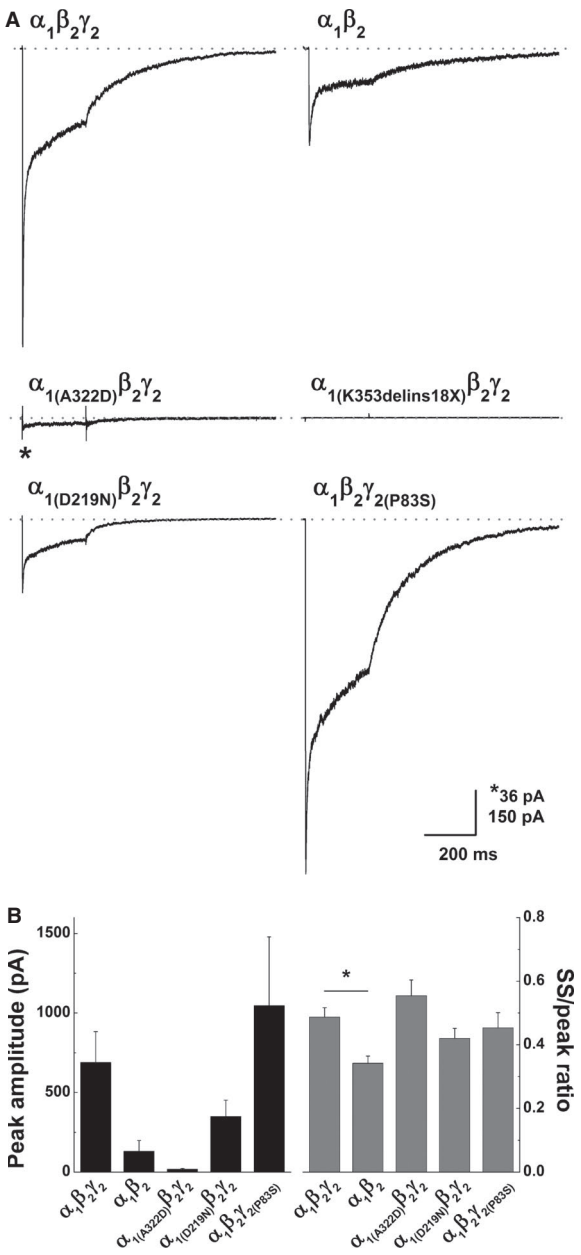


FIG. 5. Wildtype and mutant GABA-A receptors have different functional profiles. (A) Typical electrophysiological responses elicited by 1–10 mM GABA in the wildtype $\alpha1\beta2\gamma2$ (patch no. 081030p1) and $\alpha1\beta2$ (patch no. 090915p2), the α1 A322D (patch no. 081106p2), K353delins18X (patch no. 081219p3) and D219N (patch no. 090312p4) mutants, and in the γ2 P83S (patch no. 081219p1) mutant. (B) The left panel illustrates the mean peak current amplitudes obtained in patches containing each of the subunit combinations. Bars indicate means and error bars indicate SEM.

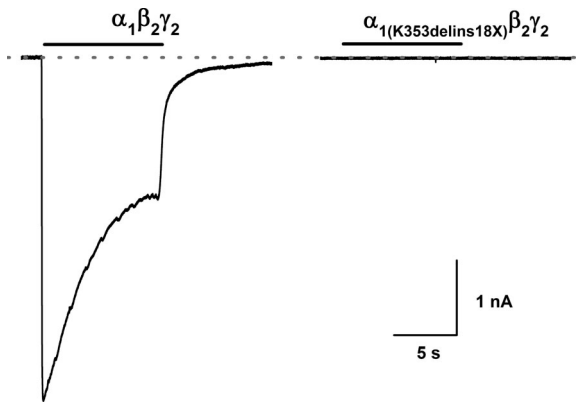


FIG. 6. α1 K353delins18X-containing $GABA_A$ receptors failed to express at the cell's surface. Typical whole-cell electrophysiological recordings of $GABA_A$ receptor responses elicited by 10-s GABA applications (100 μM) in tsA201 cells transfected with wildtype (patch no. 091001c5) or α1 K353delins18X-containing (patch no. 091001c9) receptors. All recordings in wildtype and mutant receptors were performed on the same day to control for transfection efficiency. Cells were voltage-clamped at −60 mV. Black bars indicate the duration of GABA application.

