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A Novel GABRG2 Mutation, p.R136*, in a family with GEFS+ and extended phenotypes

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Abstract

Genetic mutations in voltage-gated and ligand-gated ion channel genes have been identified in a small number of Mendelian families with genetic generalised epilepsies (GGEs). They are commonly associated with febrile seizures (FS), childhood absence epilepsy (CAE) and particularly with generalised or genetic epilepsy with febrile seizures plus (GEFS+). In clinical practice, despite efforts to categorise epilepsy and epilepsy families into syndromic diagnoses, many generalised epilepsies remain unclassified with a presumed genetic basis. During the systematic collection of epilepsy families, we assembled a cohort of families with evidence of GEFS+ and screened for variations in the $\gamma 2$ subunit of the γ -aminobutyric acid (GABA) type A receptor gene (*GABRG2*). We detected a novel *GABRG2*(p.R136*) premature translation termination codon in one index-case from a two-generation nuclear family, presenting with an unclassified GGE, a borderline GEFS+ phenotype with learning difficulties and autism spectrum disorder (ASD). The *GABRG2*(p.R136*) mutation segregates with the febrile seizure component of this family's GGE and is absent in 190 healthy control samples. *In vitro* expression assays

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Competing Interests

All authors have confirmed that there are no competing interests in connection to this work.

demonstrated that $\gamma 2(p.R136^*)$ subunits were produced, but had reduced cell-surface and total expression. When $\gamma 2(p.R136^*)$ subunits were co-expressed with $\alpha 1$ and $\beta 2$ subunits in HEK 293T cells, GABA-evoked currents were reduced. Furthermore, $\gamma 2(p.R136^*)$ subunits were highly-expressed in intracellular aggregations surrounding the nucleus and endoplasmic reticulum (ER), suggesting compromised receptor trafficking. A novel *GABRG2*(p.R136^{*}) mutation extends the spectrum of *GABRG2* mutations identified in GEFS+ and GGE phenotypes, causes GABA_A receptor dysfunction, and represents a putative epilepsy mechanism.

Keywords

GABAA receptors; epilepsy; protein truncating mutations; autism spectrum disorder

Introduction

Genetic generalised epilepsies (GGE) account for approximately 30% of all epilepsies and are considered to have a complex genetic basis. This is reflected in the recent nomenclature revision where idiopathic is deemed to be synonymous with ‘genetic’ (Berg et al., 2010). GGEs are characterised by generalised spike and wave discharges on electroencephalogram (EEG) and comprise a number of distinct and overlapping clinical syndromes that vary in severity. These include specific electro-clinical syndromes such as: childhood absence epilepsy (CAE); juvenile myoclonic epilepsy (JME), epilepsy with generalised tonic-clonic seizures on awakening, and generalised epilepsy with febrile seizures (FS) plus (GEFS+), where FS occur in association with GGE. Over the last decade genetic mutations in voltage-gated ion channels (sodium, calcium and potassium channel subunits) and in ligand-gated ion channels (nicotinic cholinergic and GABA_A receptor subunits) have been reported in a small number of GGEs (Heron et al., 2007; Macdonald and Kang, 2009).

GEFS+, also more recently termed genetic epilepsy with febrile seizures plus, is a well-described familial epilepsy syndrome, characterised by heterogeneous epilepsy phenotypes. Although initially described in large families with autosomal dominant inheritance and incomplete penetrance, most GEFS+ families are now considered to follow a complex inheritance pattern and significant overlap with GGE is observed (Scheffer and Berkovic 1997; Singh et al., 1999). GEFS+ is considered genetically-heterogeneous segregating in a small number of GEFS+ families with mutations in genes encoding neuronal voltage-gated sodium channel subunits (*SCN1A*, *SCN2A* and *SCN1B*) and ligand-gated GABA_A receptor subunits (*GABRG2* and *GABRD*) (Wallace et al., 1998; Escayg et al., 2000; Baulac et al 2001; Wallace et al 2001). The most common genetic abnormalities identified to date are missense *SCN1A* mutations, thought to account for approximately 10% of GEFS+ families (Marini et al., 2007).

GABA is the major inhibitory neurotransmitter in the mammalian brain and mutations in genes encoding $\alpha 1$, $\beta 3$, $\gamma 2$ and δ GABA_A receptor subunits have been associated with different GGE spectrum epilepsies such as simple FS, CAE and GEFS+ (Harkin et al., 2002; Kananura et al., 2002; Dibbens et al., 2004; Adenaert et al., 2006; Sun et al., 2008; Tanaka et al., 2008; Tian et al., 2013). GABA_A receptor dysfunction was first demonstrated by the

identification of the GABRG2(p.R82Q) mutation in a family with CAE and FS, and the GABRG2(p.K328M) mutation in a GEFS+ family. This was followed by further mutations described in six unrelated index-cases, underscoring the critical role of $\gamma 2$ subunits for receptor trafficking, targeting, clustering and synaptic maintenance and response to benzodiazepine modulators (Essrich et al., 1998; Moss et al 2001; Schweizer et al., 2003; Keller et al., 2004; Rathenberg and Moss 2004).

Functional studies have suggested that these mutations alter receptor biogenesis or function via impaired receptor subunit mRNA stability, protein folding and stability, or assembly and receptor trafficking (Macdonald and Kang., 2009). Recently, two cellular biological control mechanisms were proposed, nonsense-mediated decay (NMD) and ER-associated degradation (ERAD), that regulate the effects of premature translation-termination codons (PTCs) in GABA_A receptor subunit genes associated with epilepsy (Kang et al., 2009).

In this study, we screened *SCN1A* and *GABRG2* in affected index-cases from 24 families with a history of GEFS+ from our growing collection of familial epilepsy in the UK (Johnston et al., 2009; Thomas et al., 2012). A novel nonsense mutation GABRG2(p.R136*) was discovered in a borderline GEFS+/unclassified GGE family that segregates with the FS component of the phenotype, and we provide *in vitro* evidence for pathogenicity of $\gamma 2$ (p.R136*) subunits in relation to the seizure phenotype.

Methods

Clinical Evaluation and Sample Collection

Families for the genetic study were sourced through clinician tertiary referrals, from an established epilepsy clinical database and through self-referral. Written informed consent was obtained from all participants to access the relevant results from investigations such as, electrocardiogram, EEG, or brain imaging (approved by MREC for Wales, 05/MRE09/78). Clinical characteristics and genealogical information was ascertained via a semi-structured interview and a clinical examination was performed. The phenotypes were recorded according to the commission on Classification and Terminology of the International League Against Epilepsy, 1981; and of Epilepsy Syndromes, 1989 with reference to recent revisions (Commission on Classification and Terminology of the International League Against Epilepsy., 1981; Engel., 2001; Engel., 2006). We used the original definition for a GEFS+ families - two or more individuals with a GEFS+ phenotype (Scheffer and Berkovic., 1997). Sets of clinical characteristics were used to subdivide the epilepsy phenotypes into 'endophenotypes'.

