


Synaptic clustering differences due to different *GABRB3* mutations cause variable epilepsy syndromes

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GABRB3 is highly expressed early in the developing brain, and its encoded $\beta 3$ subunit is critical for GABA_A receptor assembly and trafficking as well as stem cell differentiation in embryonic brain. To date, over 400 mutations or variants have been identified in *GABRB3*. Mutations in *GABRB3* have been increasingly recognized as a major cause for severe paediatric epilepsy syndromes such as Lennox-Gastaut syndrome, Dravet syndrome and infantile spasms with intellectual disability as well as relatively mild epilepsy syndromes such as childhood absence epilepsy. There is no plausible molecular pathology for disease phenotypic heterogeneity. Here we used a very high-throughput flow cytometry assay to evaluate the impact of multiple human mutations in *GABRB3* on receptor trafficking. In this study we found that surface expression of mutant $\beta 3$ subunits is variable. However, it was consistent that surface expression of partnering $\gamma 2$ subunits was lower when co-expressed with mutant than with wild-type subunits. Because $\gamma 2$ subunits are critical for synaptic GABA_A receptor clustering, this provides an important clue for understanding the pathophysiology of *GABRB3* mutations. To validate our findings further, we obtained an in-depth comparison of two novel mutations [*GABRB3* (N328D) and *GABRB3* (E357K)] associated with epilepsy with different severities of epilepsy phenotype. *GABRB3* (N328D) is associated with the relatively severe Lennox-Gastaut syndrome, and *GABRB3* (E357K) is associated with the relatively mild juvenile absence epilepsy syndrome. With functional characterizations in both heterologous cells and rodent cortical neurons by patch-clamp recordings, confocal microscopy and immunoblotting, we found that both the *GABRB3* (N328D) and *GABRB3* (E357K) mutations reduced total subunit expression in neurons but not in HEK293T cells. Both mutant subunits, however, were reduced on the cell surface and in synapses, but the Lennox-Gastaut syndrome mutant $\beta 3$ (N328D) subunit was more reduced than the juvenile absence epilepsy mutant $\beta 3$ (E357K) subunit. Interestingly, both mutant $\beta 3$ subunits impaired postsynaptic clustering of wild-type GABA_A receptor $\gamma 2$ subunits and prevented $\gamma 2$ subunits from incorporating into GABA_A receptors at synapses, although by different cellular mechanisms. Importantly, wild-type $\gamma 2$ subunits were reduced and less clustered at inhibitory synapses in *Gabrb3*^{+/-} knockout mice. This suggests that impaired receptor localization to synapses is a common pathophysiological mechanism for *GABRB3* mutations, although the extent of impairment may be different among mutant subunits. The study thus identifies the novel mechanism of impaired targeting of receptors containing mutant $\beta 3$ subunits and provides critical insights into understanding how *GABRB3* mutations produce severe epilepsy syndromes and epilepsy phenotypic heterogeneity.

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Introduction

GABA_A receptors mediate neurotrophic signalling in the developing brain and most of fast synaptic inhibition in the mature brain. Mutations in GABA_A receptor subunit genes (GABRs) including GABRB3 (Allen *et al.*, 2013; Moller *et al.*, 2017) and GABRG2 are frequently associated with genetic epilepsy syndromes and neurodevelopmental disorders, which range from simple childhood absence epilepsy and autism to the severe epilepsies such as Dravet syndrome and Lennox-Gastaut syndrome (Kang and Macdonald 2009; Macdonald *et al.*, 2010; Petrou and Reid, 2012; Butler *et al.*, 2018). For GABRB3 alone, more than 400 mutations (<https://www.ncbi.nlm.nih.gov/clinvar/>) have been identified, underscoring the importance of GABRB3 in normal brain development and neurodevelopmental disorders.

Among GABA_A receptor subunits, the $\beta 3$ subunit encoded by GABRB3 plays a central role for GABA_A receptor assembly and trafficking to the cell surface (Connolly *et al.*, 1996; Jacob *et al.*, 2008), while the $\gamma 2$ subunit encoded by GABRG2 is required for postsynaptic receptor clustering (Allred *et al.*, 2005). Unlike other GABA_A receptor subunits that require assembly with partnering subunits for surface localization, the $\beta 3$ subunit can traffic to the cell surface and form homo-pentamers when expressed alone because of four amino acids (glycine 171, lysine 173, glutamate 179, and arginine 180) that are specific to the $\beta 3$ subunit (Taylor *et al.*, 1999), suggesting its unique ability for homo-oligomerization and membrane targeting. Of relevance to the aetiology of neurodevelopmental disorders, GABRB3 has been found to be abundant in the early brain (Laurie *et al.*, 1992) and has been reported to induce stem cell differentiation (Andang *et al.*, 2008). Importantly, it has been demonstrated that $\beta 3$ subunit-dependent phosphorylation mediates GABA_A receptor immobilization and accumulation of the inhibitory synaptic scaffold protein gephyrin at synapses, which are crucial for long-term potentiation of inhibition and are likely to modulate network excitability (Petrini *et al.*, 2014). Additionally, $\beta 3$ subunit-AP2 interactions stabilize GABA_A receptors at endocytic zones and are likely to play a key role in regulating synaptic receptor number during inhibitory synaptic

plasticity (Smith *et al.*, 2012). Together, this suggests a critical role of $\beta 3$ subunit in regulating synaptic strength and brain development.