reported in other families with *GABRG2* mutations (Helbig *et al.*, 2008). So far, reports on mutations in *GABRA1* are limited to one family with juvenile myoclonic epilepsy (Cossette *et al.*, 2002) and a single individual with childhood absence epilepsy (Maljevic *et al.*, 2006). In contrast, family B with the D219N mutation in *GABRA1* exhibits mainly febrile seizures, with or without generalized tonic–clonic and absence seizures. Family A with the K353delins18X rather exhibits late-onset, afebrile, generalized tonic–clonic seizures as well as photosensitivity. Compared to *GABRG2*, febrile seizures thus seem to be less consistently associated with *GABRA1* mutations.

Mutations in the *GABRA1* gene, K353delins18X and D219N, segregate into two unrelated French Canadian families and were associated with disrupted plasma membrane delivery of mature protein as well as signaling deficits in the $GABA_A$ receptor. These mutations would be expected to reduce the strength of inhibitory transmission in neuronal circuits and, as a result, predispose the carrier to seizures; this line of reasoning is consistent with other studies purporting a prominent role of mutant $GABA_A$ receptors in IGE (Galanopoulou, 2010; Macdonald *et al.*, 2010). In contrast, we failed to identify any noticeable functional defect in the non-synonymous mutation P83S of the *GABRG2* gene. This observation is surprising as all other IGE-associated $GABA_A$ receptor mutants described to date exhibit defective behavior (Galanopoulou, 2010; Macdonald *et al.*, 2010). Considering its high degree of penetrance in three generations of a large extended French Canadian family and significant conservation across species (Supporting Information Fig. S1), as well as its absence in control chromosomes, all suggest that P83S is a real mutation and not a misclassified SNP. Whether this mutation disrupts a more nuanced aspect of $GABA_A$ receptor signalling such as may occur at the axon initial segment (Wimmer *et al.*, 2010), or it exhibits temperature sensitivity (Kang *et al.*, 2006), will require further experimentation.

Comparison with previous studies

Prior work has catalogued a total of 16 IGE-related variants in both translated and untranslated regions of $GABA_A$ receptor genes (Macdonald *et al.*, 2010). Although the specific molecular effects associated with each mutation can vary significantly from affecting mRNA transcript stability to disruption of channel gating (Macdonald *et al.*, 2010), their overall predicted effect at the level of the neuronal circuit is to undermine inhibition imposed by $GABA_A$ receptors. Consistent with this, electrophysiological experiments revealed that the kinetic properties of receptors assembled with the α1 D219N subunit are faster. Interestingly, the equivalent residue in the Torpedo nAChR is also negatively-charged, but in this case is a Glu which is thought to form an electrostatic interaction with the adjacent Arg (Unwin, 2005). If the α1 GABA receptor subunit adopts a similar structural arrangement, we would predict that this electrostatic