Mutation Analysis

From eighty ascertained epilepsy families who had consented to genetic studies, fourteen were classified as probable GEFS+ and ten as having a borderline GEFS+ family phenotype (Thomas et al., 2012). We tested eighteen participants sourced from nine of these GEFS+ or borderline GEFS+ families for variations in *SCN1A* (OMIM: 182389) and *GABRG2* (OMIM: 137164) genes. Polymerase chain reaction was used to amplify *GABRG2* and *SCN1A* coding regions and splice sites and amplicons were sequenced with ABI capillary

technology (Foster City, CA). Population studies on identified gene-mutants were performed using LightScanner™ high-resolution melting (Idaho Technology, USA) and scrutinising bioinformatics databases.

Bioinformatics and structural modeling

Posttranslational modifications and trafficking of wild-type and *GABRG2(R136X)* sequences were predicted using PSORTII and PROSCAN (Horton and Nakai., 1997; Combet et al., 2000). Structural modelling of wild-type $\gamma 2$ and mutant $\gamma 2(p.R136^*)$ subunits was carried out using a homology modelling pipeline built with the Biskit structural bioinformatics platform, which scans the entire Protein DataBank (PDB) for candidate homologies (Grunberg et al., 2007). The best homology attained for $\gamma 2$ subunits was based on 21% identity with the crystal structure (PDB: 2BG9) of the nicotinic acetylcholine receptor from *Torpedo marmorata* (Unwin., 2005). The pipeline workflow incorporates the NCBI tools platform, including the BLAST program for similarity searching of sequence databases (Wheeler et al., 2007; Altschul et al., 1990). T-COFFEE was used for alignment of the test sequence with the template and homology models were generated over 10 iterations of the MODELLER program (Notredame et al., 2000; Eswar et al., 2003). All models were visualized using the molecular graphics program Chimera (Pettersen et al., 2004).

GABA_A receptor expression constructs

Complementary DNA (cDNA) was generated from human brain using the Superscript III First-Strand Synthesis System Supermix III (Clontech Cat no. 18080-400). Gene-specific primers (supplementary information) were used to amplify each GABA_A receptor subunit. The cDNAs encoding human *GABRA1*, *GABRB2* and *GABRG2* were cloned into the expression vector pcDNA3.1/V5-His TOPO TA vector (Invitrogen Cat no. 45-0005). The GABA_A receptor tagged or untagged cDNAs and minigenes used for flow cytometry, biochemistry, electrophysiology and confocal microscopy have been described before (Kang et al., 2009). In the transfection of $\alpha 1$, $\beta 2$ and the mutant $\gamma 2$ subunits with different ratios, the total amount of cDNA was normalized by the empty vector pcDNA. The *GABRG2(p.R136^*)* mutation was generated using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. In this paper all mutations were identified in the immature peptide, which has been the convention in the literature for $\alpha 1$, $\beta 3$ and δ subunit mutations and variants but not for $\gamma 2$ subunit mutations. In most reports, $\gamma 2$ subunit mutations have been identified in the mature peptide. For example, the $\gamma 2$ subunit mutation p.K328M has been reported as the p.K289M mutation (the signal peptide is 39 aa long).

Electrophysiology and the surface protein biotinylation

Lifted whole-cell recordings were obtained from transfected HEK 293T cells as previously described (Kang and Macdonald, 2004). Cells were voltage-clamped at -50 mV and E_{Cl} was 0 mV. Zinc (10 μ M) was co-applied with GABA (1 mM) after pre-application for 6 sec. The cells were washed with control solution for 45 sec after zinc and GABA co-application, and only currents that returned to baseline were included in the analysis. The GABA_A receptor

subunit surface biotinylation was performed as previously described (Kang and Macdonald, 2004).

Cell culture, magnetofection and immunocytochemistry

PC12 cells were grown in DMEM with sodium pyruvate, pyridoxine and without L-glutamate (Gibco #21969-035) containing 10% foetal bovine serum (Gibco #10106-110), 5% horse serum (Sigma #H1270), 1x penicillin, streptomycin, and L-glutamate (Sigma #G1146) and 1% 1M HEPES, pH 7.4 (Fisher #H/2230/48). The cells were plated onto collagen-coated glass coverslips and 24 hours later were transfected using a magnetofection protocol (Buerli et al., 2007). Sixty hours after magnetofection, the medium was replaced with differentiation medium (DMEM with 1% horse serum, 1x penicillin, streptomycin, and L-glutamate and 1% 1M HEPES, pH 7.4) containing 100 ng/ml nerve growth factor (NGF, Calbiochem #480354) inducing neuronal differentiation (Shafer et al., 1991a, 1991b). Seventy-two hours later, PC12 cells were fixed with ice-cold 100% methanol for 3 minutes, followed by washing with PBS and permeabilization for 10 minutes at room temperature using 0.5% Triton-X 100 in PBS. Fixed cells were rinsed in PBS and incubated for 10 minutes at room temperature in blocking solution (0.1% Triton-X 100 in PBS and 2% BSA). Cells were incubated for 90-120 minutes at room temperature with the following primary antibodies diluted in blocking solution: rabbit polyclonal anti- $\gamma 2$ subunit (Sigma #G0545, diluted 1:50), and mouse monoclonal anti- $\beta 2,3$ subunit (bd17, Chemicon #MAB341, diluted 1:75). Cells were subsequently rinsed and incubated with secondary antibody coupled to AlexaFluor 488 or 568 (Molecular Probes, diluted 1:400) for 30-60 minutes at room temperature in blocking solution. Cells were dried, mounted in DAPI (Vector laboratories) and stored at 4°C. The Cos-7 cells or rat cortical neurons were cultured and transfected with $\gamma 2^{YFP}$ or $\gamma 2(p.R136^*)^{YFP}$ using the Ca^{2+} -phosphate precipitation method. Live Cos-7 cells were visualized at 2 days after transfection. Live neurons were transfected at day 7 in culture and were directly visualized at 8 days after transfection (15 days in culture) (Kang et al., 2010). All samples and controls were analysed and imaged by confocal laser-scanning microscopy using a Zeiss LSM510 META confocal system.

Results

Molecular Genetics

A novel *GABRG2* mutation was identified in a borderline GEFS+/GGE family (Fig. 1A) and no *SCN1A* variation was identified beyond known SNPs. A novel heterozygous *GABRG2* mutation was generated by a C>T substitution at position 406 in the nucleotide sequence, (c. 406C>T), and introduced a premature stop codon (TGA) at a highly-conserved arginine residue at position 136 in the immature *GABRG2* polypeptide sequence (p.R136*). This results in a truncated *GABRG2* protein with loss of all four transmembrane domains and C-terminus and stunted retention of the N-terminus elements (Fig. 1B,C). This novel *GABRG2*(p.R136*) mutation was subsequently found in three other nuclear family members segregating strongly with the FS component of the clinical presentation (Fig. 1D). The *GABRG2* c.406C>T genotype joins a small compendium of other *GABRG2* mutants (Fig. 1E) in the literature and was not present in 190 healthy unrelated control samples or database resources.