Mutations in GABRB3 have long been associated with autism and childhood absence epilepsy (Tanaka *et al.*, 2008; Delahanty *et al.*, 2011). Additionally, GABRB3 knockout mice have been reported to display seizures and autism-like behaviours and have thus been proposed to be a model of Angelman syndrome, a severe neurodevelopmental disorder, as well as autism (DeLorey and Olsen, 1999; DeLorey, 2005), suggesting a role for GABRB3 mutations in seizures and neurodevelopment. However, the correlation of GABRB3 mutations and severe epilepsy with developmental disorders was not established until the recent identification of GABRB3 mutations in Lennox-Gastaut syndrome by the Epilepsy Phenome/Genome Project (Epi4K) (Allen *et al.*, 2013). Following this report, multiple groups identified a substantial number of mutations in GABRB3 associated with intellectual disability and various epilepsy syndromes, including Lennox-Gastaut syndrome, infantile spasms, early onset epileptic encephalopathy and Dravet syndrome (Hamdan *et al.*, 2014; Le *et al.*, 2017). Prominent features of these epilepsy syndromes include an early childhood manifestation and cognitive impairment. This thus suggests that GABRB3 is a critical gene for normal brain development and impairment in the function of the gene would lead to neurodevelopment-related disorders such as epilepsy.

In this study, we evaluated several GABRB3 mutations associated with different epilepsy syndromes and compared two novel mutations (N328D and E357K) in GABRB3 in detail. The GABRB3 (N328D) mutation is associated with Lennox-Gastaut syndrome whereas the GABRB3 (E357K) mutation is associated with juvenile absence epilepsy. We characterized the functional impairment of the two mutations in both non-neuronal cells and rat and mouse neurons with multidisciplinary approaches such as electrophysiology recordings, confocal microscopy, and surface biotinylation and validated the findings in the mouse model. The study identified a common pathophysiological mechanism associated with impairment of $\beta 3$ subunits associated with different epilepsy syndromes including, Lennox-Gastaut

syndrome, infantile spasms, early onset epileptic encephalopathy, childhood absence epilepsy and juvenile absence epilepsy and provided critical insights into understanding phenotypical heterogeneity.

Materials and methods

Patients

Both patients were from a cohort of 157 idiopathic generalized epilepsies in the Epilepsy Center of the second affiliated hospital of Guangzhou Medical University in China. The patients were diagnosed according to the criteria of the Commission on Classification and Terminology of the International League Against Epilepsy (ILAE) (1981, 1989, 2001, 2010).

Ethics statement

Parents of each patient provided signed informed consent using a protocol approved by the Ethics Review Committee of Guangzhou Medical University. All animals and related experiments in this study were approved by the Vanderbilt University IACUC. The animal protocol number is M1600102-00 (to J-Q.K.).

Whole exome sequencing and analysis

Genomic DNA was extracted from peripheral blood obtained from the patients and their parents using the QIAamp® blood mini kit (Qiagen). Exome sequencing was performed by Beijing Genomics Institute (BGI) (Shenzhen, China). The exome was captured from fragmented genomic DNA samples using the SureSelect Human All Exon 50 Mb kit (Agilent Technologies) for enrichment, and 100-bp paired-end sequencing was carried out on an Illumina HiSeq 2000 instrument according to the manufacturer's protocols. Raw image files were processed with Illumina Basecalling Software 1.7 for base calling with default.

Bioinformatic analyses and variant filters were carried out using the house pipeline developed by BGI in Shenzhen (Mancini *et al.*, 2015). In brief, high-quality DNA sequence reads were mapped to the human reference genome (Hg19/GRCh37) using SOAP aligner (<https://github.com/ShujiaHuang/SOAPaligner>). After filtering out duplicate reads, the consensus sequences in target regions were called by SOAP (Short Oligonucleotide Analysis Package) snp (see URLs) using uniquely mapped reads. The Genome Analysis Toolkit (Li and Durbin, 2009) was used to identify indels with BWA-aligned high-quality reads (McKenna *et al.*, 2010). To eliminate non-pathogenic variants, public databases including dbSNP, the 1000 Genomes Project, ExAC, ExAC-East Asian Population, and ESP, as well as a BGI in-house database, were used to filter variants with a minor allele frequency (MAF) > 0.5%. Software for functional prediction (e.g. SIFT and PolyPhen-2) and conservation degree (e.g. GERP) were interrogated.