TABLE 2. Desensitization and off-kinetics of WT and mutant GABAA receptors

		Desensitization							Off-kinetics						
Comps	Receptor	τ fast	%	τ int.	%	τ slow	%	*n*	τ fast	%	τ int.	%	τ slow	%	*n*
2	α1β2γ2	2.8 ± 0.4	49	N/A		163 ± 39	51	13	27 ± 0	40	N/A		206 ± 26	60	21
	α1(D219N)β2γ2	2.2 ± 0.2	43	N/A		143 ± 52	57	4	6 ± 4	34	N/A		58 ± 6	66	2
	α1β2γ2(P83S)	2.3 ± 0.8	43	N/A		93 ± 36	57	5	34 ± 8	32	N/A		212 ± 38	69	7
3	α1β2γ2	2.4 ± 0.2	41	14 ± 2	22	279 ± 82	37	9	27 ± 0	17	70 ± 0	73	285 ± 0	10	1
	α1(D219N)β2γ2	1.3 ± 0.4	19	9 ± 4	35	121 ± 22	46	5	2.3 ± 0.4	21	26 ± 4	42	146 ± 18	37	7
	α1β2γ2(P83S)	2.4 ± 0.4	36	21 ± 5	31	287 ± 92	33	5	8 ± 2	4	66 ± 5	41	380 ± 102	55	3

Mean values for time constants (τ) are in ms and are ±SEM. Comps, number of exponential components; int, intermediate; %, percent contribution of component.

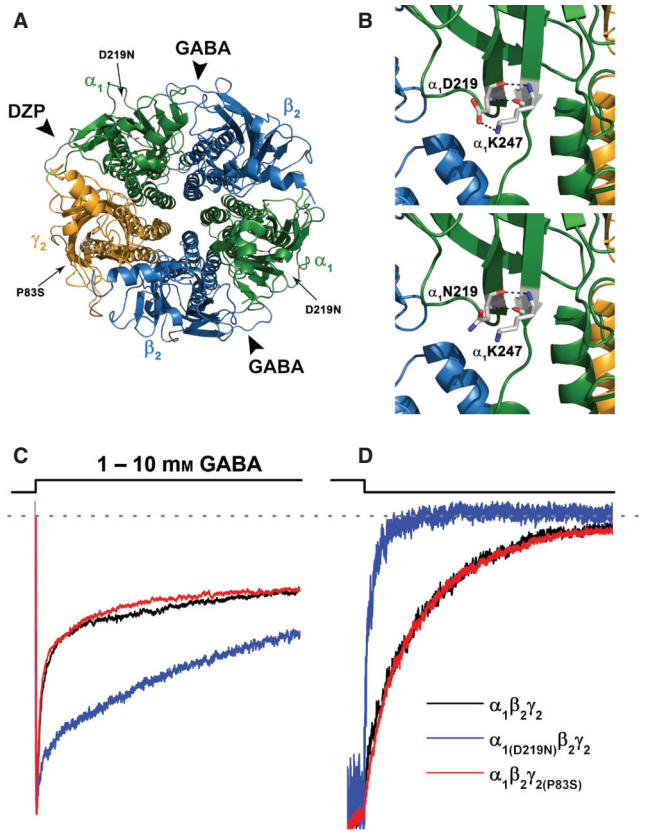


FIG. 7. The α1 D219N subunit disrupted receptor re-equilibration kinetics. (A) Structural model of the $GABA_A$ receptor (Ernst *et al.*, 2005) highlights the organization of the subunits within a pentameric complex as well as the proposed GABA and diazepam (DZP) binding sites. (B) Panels illustrate the possible location of the D219 and N219 residues at the interface between the putative transmembrane and extracellular domains using the *Torpedo marmorata* nAChR X-ray crystal structure [Protein Data Bank (PDB) accession number 2BG9]. (C) Typical electrophysiological responses elicited by 1–10 mM GABA in the wildtype α1β2γ2 (black, patch no. 081030p1), α1 D219N (blue, patch no. 081212p2) and γ2 P83S (red, patch no. 090914p2) mutants. The rates into desensitization for α1 D219N responses appear slower because of the increased contribution of the slow exponential component for this receptor. (D) Typical electrophysiological recordings showing the off-kinetics in the wildtype (patch no. 081030p1) and mutant (α1 D219N, patch no. 090312p2; γ2 P83S, patch no. 090803p1) receptors.

interaction is established between Asp219 and Lys247 (Fig. 7B). Replacement of Asp219 with an Asn would reduce the strength of this interaction and therefore may account for the accelerated $GABA_A$ receptor kinetics observed in our experiments.