Clinical Segregation Analysis

From the family pedigree and clinical characteristics (Fig. 2, Table 1), the *GABRG2*(p.R136*) mutation does not segregate purely with those clinically-affected with epilepsy. Further analysis through seizure-type endophenotyping was performed considering six main seizure presentations including FS, afebrile seizures, generalised tonic-clonic seizures, absence seizures, myoclonic jerks and eyelid myoclonia. Endophenotyping of *GABRG2*(p.R136*) suggested that the mutation segregates most closely with presentation of FS (Fig. 2).

Clinical Summaries of the Affected Family Members

Individual II-1 has a history of childhood seizures suggestive of FS and atypical eyelid myoclonia. Suffers from Migraine.

Individual II-2 is not clinically affected.

Individual III-3 is the index case harbouring the *GABRG2* p.R136* mutation and suffers from generalised epilepsy, not typical of classical GGE. He has a history of FS and afebrile GTCS seizures. He also has intellectual disability and autism spectrum disorder (ASD). Educationally he requires a high level of one-to-one support. He is treated with sodium valproate and levetiracetam.

Individual III-4 has an unclassified generalised epilepsy with moderate to severe intellectual disability and ASD. She has an educational statement and is in special needs schooling. She is treated with sodium valproate and levetiracetam.

Individual III-5 has an unclassified generalised epilepsy and mild intellectual disability. She also has an educational statement at school and requires full time learning support; her main difficulties are in literacy and numeracy skills. She is treated with carbamazepine and levetiracetam.

Individual III-6 has an unclassified generalised epilepsy. She has been assessed by a community paediatrician and had a Connors scale assessment, however, does not meet with the criteria for ASD or attention deficit hyperactivity disorder (ADHD). She is treated with carbamazepine.

Individual III-7 has joint hypermobility and mild delay in motor skills but does not have epilepsy at present.

All of the children with epilepsy continue to have multiple seizures and are so far refractory to medication despite trying different AEDs, alone or in combination.

Molecular Modeling and Bioinformatics

The full-length 467aa wild-type *GABRG2* subunit and the novel mutant *GABRG2*(p.R136*) subunit were modelled by 21% homology with the crystal structure of the nicotinic acetylcholine receptor (Fig. 1C).³¹ N-terminal regions are characteristically highly flexible, and the modelled structure of residues 1-97 appear highly unravelled. Residues 1-25 could

not be reliably modelled due to a lack of coverage for this section by homologues in the PDB. The truncated mutant would be expected to run to the middle of the main beta sheet of the N-terminal region. Bioinformatic analysis of the mutant $\gamma 2(p.R136^*)$ subunit demonstrated an ER retention signal KKXX-like motif (IKVL) in its C-terminal section in contrast to wild-type subunits. The truncated protein following the signal cleavage site was also rich in sites for at least 9 potential posttranslational modifications including 2 N-glycosylation sites (13-16 and 90-93), 3 protein kinase C phosphorylation sites (12-14, 19-21, 92-94), 2 casein kinase II phosphorylation sites (3-6 and 81-84), one tyrosine kinase phosphorylation site (2-10) and one N-myristoylation site (47-52).

In vitro Functional Analysis of the $\gamma 2(R136X)$ Subunit

The cellular fate of mutant $\gamma 2(p.R136^*)$ subunits was studied using both recombinant subunit cDNAs and nonsense-mediated mRNA decay (NMD) competent minigene strategies. Since nonsense mutations in early exons may activate NMD and the R136X mutation is located in one of the early exons, it could activate NMD to degrade the mutant $\gamma 2$ subunit mRNA. We thus included a minigene strategy to study the $\gamma 2(p.R136^*)$ subunit mutation as previously used for $\gamma 2$ subunit truncation mutations.²² We have demonstrated in our previous study that the minigene strategy is feasible to study GABA_A receptor truncation mutations because it contains an intron that is required for mRNA processing if nonsense-mediated mRNA decay is involved.

Mutant $\gamma 2(R136X)$ subunits were produced but were not trafficked to the cell surface

HEK 293T cells were co-transfected with $\alpha 1$, $\beta 2$ and wild-type $\gamma 2S^{HA}$ or mutant $\gamma 2S(p.R136^*)^{HA}$ subunit cDNAs. Surface expression of wild-type $\gamma 2S^{HA}$ or mutant $\gamma 2S(p.R136^*)^{HA}$ subunits was measured with the quantitative method of flow cytometry using Alexa 647 fluorophores (Fig. 3A). Mutant $\gamma 2S(p.R136^*)^{HA}$ subunits only had minimal surface-expression relative to wild-type $\gamma 2S^{HA}$ subunits ($0.05 \pm 0.04\%$ of wild-type fluorescence) (Fig. 3B). The lack of surface expression of mutant $\gamma 2S(p.R136^*)^{HA}$ subunits could be due to compromised subunit trafficking or due to activation of NMD by the p.R136* PTC since it was located in an early exon. We then studied the total protein expression of $\gamma 2(p.R136^*)$ subunits with both intronless cDNAs and intron-containing minigenes. We transfected $\alpha 1$ and $\beta 2$ with $\gamma 2S^{HA}$ or $\gamma 2S(p.R136^*)^{HA}$ subunits in cDNAs (Fig. 3C, left) and with $\gamma 2$ or $\gamma 2(p.R136^*)$ subunit minigenes (Fig. 3C, right). As expected, wild-type $\gamma 2^{HA}$ subunits migrated at about 50 KDa. Interestingly, mutant $\gamma 2S(p.R136^*)^{HA}$ subunits migrated in the 10-30 KDa area as multiple bands. The molecular mass of the mutant truncated $\gamma 2(p.R136^*)$ subunit was predicted to be 11.8 KDa after signal peptide cleavage. The nature of these multiple bands is unclear but is likely to be due to different glycosylation forms of $\gamma 2(p.R136^*)$ subunits as monomers or dimers. Further study is required to determine whether mutant $\gamma 2(p.R136^*)$ subunits dimerize or form oligomers as observed in other $\gamma 2$ truncated subunits (Kang and Macdonald, 2009). Nevertheless, compared to wild-type subunits, levels of both mutant $\gamma 2S(p.R136^*)^{HA}$ subunit cDNA (1 vs 0.67 ± 0.05 , $n = 4$) and $\gamma 2(p.R136^*)$ subunit minigene (1 vs 0.42 ± 0.02 , $n = 4$) were reduced (Fig. 3D).