Expression vectors with GABA_A receptor subunits

The cDNAs encoding human GABA_A receptor α 1, β 3, γ 2, and haemagglutinin (HA)-tagged β 3 or γ 2 subunits were as described previously (Delahanty *et al.*, 2011; Kang *et al.*, 2013). The HA tag is inserted between the fourth and fifth amino acid of the mature protein peptide for both the human β 3 and γ 2 subunits. The protein expression pattern and biophysical properties of the HA-tagged constructs were unaltered compared with those of native β 3 or γ 2 subunits. *GABRB3* (N328D) and *GABRB3* (E357K) variants were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and were confirmed by DNA sequencing. The short form of the γ 2 subunit (γ 2S) was used throughout in this study.

Transfection of GABA_A receptor subunits and measurement of cell surface GABA_A receptor subunit expression

Transfection of human GABA_A receptor subunit cDNAs was based on our standard laboratory protocol (Kang *et al.*, 2009b). Briefly, HEK293T cells were transfected using polyethylenimine (PEI) reagent (40 kD, Polysciences) at a DNA:PEI ratio of 1 μ g:2.5 μ l and harvested 48 h after transfection. Total 3 μ g of cDNA of GABA_A receptor α 1, β 3 and γ 2s subunits were transfected at a 1:1:1 cDNA ratio. For mixed condition, the wild-type and the mutant β 3 subunits were mixed at a 1:1 cDNA ratio with 0.5 μ g for each. The same transfection protocol was used for flow cytometry, immunocytochemistry, western blot and patch clamp recordings in HEK293T cells. Measurement of surface expression of GABA_A receptor subunits using flow cytometry has been described previously (Kang *et al.*, 2009b). The transfected HEK293T cells were removed from the dishes by trypsinization and then resuspended in FACS buffer [phosphate-buffered saline (PBS) supplemented with 2% foetal bovine serum and 0.05% sodium azide]. Following washes with FACS buffer, cells were incubated with mouse monoclonal anti- β 2/3 antibody (1:200) or anti-HA for HA-tagged γ 2 subunits for 2 h and then incubated with fluorophore Alexa-488 conjugated goat anti-mouse secondary antibody (1:2000) for 1 h at 4°C. Cells were then washed with FACS buffer and fixed with 2% paraformaldehyde. The acquired data were analysed using FlowJo 7.1 (Tree Star, Inc.).

Neuronal cultures and transfection in neurons

Rat hippocampal and mouse cortical neurons were prepared as described previously (Kang *et al.*, 2009b, 2010). Rat neurons were used for immunohistochemistry experiments and mouse neurons were used for western blot. Rat hippocampal neurons were cultured from embryonic Day 18 rat pups. Mouse cortical neurons were cultured from postnatal Day 0 mouse pups. The neurons were plated at a density of 0.5–1 \times 10⁵ for immunohistochemistry and 2 \times 10⁵ for western blot in plating

media that contained Dulbecco's modified Eagle medium 420 ml, F12 40 ml, foetal bovine serum 40 ml, penicillin and streptomycin 1 ml and L-glutamine (200 mM) 0.2 ml for 4 h and then maintained in NeurobasalTM media that contained B27 supplement (50:1), L-glutamine (200 mM) and penicillin and streptomycin 1 ml. Neurons were transfected with 15 µg cDNA at Day 5–7 in culture with calcium phosphate and were harvested 8–10 days after transfection. Four 100 mm² dishes of neurons were transfected with each subunit in each experiment to ensure enough proteins for immunoblotting assay due to low transfection efficiency in neurons.

Gabrb3 heterozygous knockout mice

The *Gabrb3*^{+/-} mouse line was generated previously (DeLorey *et al.*, 1998) and was obtained from Jackson Laboratory. Genotyping was performed following the instructions from Jackson Laboratory. Wild-type × heterozygous breeding was carried out in C57BL/6J background. All the experimental procedures in mice were approved by Vanderbilt University Division of Animal Care.

Electrophysiology

Expression of recombinant GABA_A receptors and subsequent whole cell recordings from lifted cells were conducted as described previously (Kang *et al.*, 2006). The experimental procedures are detailed in the Supplementary material.

Immunocytochemistry and related quantifications

HEK293T cells expressing wild-type α1β3^{HA}γ2, mutant α1β3 (N328D)^{HA}γ2s or mutant α1β3 (E357K)^{HA}γ2s receptors were fixed with 4% paraformaldehyde and permeabilized with 0.5% TritonTM X-100 for total protein expression or non-permeabilized for surface protein expression. Cells were then immunostained with one or two antibodies based on each specific experimental design. A list of catalogues and dilutions for primary antibodies is provided in Supplementary Table 1. Rhodamine-conjugated mouse IgG alone or in combination with Alexa-488-conjugated rabbit IgG was used to visualize the wild-type or mutant subunits. The images were acquired using a LSM 510 invert confocal microscope with 63× objective and analysed by Metamorph or ImageJ.