The K353delins18X frameshift mutation alters the downstream amino acid sequence and results in the introduction of a premature translation–termination codon. This results in a truncation of the fourth transmembrane domain of the subunit and its retention in the ER (Figs 3 and 4). Other frameshift mutations in the coding sequence of *GABRA1* have been reported (975delC and S326fs328X; Kang *et al.*, 2009). Kang *et al.* suggest that premature translation can produce mRNA degradation through the activation of molecular pathways such as nonsense-mediated decay and reduced mutant α1 subunit mRNA. Because the α1 subunit is essential for the formation of functional $GABA_A$ receptors, a decrease in this subunit will also result in a decrease in surface $GABA_A$ receptors (Mizielinska *et al.*, 2006). In an intact neuronal system, the two novel *GABRA1* mutants, D219N and K353delins18X, could prove to be deleterious by causing an imbalance between excitation and inhibition. A decrease in function or expression could also lead to haploinsufficiency if the intact allele is not sufficient to produce the wildtype phenotype.

The paradox of the γ2 P83S subunit mutant

The $GABA_A$ receptor γ2 subunit fulfills a number of roles including the clustering of receptors and postsynaptic recruitment of the scaffolding protein gephyrin (Essrich *et al.*, 1998) as well as determining sensitivity to allosteric modulators such as Zn^{2+} and benzodiazepines (Olsen & Sieghart, 2009). The binding site for benzodiazepines occurs at the interface between α_1 and γ_2 (Sigel & Buhr, 1997; Boileau *et al.*, 1998) whereas the γ_2/β_2 subunit interface is the location of the γ_2 R82Q mutation that is implicated in childhood absence epilepsy and febrile seizures (Wallace *et al.*, 2001). As $GABA_A$ receptors containing the R82Q mutation exhibit altered kinetics and reduced benzodiazepine sensitivity (via a long-distance effect) as well as defective trafficking (Bowser *et al.*, 2002; Kang *et al.*, 2006; Goldschen-Ohm *et al.*, 2010), we anticipated a marked phenotype for the adjacent P83S mutant. Instead, we failed to observe any defective behaviour in terms of functional expression levels or sensitivity to benzodiazepines or Zn^{2+}. Given this, we conclude that Pro83 does not participate in the stability of the salt-bridge proposed to be a critical determinant of benzodiazepine efficacy (Goldschen-Ohm *et al.*, 2010). It would be interesting in future experiments to determine whether an appreciable phenotype can be assigned to the P83S mutant when $GABA_A$ receptors are assembled from β1 or β3 subunits rather than the β2 subunit used in the present study.

Working towards the molecular basis of IGE

Although the cause of the common IGE syndromes is unknown, there are increasing examples of familial cases that are genetic in origin