Mutant $\gamma 2(p.R136^*)$ subunits did not assemble into functional receptors, and the receptors formed were likely $\alpha 1\beta 2$ receptors

$\gamma 2$ subunits are not required for GABA_A receptor assembly (Angelotti et al., 1993a). When $\gamma 2$ subunits are absent or have impaired trafficking, $\alpha 1\beta 2$ receptors will form that have smaller channel conductance and current amplitude and different pharmacological properties (Angelotti and Macdonald, 1993b). We compared the current amplitudes from HEK 293T cells transfected with $\alpha 1$, $\beta 2$ and $\gamma 2$ subunit or $\alpha 1$, $\beta 2$ and $\gamma 2(p.R136^*)$ subunit cDNAs in a 1:1:1 cDNA ratio (Fig. 4A). Peak amplitudes of $\alpha 1\beta 2\gamma 2S(p.R136^*)$ receptor currents (907 ± 238 , $n = 14$) were smaller than those of wild-type currents (4563 ± 467 , $n = 9$) (Fig. 4B). As demonstrated in Figure 3, mutant $\gamma 2S(p.R136^*)$ subunits had minimal surface-expression, suggesting that currents recorded with co-expression of $\alpha 1$, $\beta 2$ and $\gamma 2S(p.R136^*)$ subunits were likely from $\alpha 1\beta 2$ receptors, which are very sensitive to zinc inhibition. With 6 sec co-applications of GABA (1 mM) and zinc (10 μ M), peak amplitudes of currents recorded from receptors formed with wild-type $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits had minimal reduction compared with control currents (Fig. 4C) ($7.2\% \pm 3.4\%$, $n = 4$). However, currents recorded from receptors formed with $\alpha 1$, $\beta 2$ and $\gamma 2(p.R136^*)$ subunits were almost abolished with co-application of GABA (1 mM) and zinc (10 μ M) ($93.4\% \pm 17\%$, $n = 4$, suggesting that $\alpha 1\beta 2$ receptors are the major receptor present with co-expression of $\alpha 1$, $\beta 2$ and $\gamma 2(p.R136^*)$ subunits. The formation of $\alpha 1\beta 2$ receptors was further supported by the evidence that the $\alpha 1$ subunit surface expression was not altered in the mutant $\alpha 1\beta 2\gamma 2(p.R136^*)$ receptors compared with the wildtype $\alpha 1\beta 2\gamma 2$ receptors (Figure 4D, E). This also suggests that there is no dominant negative suppression from the mutant $\gamma 2(p.R136^*)$ subunits on the wildtype partnering subunits like $\alpha 1$. With the increased mutant $\gamma 2(p.R136^*)$ subunits, the total expression of $\alpha 1$ and $\beta 2$ subunits was reduced at the ratio of 1:1:5 ($\alpha 1:\beta 2:\gamma 2(p.R136^*)$) (Figure 4F to I), suggesting the dominant negative effect of the mutant $\gamma 2(p.R136^*)$ subunits on the wildtype partnering subunits only at a high amount. This dominant negative suppression of $\gamma 2(p.R136^*)$ subunit at high amounts was smaller compared with $\gamma 2(Q390X)$, another mutant subunit associated with a more severe epilepsy phenotype Dravet syndrome (Kang et al., 2009; Harkin et al., 2002) (Figure 4F to I).

After co-expression of $\alpha 1$, $\beta 2$, and wild-type $\gamma 2$ or mutant $\gamma 2(p.R136^*)$ subunits in PC12 cells, we determined the surface and intracellular distribution of $\gamma 2(p.R136^*)$ and $\gamma 2$ subunits by co-labelling with anti- $\gamma 2$ (red) and anti $\beta 2,3$ antibodies (green) and using confocal microscopy. Wild-type, $\gamma 2$ subunit immuno-fluorescence had a smooth uniform distribution with clusters that could be detected mainly on the surface (cell membrane), with less prominent intracellular staining (Fig. 5 A, B) and co-localizing with $\beta 2$ subunit immunoreactivity (Fig. 5C, D). Expression of $\gamma 2(p.R136^*)$ subunits resulted in less cell-surface immunoreactivity and more diffuse labelling (Fig. 5E). We also observed the presence of intracellular aggregates, consistent with staining within ER and peri-nuclear regions (Fig. 5F-H).

Consistent to what has been observed in PC12 cells, the coexpression of $\alpha 1$, $\beta 2$ and wild-type or the mutant $\gamma 2(p.R136^*)$ subunits tagged with the yellow fluorescent protein (YFP) in COS-7 had similar expression pattern as seen in PC12 cells. The subcellular localization of $\alpha 1\beta 2\gamma 2^{YFP}$ receptors in COS-7 cells were determined with confocal microscopy 2 days later

after transfection. Similar to the the observation in PC 12 cells, wild-type $\gamma 2^{YFP}$ subunits were present both intracellularly and on the cell-surface while mutant $\gamma 2(p.R136^*)^{YFP}$ subunits were localized around the nuclear region in aggregated fluorescence dots without cell surface-expression (Fig. 6A). Total fluorescence intensity was lower for mutant $\gamma 2(p.R136^*)^{YFP}$ subunits than for wild-type $\gamma 2^{YFP}$ subunits (0.61 ± 0.077 vs 1). We then transfected the wild-type $\gamma 2^{YFP}$ or mutant $\gamma 2(p.R136^*)^{YFP}$ subunits in cultured cortical neurons and visualized the neurons 8 days after transfection (Fig. 6B). Wild-type $\gamma 2^{YFP}$ subunits had widespread distribution in both somata and neuronal processes including dendrites and axons while mutant $\gamma 2(p.R136^*)^{YFP}$ subunits were distributed only in neuronal somata. Compared with wild-type $\gamma 2^{YFP}$ subunits, the fluorescence intensity ratio (process divided by soma fluorescence intensities) was much lower in neurons expressing $\gamma 2(p.R136^*)^{YFP}$ subunits (1.4 ± 0.063 vs 0.09 ± 0.05). However, both the wild-type and the mutant $\gamma 2$ subunits appeared as puncta in neurons.