Surface biotinylation, subcellular fractionation and isolation of synaptosomes and western blot analysis

Cell surface protein biotinylation and western blot procedures were modified from a previous protocol (Kang *et al.*, 2009a, 2010). The procedures of subcellular fractionation were modified from a previous study for synaptosome preparation (Kang *et al.*, 2015; Warner *et al.*, 2016). The synaptosome layer was at the 1.0/1.2M sucrose interface. To prepare postsynaptic densities, the synaptosome layer fraction was diluted to 0.32M sucrose by adding 2.5 volumes of 4mM HEPES (pH 7.4) and balanced with HEPES-buffered sucrose. The diluted synaptosome layer

preparation was then centrifuged at 150 000g for 30 min (TH 641: 29 600 rpm). After centrifugation, the pellet was collected and suspended by adding 4 ml 0.5% TritonTM X-100 solution containing 50 mM HEPES, 2 mM EDTA and protease inhibitors rotated for 15 min. The experimental procedures are detailed in the Supplementary material.

Data analysis

Numerical data were expressed as mean ± SEM. Proteins were quantified by Odyssey software and data were normalized to loading controls and then to wild-type subunit proteins, which was arbitrarily taken at a value of 1 in each experiment. Fluorescence intensities from confocal microscopy experiments were determined using MetaMorph imaging software and the measurements were carried out in ImageJ as modified from previous description (Kang and Macdonald 2004; Kang *et al.*, 2010, 2015; Warner *et al.*, 2016). For statistical significance, we used one-way ANOVA with Newman-Keuls test or Student's unpaired *t*-test. In some cases, one sample *t*-test was performed (GraphPad Prism, La Jolla, CA), and statistical significance was taken as *P* < 0.05.

Data availability

The data supporting the findings of this study are available within the article and its Supplementary material.

Results

Reduced cell surface expression of γ2 subunits is a common phenomenon among GABRB3 mutations

More than 400 variants and mutations in *GABRB3* have been identified to date and these variants/mutations are associated with various epilepsy syndromes including Lennox-Gastaut syndrome, infantile spasms and Dravet syndrome, autism and intellectual disability (<https://www.ncbi.nlm.nih.gov/clinvar/>) (Fig. 1A and Supplementary Fig. 1). *GABRB3* (N110D) is associated with infantile spasms and has been previously investigated (Janve *et al.*, 2016), thus it was not included in this study. It is known that β3 subunits alone can traffic to surface by forming homopentamers. However, γ2 subunits are required for clustering receptors at synapses (Allred *et al.*, 2005), thus it could serve as a marker for synaptic receptor expression level. We thus determined the trafficking of β3 and γ2 subunits for multiple mutations associated with different epilepsies and autism by co-expressing human α1, β3 and γ2 subunits. HA-tagged γ2 subunits were validated to have similar expression and function as the native subunit and were used for high throughput flow cytometry assay. We found that surface expression of β3 subunits was variable (Fig. 1B and C). However, the surface expression of all γ2 subunits was reduced except for one mutation, *GABRB3* (T288N) associated with epileptic encephalopathy (Hernandez *et al.*, 2017) that was unchanged,