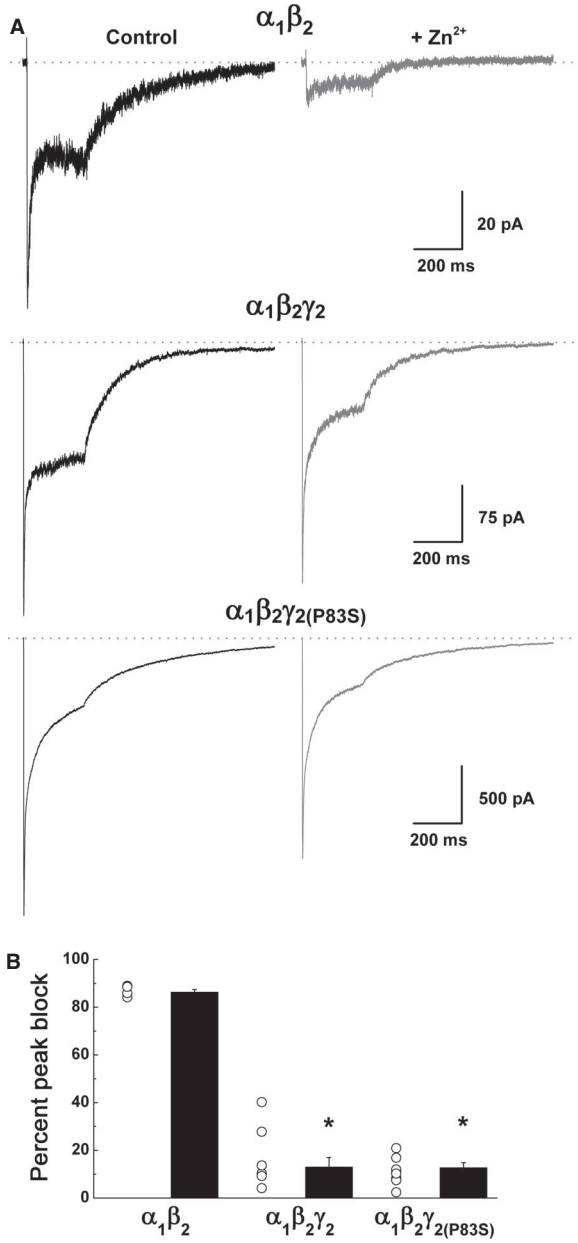


FIG. 8. The γ2 P83S mutation does not affect Zn^{2+} block of GABA-A receptors. (A) Typical electrophysiological responses elicited by 1 mM GABA in the absence (black) or presence (grey) of 10 μM Zn^{2+} for the wildtype $\alpha1\beta2$ (top row, patch no. 090914p1) and $\alpha1\beta2\gamma2$ (middle row, patch no. 090721p1) receptors, as well as the γ2 P83S-containing receptors (lower row, patch no. 090924p2). (B) This panel shows the mean percentage of peak current that was blocked by 10 μM Zn^{2+}. Bars indicate means and error bars indicate SEM. Circles are individual data.