Discussion

Families with evidence of GEFS+ or FS have been collected, from which a panel of index-cases were assembled. By mutation screening the coding regions of *SCN1A* and *GABRG2*, we discovered a novel p.R136* PTC mutation in the $\gamma 2$ subunit of the GABA_A receptor segregating with the familial FS endophenotype. The *in vitro* functional characterisation of the novel *GABRG2(p.R136*)* mutation provides cellular evidence in support of GABA_A receptor functional impairment. The co-expression of mutant $\gamma 2(p.R136^*)$ subunits with wild-type $\alpha 1$ and $\beta 2$ subunits, demonstrated that receptors formed had reduced current amplitudes and reduced cell surface expression levels with greater intracellular retention compared to wild-type $\gamma 2$ subunits. This suggests that the *GABRG2(p.R136*)* mutation impairs receptor channel function and alters receptor trafficking. The increased zinc sensitivity of currents recorded with co-expression of the mutant $\gamma 2(p.R136^*)$ subunit with wild-type $\alpha 1$ and $\beta 2$ subunits suggests that $\alpha 1\beta 2$ receptors were trafficked to the surface rather than $\alpha 1\beta 2\gamma 2(p.R136^*)$ receptors. A similar observation of $\alpha 1\beta 2$ receptor formation has been made in other $\gamma 2$ subunit truncation mutations (Kang, et al., 2013) This is also consistent with the previous finding that the formation of $\alpha\beta$ receptors in the *GABRG2* heterozygous knockout condition (Crestani et al., 1999). Furthermore, $\gamma 2(p.R136^*)$ subunit immunoreactivity was localised to the nuclear membrane and the ER providing evidence to support the bioinformatic structural model that predicted a novel ER retention motif in the C-terminus of the $\gamma 2(p.R136^*)$ subunit.

The functional assays suggested that co-expression of the mutant $\gamma 2(p.R136^*)$ subunit rather than the wild-type $\gamma 2$ subunit resulted in reduced channel function due to impaired incorporation of $\gamma 2(p.R136^*)$ subunits into receptors trafficked to the cell-surface. What is the basis of the reduced mutant subunit surface-expression? The structural model suggests that the signal peptide, possessed by both wild-type and mutant subunits, would traffic both proteins to the ER where following enzymatic cleavage of the 39 amino-acid long signal peptide, the 97 amino acid polypeptide would remain. Wild-type $\gamma 2$ subunits would thereafter be oligomerized, assembled into receptors and trafficked to the cell-surface membrane to form functional GABA_A receptors. However, the putative KKXX-like retention motif would retain mutant $\gamma 2(p.R136^*)$ subunits within the ER, leading to

accumulation and eventual degradation of the truncated subunits. Short linear sequences similar to this motif, which occupy the extreme COOH-terminal position of transmembrane ER proteins, have been implicated as retention signals (Nilsson et al., 1989). This suggests that despite potential instability, the $\gamma 2(p.R136^*)$ subunit polypeptide may accumulate appreciably in the ER. Bioinformatic analysis also demonstrated that the truncated $\gamma 2(p.R136^*)$ subunit following the signal cleavage site would be rich in posttranslational modification sites and given the predicted unravelled structure of the $\gamma 2(p.R136^*)$ subunit, it is likely that all of these posttranslational modification sites would be accessible (Fig. 1D).

The *in vitro* experiments also suggest that $\gamma 2(p.R136^*)$ subunits have the capacity to assemble with $\beta 2$ subunits. The co-localisation of $\gamma 2$ and $\beta 2,3$ subunit immunoreactivity, which was especially prominent in the intracellular retentions, suggests that $\gamma 2(p.R136^*)$ subunits may oligomerize with $\beta 2$ subunits, which are potentially retained. It is known that N-terminal sequences are critical for the proper assembly of GABA_A receptors and arginine in particular has been reported to be involved in the regulation of ER export (Kang and Macdonald, 2004). The $\gamma 2$ subunit is also considered to be critical for receptor trafficking, clustering and synaptic maintenance (Crestani et al., 1999). Therefore, it is likely that any disruption of the $\gamma 2$ subunit would have consequences potentially on the surface-expression of other GABA_A receptor subunits and subsequently impair GABAergic inhibition and lower seizure threshold. The impaired GABA_A receptor function is due to the loss of the mutant $\gamma 2(p.R136^*)$ subunits on the cell surface and synapse and potentially due to the reduced functional wild-type subunits and altered receptor composition.

Most GABA_A subunit missense mutations impair surface-expression of GABA_A receptors or impair channel kinetics. Similar *in vitro* experimental outcomes of impaired or altered receptor trafficking are observed with other GABRG2 mutations, including K328M and Q390X found in GEFS+ and Dravet syndrome families and the GABRG2 mutation R82Q found in a family with CAE and FS (Harkin et al., 2002; Kang et al., 2006). In both COS cells and hippocampal neurons, the GABRG2(p.R82Q) mutation reduced cell-surface subunit expression and exhibited impaired temperature-dependent trafficking (Frugier et al., 2007). Cell-surface subunit expression was also reduced with the nonsense GABRG2(p.Q390X) mutation, with ER retention of the mutant protein, no GABA-evoked currents recorded from injected oocytes and reduced GABA-evoked current in transfected HEK 293T cells (Kang et al., 2009). The GABRG2(p.Q40X) and GABRG2(p.W429X) nonsense mutations cause PTCs and may trigger NMD and cause epilepsy by haploinsufficiency. The *GABRG2(IVSG+2T-G)* splice donor site mutation also has the potential to trigger NMD and cause epilepsy by haploinsufficiency (Kananura et al., 2002).

The heterozygosity of the GABRG2(p.R136^{*}) mutation would be expected to reduce the number and density of functional GABA_A receptor complexes at inhibitory synapses because the mutant $\gamma 2(p.R136^*)$ subunits were retained inside somata regions and could not be trafficked to dendrites and synapses as shown in Figure 5B. This potentially would cause a decrease in inhibitory responses to GABA, giving increased neuronal excitability and susceptibility to seizures in individuals harbouring the mutation. However, we cannot rule out the possibility that *in vivo* expression of both wild-type and mutant $\gamma 2$ subunits may differ from the *in vitro* effects observed. Functional analysis of the previously-identified

GABRG2(p.Q390X) mutation showed a dominant-negative suppression of the biogenesis of wild-type $\gamma 2$ subunits (Kang et al., 2006; 2009). We suggest that similar cellular control mechanisms may affect $\gamma 2$ (p.R136*) subunit expression.

The discovery of this novel GABRG2(p.R136*) mutation extends the spectrum of *GABGR2* mutations to a wider GGE phenotype. Within the studied family there was a broad-range of seizure types including FS, afebrile seizures, generalised tonic-clonic seizures, absence seizures, myoclonic jerks and eyelid myoclonia, with marked interfamilial phenotypic variability – which is typical of GEFS+. Given the small family size and the adoption of individual II-1, it is difficult to draw conclusive genotype-phenotype correlations; however, consistent with previous findings, the GABRG2(p.R136*) mutation segregated most closely with FS. The epilepsy trait in this family cannot simply be explained by autosomal dominant inheritance. The presence of two epilepsy phenocopies in the nuclear family (Fig. 2 individuals III5 and III6); individuals diagnosed with epilepsy yet who tested negative for the GABRG2(p.R136*) mutation, serves to illustrate that the mutation does not exert a pure dominant effect. Phenocopies, although not typical of *GABGR2* gene-positive families, have previously been described (Wallace et al., 1998; Baulac et al., 2001; Harkin et al., 2002). The asymptomatic individual in this studied family harbouring the novel GABRG2(p.R136*) mutation (Fig. 2; individual III7) illustrates that the mutation is either partially-penetrant or potentially that the individual has not been exposed to the necessary environmental triggers to cause seizures, such as an appropriate temperature change to potentially impair receptor subunit trafficking. Since this study was a focussed candidate gene approach, it is possible that *GABRG2* is only one of several genes in interplay in this family and that genetic loading across several loci is producing a varied phenotype and skewed segregation analysis. Nevertheless, we add here the in-depth analysis of a rare *GABRG2* PTC mutation that has at best a major effect on the affected cases in the family or at least is contributory to the overall phenotypic spectrum.