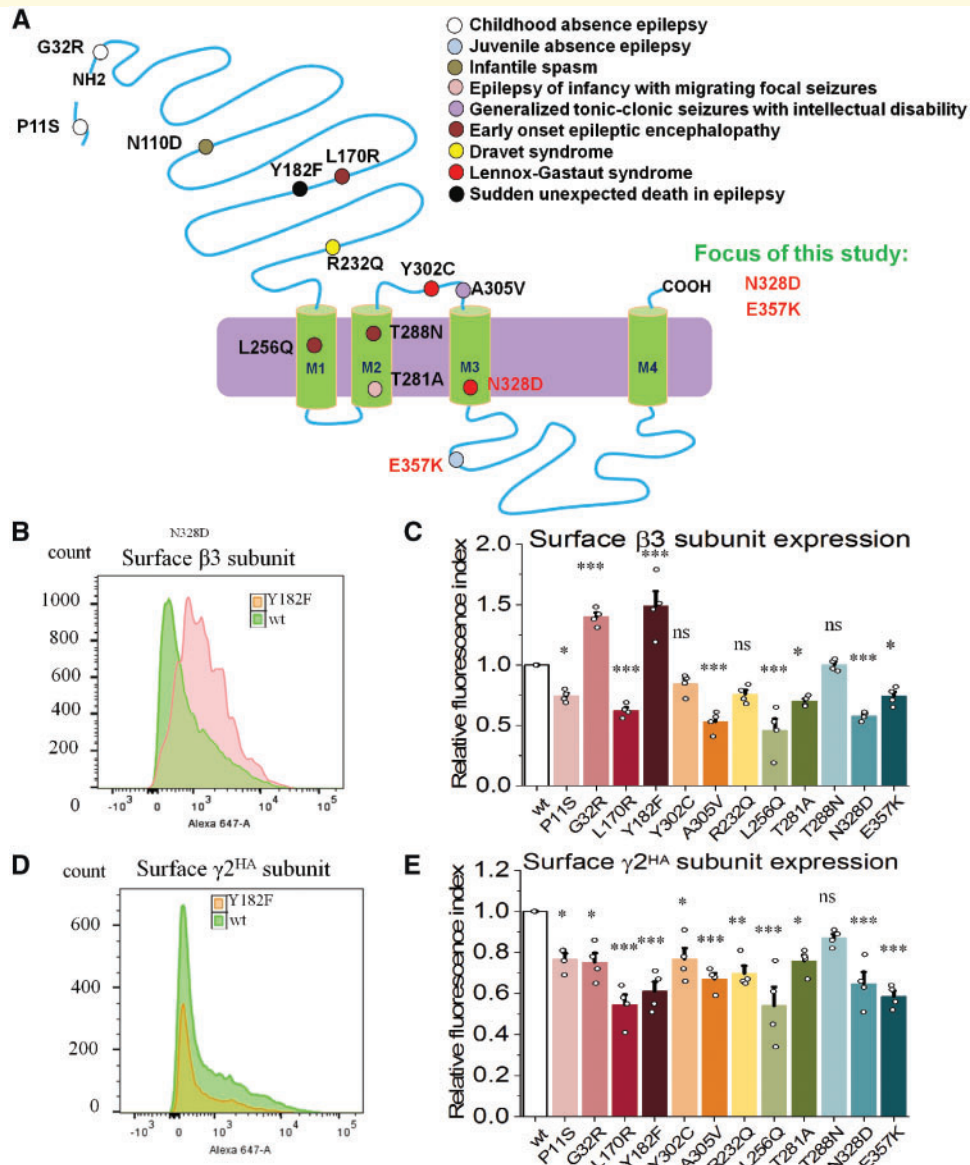


Figure 1 Reduced partnering $GABA_A$ receptor γ_2 subunit at cell surface is a common phenomenon across *GABRB3* epilepsy mutations. **(A)** Schematic presentation of β_3 subunit protein topology and locations of mutations in human *GABRB3* associated with various epilepsy syndromes and neurodevelopmental disorders. These mutations are distributed in various locations and domains of the β_3 subunit protein peptide. The coloured dots represent the relative locations of the epilepsy related mutations. **(B–E)** The flow cytometry histograms depict surface β_3 **(B)** or HA-tagged γ_2 subunit (γ_2^{HA}) **(D)** expression levels on HEK293T cells co-expressing the human wild-type or the mutant β_3 subunit cDNAs with the $\alpha 1$ and HA-tagged γ_2 subunit cDNAs for 48 h. The expression levels of the β_3 or γ_2^{HA} subunits in the mutant $\alpha 1\beta_3(Y182F)\gamma_2^{HA}$ receptors was chosen as example. The HA-tagged γ_2 subunit protein was immunostained with a mouse anti-HA antibody while β_3 subunits were immunostained with the mouse anti- β_2/β_3 antibody (BD17). The relative surface levels of each mutant β_3 subunit were normalized to the wild-type β_3 subunit when co-expressed with $\alpha 1$ and γ_2^{HA} cDNAs. The relative subunit expression level of β_3 **(C)** or γ_2^{HA} **(E)** subunits were normalized to those obtained with co-expression of γ_2^{HA} subunit with $\alpha 1$ and the wild-type β_3 subunit cDNAs. In **C** and **E**, $n = 4$ different transfections, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$, one-way ANOVA with *post hoc* Tukey test.

with the reductions ranging from 15% to 40% compared with wild-type (Fig. 1D and E). The variation of β_3 subunit surface expression was likely due to the ability of β_3 subunits to form homo-pentamers and to traffic to the cell surface. However, the significance of β_3 homo-pentamers for their function is unknown.