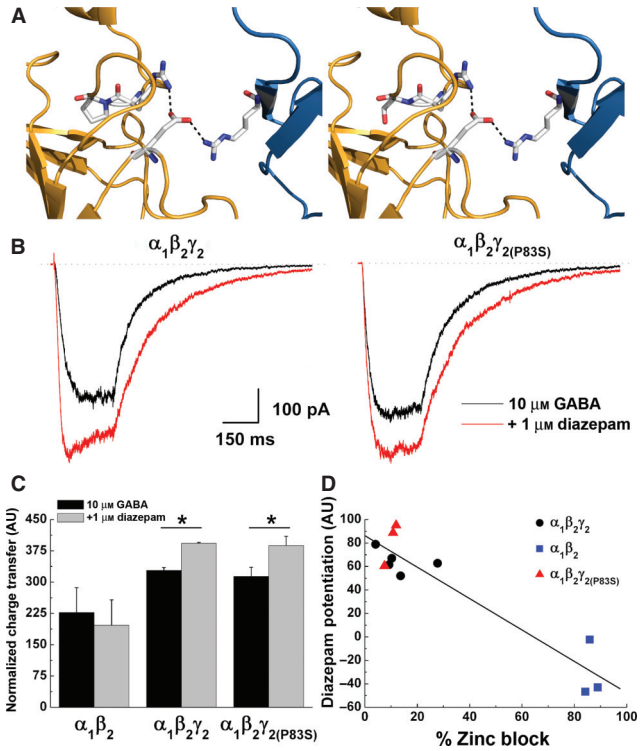


FIG. 9. The γ2 P83S mutation does not affect diazepam regulation of GABA-A receptors. (A) Homology model showing the possible conformation of the P83 and S83 residues at the interface between the γ2 and β2 subunits (PDB 2BG9). The three residues thought to allosterically modulate the benzodiazepine-binding site (γ2R82, γ2E178 and β2R117) are also shown, and do not appear to be disrupted by the P83S mutation. (B) Example electrophysiological traces in wildtype (patch no. 090713p4) and γ2 P83S-containing receptors (patch no. 090724p1) showing the response modulation by 1 μM diazepam. Mutant responses were indistinguishable from wildtype responses. (C) Diazepam potentiation was characterized by measuring the area under the curve for each response. This panel summarizes the mean charge transfer (in arbitrary units) for the $\alpha1\beta2$, $\alpha1\beta2\gamma2$ and $\alpha1\beta2\gamma2$(P83S) receptors. Bars indicate means and error bars indicate SEM. Responses from γ2-containing receptors were significantly potentiated by diazepam. (D) This graph demonstrates the relationship between Zn^{2+} block and diazepam potentiation for $\alpha1\beta2$, $\alpha1\beta2\gamma2$ and $\alpha1\beta2\gamma2$(P83S) receptors. Receptors containing a γ2 subunit (wildtype or mutant) exhibited substantial diazepam potentiation and little Zn^{2+} block, while receptors lacking a γ2 subunit showed the opposite relationship.

(Scheffer & Berkovic, 2003), most probably involving several interacting factors. It has also been proposed that it represents a 'channelopathy' given the preponderance of genetic defects found in ion channels expressed by individuals with familial IGE (Scheffer & Berkovic, 2003; Hahn & Neubauer, 2009; Catterall *et al.*, 2010; Macdonald *et al.*, 2010; Zamponi *et al.*, 2010). Understanding the molecular basis of IGE, as it relates to the $GABA_A$ receptor, also needs to take into account the effect of genetics on the susceptibility of individual neuronal cell types and their neuronal circuits as has emerged from studying $Na_V1.1$ channels in epilepsy (Catterall *et al.*, 2010). By comparing the phenotypic properties of homozygous null or heterozygous $Na_V1.1$ mice, several groups have noticed that lesions in the hippocampal circuit primarily affect the excitability of GABAergic interneurons, which control circuit inhibition, whilst having little or no effect on the firing properties of the excitatory pyramidal cells (Yu *et al.*, 2006; Ogiwara *et al.*, 2007). In other words, although all neuronal cell types either lack or have reduced $Na_V1.1$ signaling in either null or heterozygous mice respectively, the defect almost exclusively affects GABAergic interneurons, an observation that could not have been appreciated from genetic studies alone.

A similar strategy to examine the $GABA_A$ receptor γ2 subunit mutant R82Q, which is associated with familial childhood absence epilepsy, has also led to unexpected insights. Here, the onset of seizures in heterozygous mice occurs abruptly during development (around postnatal day 20) as occurs in the human condition (Tan *et al.*, 2007). In support of this, seizure susceptibility was significantly reduced when expression of the γ2 (R82Q) subunit was delayed until after development (Chiu *et al.*, 2008), confirming that the onset of symptoms is dependent on a critical period. Interestingly, more recent

work shows that, unlike its wildtype counterpart, the γ2 (R82Q) subunit is excluded from the axon initial segment (Wimmer *et al.*, 2010), the site of action potential initiation in neurons (Stuart *et al.*, 1997). Clearly, further insight into the three novel $GABA_A$ receptor mutants described in this study will require careful consideration of how they disrupt neuronal circuit behavior, from an appreciation of their developmental expression pattern to their distribution within distinct populations of neurons.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Peptide sequence alignment of a portion of the GABRA1 and GABRG2 proteins from different species.

Table S1. Clinical manifestations of the 16 affected individuals carrying novel GABAA receptor mutations.

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Abbreviations

ER, endoplasmic reticulum; GABA, γ-amino-butyric acid; IGE, idiopathic generalized epilepsy; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; SNP, single-nucleotide polymorphism.

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