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Abbreviations

CAE	childhood absence epilepsy
EEG	electroencephalogram
ER	endoplasmic reticulum
FS	febrile seizures
GEFS+	generalised epilepsy with febrile seizures plus
GGE	genetic generalised epilepsy
JME	juvenile myoclonic epilepsy

PTC	premature translation termination codon
GABRG2	$\gamma 2$ subunit gene of the γ -aminobutyric acid type A receptor

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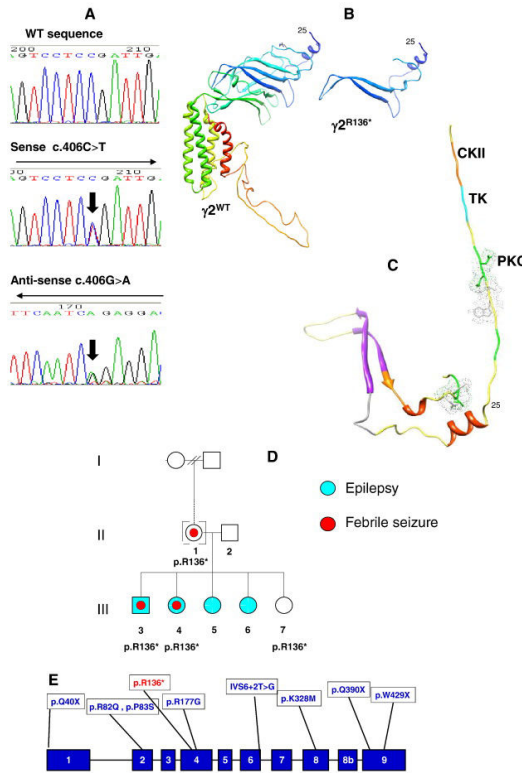


Figure 1. A novel GABRG2 gene-mutation

A) Sequencing of exon 4 of *GABRG2* in a control (a) and affected index -case DNA (b+c) reveals a new *GABRG2* gene-mutant (c.406C>T) resulting in a nonsense mutation outcome - *GABRG2*(p.R136*). This is illustrated by the double nucleotide peak and marked with an arrow; **B)** Structural modelling of $\gamma 2$ WT illustrating the position of R136 and posttranslational modification sites of the truncated *GABRG2*(p.R136*) subunit. Residues 1-25 could not be reliably modelled due to a lack of coverage for this section by homologues in the Protein Data Bank. The truncated *GABRG2*(p.R136*) subunit section composes several sections of beta turn, two short regions of alpha helix, retention of functional domains, and two beta strands. **C)** Structural modelling of $\gamma 2$ WT illustrating the posttranslational modification sites of the truncated *GABRG2*(p.R136*). CKII = casein kinase II phosphorylation (orange); TK = tyrosine kinase phosphorylation (cyan); PKC = protein kinase C phosphorylation (green); and the side chains for N-glycosylation and N-myristoylation are shown. **D)** Pedigree structure and outcomes of *GABRG2* sequencing. A pedigree of the nuclear GEFS+ family harbouring the novel *GABRG2*(p.R136*) mutation and the affection status for epilepsy (cyan) or febrile seizures (red dot) indicated. **E)** Spectrum of known mutations in *GABRG2* relative to the novel *GABRG2*(p.R136*) mutation. An exonic representation of the *GABRG2* gene illustrates the relative positions of the *GABRG2*(p.R136*) mutation to previously-identified *GABRG2* mutations.¹¹⁻²¹

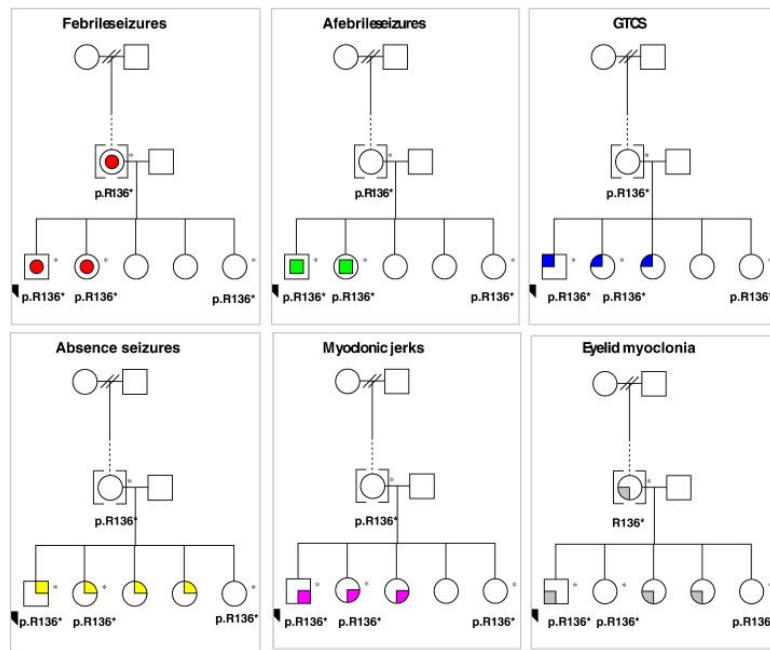


Figure 2. Endophenotyping of the GEFS+ Family and the segregation with the GABRG2 p.R136* mutation

Each presenting phenotype within the multiplex family is represented in a panel, including febrile seizures (red circles), afebrile seizures (green squares), generalised tonic-clonic seizures (GTCS – blue quarter), absence seizures (yellow quarter), myoclonic jerks (magenta quarters), and eyelid myoclonia (grey quarters).