Patient phenotypes, molecular genetics and EEG for two novel mutations

We then focused on two new mutations (N328D and E357K) in *GABRB3* identified by the whole exome

sequencing. The patient's detailed history and clinical features are documented in Supplementary Table 2. The patient with the *GABRB3* (N328D) mutation, a 10-year-old male, had his first seizure at 1 year and 2 months of age with a 39°C fever. It was a generalized tonic-clonic seizure (GTCS) lasting for ~1–2 min. Similar seizures subsequently occurred. Other less severe seizures such as myoclonic seizures also occurred several times a day. Valproate was administered with a dose of 15 mg/kg/d, but the seizures continued without improvement. Five months later, valproate was increased to 29 mg/kg/d, but the tonic and myoclonic seizures did not change. Lamotrigine was then titrated up to 4 mg/kg/d, and the patient has been seizure-free since 7 years of age. The initial EEG examination demonstrated an ictal 2.0–2.5 Hz generalized poly-spike-wave discharges during myoclonic seizures; interictal spike-and-slow wave complexes and irregular fast rhythms with slow waves were recorded during wakefulness (Fig. 2A) and sleep (Fig. 2B). A recent EEG examination presented irregular 4–5 Hz theta activities bilaterally in the temporal lobes. The patient had normal development before seizure onset and had moderate intellectual disability and speech slowing after seizure onset.

The patient with the *GABRB3* (E357K) mutation was an 18-year-old male, who, at 14 years of age, had his first GTCS, which lasted 2–3 min. Subsequent GTCSs occurred once per year. Subsequently, he suffered from absence and myoclonic seizures that occurred several times per day. When valproate was administered at a dose of 750 mg/d (12.5 mg/kg/d), no GTCSs occurred, absence seizures decreased to several times per month and sporadic myoclonic seizures remained. One year later, with the add-on of lamotrigine at 100 mg/d (1.7 mg/kg/d), the patient became seizure-free. The initial EEG examination supported absence seizures. As shown in Fig. 1C and D, an ictal EEG of an absence seizure showed paroxysmal 3.0–3.5 Hz generalized spike-and-wave discharges, lasting for ~5 s (Fig. 2C) and an interictal EEG of the patient showed single generalized spike or spike-and-waves (Fig. 2D). A recent EEG indicated subclinical generalized spike-and-wave discharges in a normal background. This patient had normal development.

With whole exome sequencing, two novel mutations, *GABRB3* (N328D) and *GABRB3* (E357K) were identified in the epilepsy patients. *GABRB3* (N328D) is a *de novo* mutation while *GABRB3* (E357K) was also identified in the father, who is apparently unaffected (Fig. 2E). Based on TMHMM Server V 2.0 software, the amino acid residue N328 is located just outside the TM3 α helix while E357 is located in the intracellular loop between TM3 and TM4 of the $\beta 3$ subunit (Fig. 2F). Both amino acid residues coded by the mutations are conserved across species (Fig. 2G). We consulted the genome Aggregation Database (GnomAD)

allele frequency and excluded the possibility of other genetic causes such as variations in *ALG13* and *PATL1* (Supplementary Table 3).

Mutations differentially reduced GABA-evoked receptor channel currents and altered desensitization

We determined the functional consequences of both $\beta 3$ subunit mutations by measuring macroscopic GABA-evoked currents in transfected HEK293T cells (Fig. 3A). Compared with wild-type receptors, both mixed and mutant subunit-containing $\alpha 1\beta 3\gamma 2$ receptors had decreased current amplitudes when evoked with a saturating concentration of GABA (1 mM). However, the $\alpha 1\beta 3$ (N328D) $\gamma 2$ receptor currents were more decreased than the $\alpha 1\beta 3$ (E357K) $\gamma 2$ receptor currents (Fig. 3B) (4564 ± 345 pA for wild-type; 2154 ± 201 pA for mixed N328D and 967 ± 131 pA for mutant N328D; 3067 ± 245 pA for mixed E357K and 1752 ± 197 pA for mutant E357K). For both mutations, the mutant conditions were more reduced than the mixed conditions.

We then determined the zinc sensitivity of the receptors containing mutant $\beta 3$ subunits as reduced sensitivity to zinc would reflect the incorporation of $\gamma 2$ subunits. The current loss of the $\alpha 1\beta 3$ (E357K) $\gamma 2$ channels during the 6 s co-application of zinc (10 μ M) and GABA (1 mM) was greater relative to the wild-type receptor current and the mutant $\alpha 1\beta 3$ (N328D) $\gamma 2$ receptor current (Figure 3C) ($7.8\% \pm 3.1\%$ for wild-type versus $13.5\% \pm 4.5\%$ for mixed N328D and $15.7\% \pm 3.2\%$ for mutant N328D; $19.9\% \pm 3.6\%$ for mixed E357K and $34.7\% \pm 4.7\%$ for mutant E357K). Increased zinc sensitivity has been observed in $\alpha 1\beta 1-3$ receptors when $\gamma 2$ subunits are absent or impaired (Kang, *et al.*, 2013). This thus suggests that the mutation may result in altered receptor assembly with impaired partnering with $\gamma 2$ subunits.

Both GABRB3 mutations decreased surface levels of $\beta 3$, $\alpha 1$, and $\gamma 2$ subunits but to different extents

Reduced current amplitude of $\beta 3$ (N328D) and $\beta 3$ (E357K) subunit-containing receptor channels could be due to reduced receptor surface expression, altered receptor stoichiometry, or altered channel gating. To explore these options, we co-transfected the wild-type $\beta 3^{\text{HA}}$, mutant $\beta 3$ (N328D) $^{\text{HA}}$ or mutant $\beta 3$ (E357K) $^{\text{HA}}$ subunits with $\alpha 1$ and $\gamma 2$ subunits in HEK293T cells. The cell surface expression of each subunit 48 h after transfection was determined with surface biotinylation (Fig. 4A). The different reduction levels for the two mutations suggest differentially impaired receptor assembly in the mutant conditions. It is worth noting that $\beta 3$ subunit surface expression in mixed and

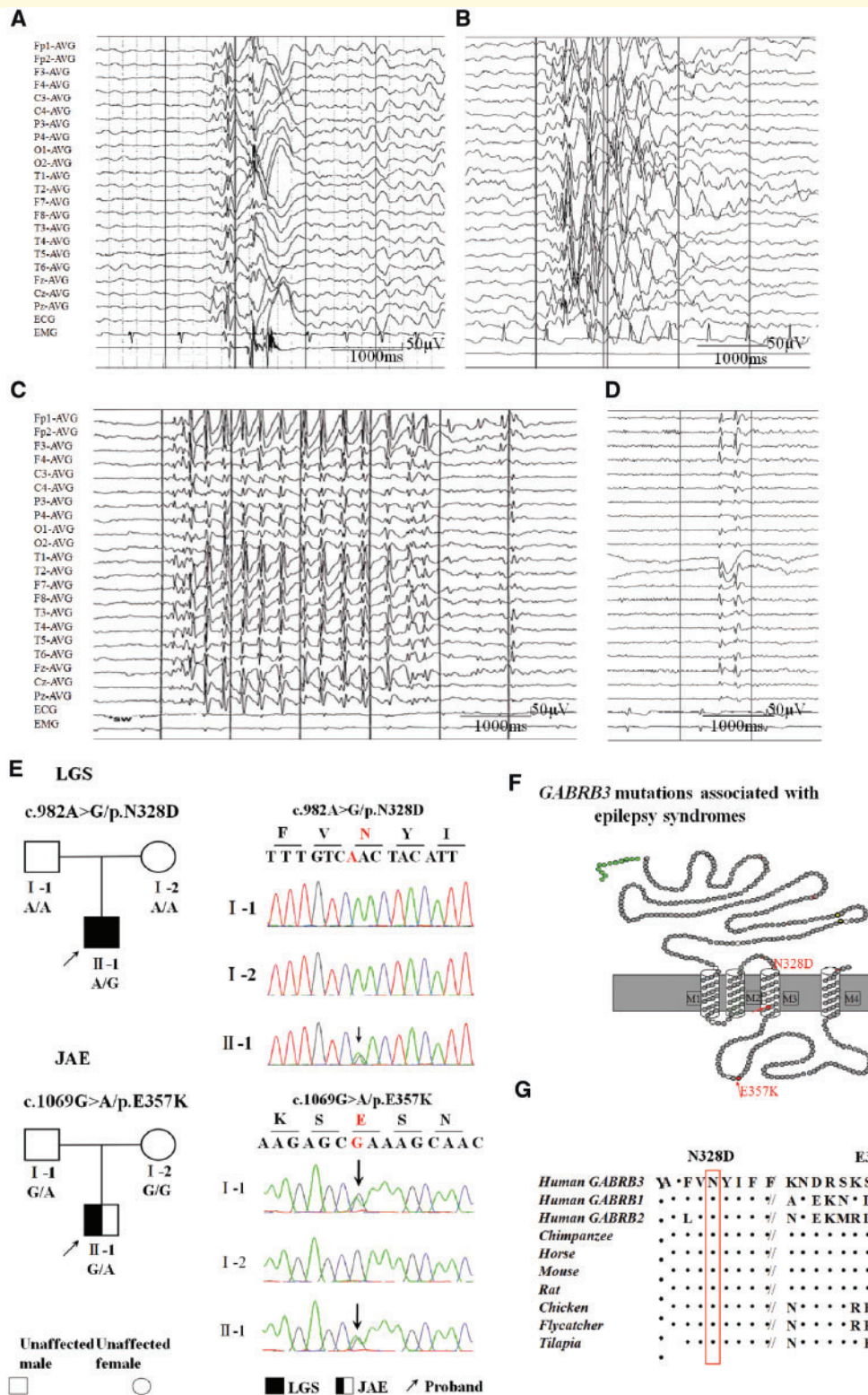


Figure 2 Clinical and molecular genetic findings in GABRB3 in patients with Lennox-Gastaut syndrome (LGS) or juvenile absence epilepsy (JAE). (A and B) Representative EEGs from each patient are presented. The top traces show EEG recordings from the patient carrying the GABRB3 (N328D) variation, showing a generalized poly-spike-waves discharge, accompanied by time-locked muscle activity on the EMG recording (A), and an interictal EEG of a fast rhythm that was superimposed on the following irregular slow waves during sleep (B). (C and D) EEG traces show an ictal EEG with paroxysmal 3.0–3.5 Hz generalized spike-and-wave discharges with behavioural arrest, lasting for ~5 s (C) and an interictal EEG of