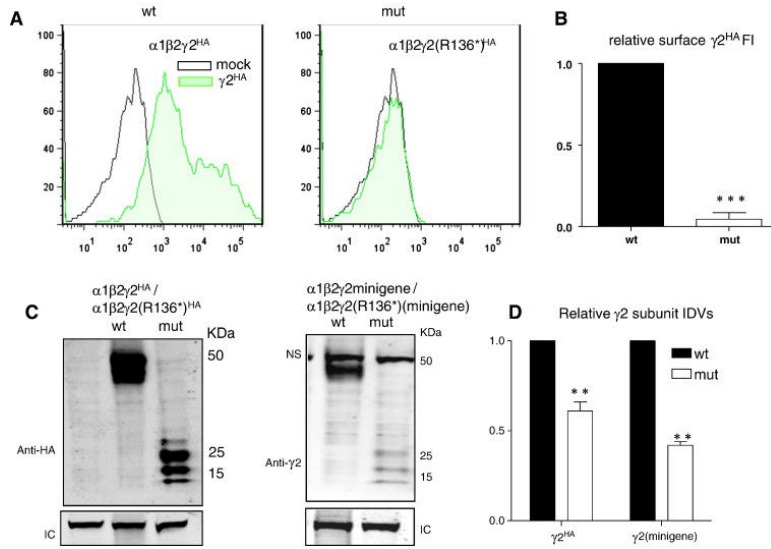


Figure 3. The mutant $\gamma 2S(p.R136^*)$ subunit protein was produced and presented as multiple bands but had minimal surface expression

HEK 293T cells were co-transfected with $\alpha 1$ and $\beta 2$ subunits and $\gamma 2S^{HA}(wt)$ or $\gamma 2S(p.R136^*)^{HA}$ (mut) subunits or co-transfected with $\alpha 1$ and $\beta 2$ subunits and $\gamma 2$ (wt) or $\gamma 2(p.R136^*)$ (mut) minigenes. **A**) The flow cytometry histograms depict surface expression levels of wild-type $\gamma 2S^{HA}$ or mutant $\gamma 2S(p.R136^*)^{HA}$ subunits from HEK 293T cells expressing either $\alpha 1$, $\beta 2$ and $\gamma 2S^{HA}$ or $\alpha 1$, $\beta 2$ and $\gamma 2S(p.R136^*)^{HA}$ subunits. The surface wild-type and mutant $\gamma 2S$ subunits were fluorescently conjugated with anti-human HA antibody (HA–Alexa Fluor-647). **B**) The relative fluorescence intensity of HA signals of from mutant $\gamma 2S(p.R136^*)^{HA}$ subunits were normalized to those obtained with wild-type $\gamma 2S^{HA}$ subunits. **C, D**) The total lysates of HEK 293T cells expressing $\alpha 1$, $\beta 2$ and $\gamma 2S^{HA}$ subunits from expression of cDNAs (C, left panel) or minigenes (C, right panel) were analyzed by SDS-PAGE. LC stands for loading control. NS stands for nonspecific band. **D**) The total IDVs of $\gamma 2S^{HA}$ subunit cDNA or minigene transfections were quantified, and the data were presented after normalized to the total wild-type $\gamma 2^{HA}$ or $\gamma 2$ subunits obtained with expression of minigenes (In B and D, **p < 0.001 ***p < 0.001 vs wt).

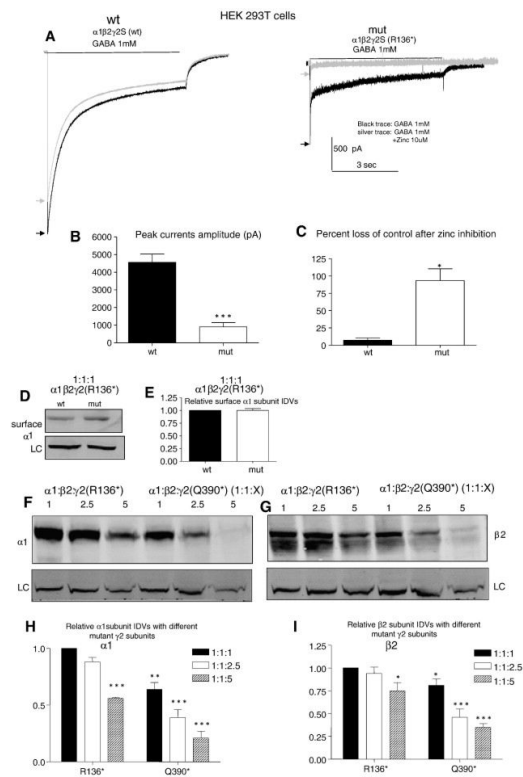


Figure 4. Current amplitudes recorded with co-expression of $\alpha 1$ and $\beta 2$ subunits with mutant $\gamma 2S(p.R136^*)$ subunits had reduced peak current amplitudes and were more sensitive to zinc inhibition while the mutant $\gamma 2S(p.R136^*)$ subunits reduced the $\alpha 1$ and $\beta 2$ subunit expression at high amounts

A,B) GABA_A receptor currents were obtained from HEK 293T cells expressing wild-type $\alpha 1$ and $\beta 2$ subunits with $\gamma 2S$ (1:1:1 cDNA ratio; wt) or $\gamma 2S(p.R136^*)$ (1:1:1 cDNA ratio, mut) subunits with application of 1 mM GABA applied for 6 sec. The representative traces of GABA_A receptor currents were evoked with application of 1 mM GABA for 6 sec (black trace, black arrow) and co-application of 1 mM GABA with 10 μ M zinc after 6 sec pre-application of 10 μ M zinc (silver traces, silver arrow). **(B)** The amplitudes of GABA_A receptor currents from **(A)** were plotted. Values were mean \pm SEM (n = 9-14 patches from 4 different transfections). **(C)** The percent loss of control current or reduction of current amplitude after coapplication of zinc and GABA was presented by the currents evoked by GABA subtracting the currents evoked by GABA and zinc (*p < 0.05***p < 0.001 vs wt, n = 4). **D,E)** The surface $\alpha 1$ subunit was extracted from HEK 293T cells expressing wild-type $\alpha 1$ and $\beta 2$ subunits with $\gamma 2S$ (1:1:1 cDNA ratio; wt) or $\gamma 2S(p.R136^*)$ (1:1:1 cDNA ratio, mut) subunits by surface biotinylation and analyzed by SDS-PAGE. The membranes were immunoblotted with a mouse anti- $\alpha 1$ antibody. **(E)** The relative amount of the $\alpha 1$ subunit expression in the mutant $\alpha 1\beta 2\gamma 2S(p.R136^*)$ receptors was normalized to the wildtype, which is arbitrarily taken as 1. **F, G, H, I)** The total lysates from HEK 293T cells expressing wild-type $\alpha 1$ and $\beta 2$ subunits with $\gamma 2S(p.R136^*)$ or $\gamma 2S(p.Q390X)$ at 1:1:1, 1:1:2.5 and 1:1:5 cDNA ratio were harvested and analysed by SDS-PAGE. The membranes were then immunoblotted with either mouse anti- $\alpha 1$ or rabbit anti $\beta 2$ antibody. The relative amount of the $\alpha 1$ or $\beta 2$ subunit expression in other conditions were normalized to the

$\alpha 1\beta 2\gamma 2S(p.R136^*)$ receptors at 1:1:1 which was arbitrarily taken as 1 (in H and I, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs $\alpha 1\beta 2\gamma 2S(p.R136^*)$ at 1:1:1, $n = 4$).

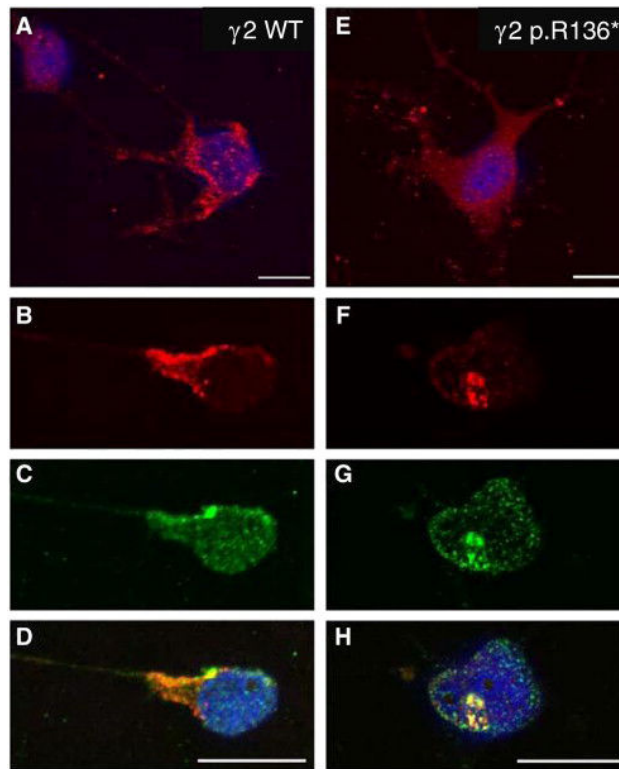


Figure 5. Expression analysis of $\gamma 2(p.R136^*)$ in vitro using differentiated PC12 cells GABA_A receptor subunits $\alpha 1$, $\beta 2$ and $\gamma 2$ -WT (A-D) or $\gamma 2$ p.R136* mutant (E-H) subunits were transiently co-transfected in PC12 cells, and immunocytochemically stained with anti- $\gamma 2$ and anti- $\beta 2,3$ -antibodies as analysed by confocal microscopy. $\alpha 1\beta 2\gamma 2$ receptor immunoreactivity (IR) revealed an even distribution with punctuate staining on cell-surface, less prominent intra-cellular staining (A, B) and co-localized with GABA_AR $\beta 2$ -IR (C, D). Expression of $\alpha 1\beta 2\gamma 2(p.R136^*)$ receptors resulted in more diffuse labelling with little cell-surface IR (E) and was predominantly localised within intracellular domains (F), whilst retaining co-labelling patterns for GABA_AR $\beta 2$ -IR (G, H). Scale bars = 20 μ m.

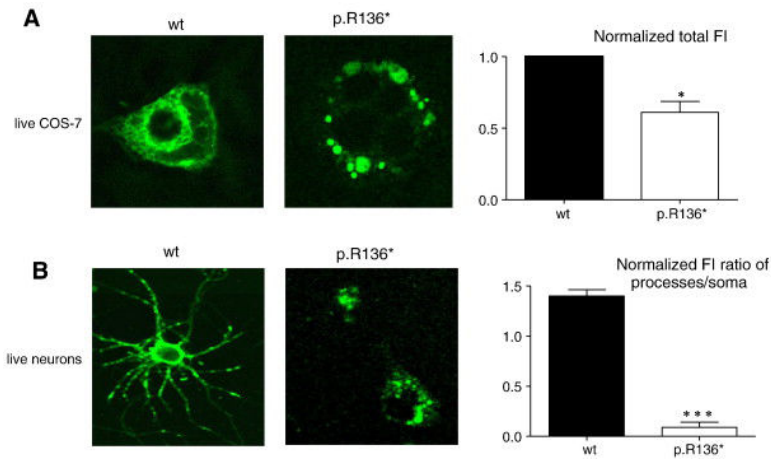


Figure 6. $\gamma 2S(p.R136^*)^{YFP}$ subunit protein was reduced and confined to intracellular region or soma in live COS-7 cells and cortical neurons

COS-7 cells or cultured cortical neurons were cotransfected with wild-type $\gamma 2S^{YFP}$ (wt) or mutant $\gamma 2S(p.R136^*)^{YFP}$ (mut) subunits with (in COS-7 cells) or without (in neurons) $\alpha 1$ and $\beta 2$ subunits using the calcium phosphate precipitation method with different amounts of subunit cDNAs. Images were acquired 2 days (COS-7) or 8 days (neurons) after transfection. The total fluorescence intensities of cells were measured using the MetaMorph imaging software. In the upper panel, the total fluorescence from COS-7 cells expressing wild-type $\gamma 2S^{YFP}$ subunit-containing receptors (wt) was arbitrarily taken as 1, and the total fluorescence from cells expressing mutant $\gamma 2S(p.R136^*)^{YFP}$ subunit-containing receptors (mut) was normalized to wt levels. In lower panel, the total fluorescence of both neuronal somata and processes (including both axon and dendrites) were measured, and the fluorescence intensity ratios of the areas of processes over the somata were quantified. (n = 8 for COS-7 and for neurons from three different transfections; *p < 0.05; ***p < 0.001 vs wt).

Table 1

Clinical characteristics of the nuclear family members.

Pedigree Reference	Age [†]	Neurological Examination	Epilepsy Diagnosis	Seizure Onset	Seizure type	EEG	RI	Treatment	Other diagnoses	GABRG2 (p.R136*)
II-1	33	N	No	NK	FS, unw EyeM	Not done	ND	-	Migraine	Positive
II-2	41	N	No	-	-	Not done	ND	-	-	Negative
III-3	8	LD	Yes	9 months	A, FS+, GTCS, MJ, EyeM	Atypical SW, GSW	AD	VAL LEV	Autistic Spectrum	Positive
III-4	7	LD	Yes	18 months	A, FS, FS+, GTCS, MJ, Uncl sz	Background SW+spikes	ND	VAL LEV	Autistic spectrum	Positive
III-5	6	LD	Yes	24 months	A, GTCS, EyeM, MJ, Uncl sz	Background SW+spikes	ND	CBZ LEV	Educational needs	Negative
III-6	2	N	Yes	12 months	A, CPS, EyeM, Uncl sz	ND	AD	CBZ	-	Negative
III-7	19 months	N	No	-	-	ND	ND	-	-	Positive

FS = febrile seizures; FS+ = febrile seizures plus; A = absences; CPS = complex partial seizures; DA = drop attacks; EyeM = eyelid myoclonia; GTCS = generalised tonic-clonic seizures; MJ = myoclonic jerks; LD = learning difficulties; GSW = generalised spike and wave; SW = slow waves; CBZ = carbamazepine LEV = levetiracetam; VAL = sodium valproate; N = normal; NAD = no abnormality detected; NK = not known; ND = Not Done; Uncl = unclassified; unw = unwitnessed

[†] in years at study onset.