a single generalized spike or spike-and-waves discharge in the patient carrying the GABRB3 (E357K) variation (D). (E and F) Two novel mutations were identified in patients with a severe epilepsy syndrome (Lennox-Gastaut syndrome) with intellectual disability or a mild epilepsy syndrome (juvenile absence epilepsy) without intellectual disability. (F) Cartoon illustrates that the location of mutations in GABRB3 in the subunit protein topology. (G) The amino acids coded by both variations are conserved across species as showing in the boxed regions.

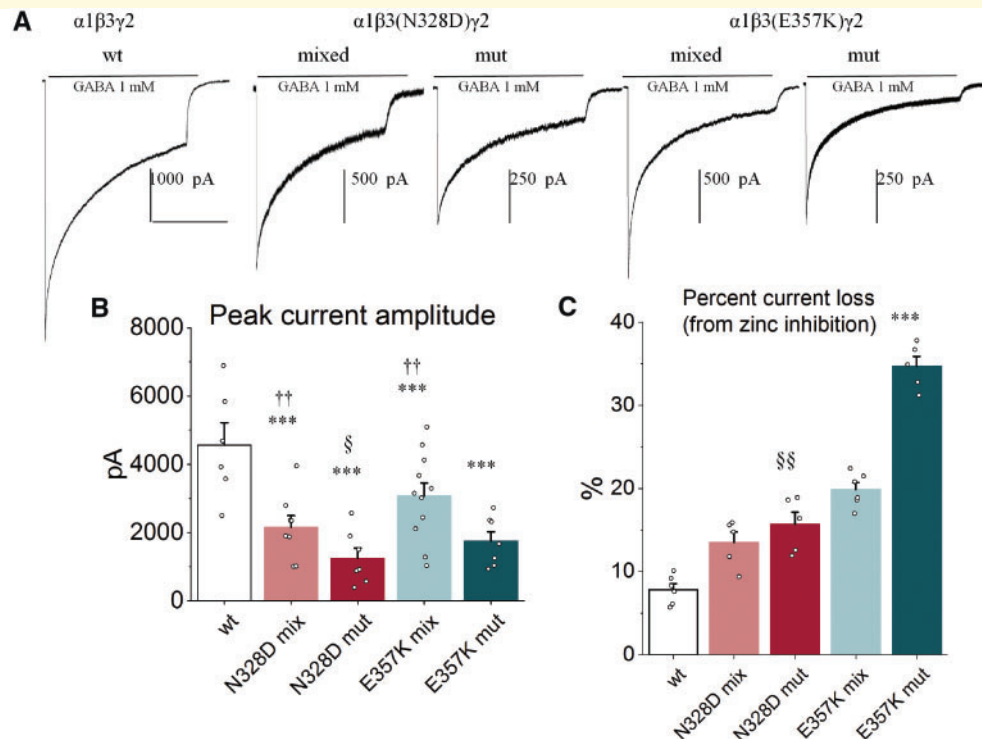


Figure 3 Mutant $\alpha 1\beta 3\gamma 2$ receptors showed decreased GABA-evoked whole-cell currents and altered zinc inhibition. (A–C) HEK 293 T cells were transfected with human $\alpha 1$ and $\gamma 2$ subunits in combination with the wild-type or mutant $\beta 3$ (N328D) or $\beta 3$ (E357K) subunits for 48 h. EGFP cDNA was included as a marker for positive transfection. Representative GABA-evoked current traces were obtained following rapid application of 1 mM GABA for 6 s to lifted HEK293T cells voltage clamped at -50 mV (A). (B) Bar graphs showing average peak current amplitude from the wild-type or the mutant $\alpha 1\beta 3\gamma 2$ receptors. Values are expressed as mean \pm standard error of the mean (SEM). (C) Per cent current loss was measured by subtracting the remaining current during GABA and zinc co-application from the peak current during GABA application. In B, $n = 6$ patches for wild-type, $n = 8$ for N328D mix, $n = 9$ for N328D, $n = 11$ for E357K mix and $n = 7$ for E357K. In C, $n = 6$ patches for wild-type, $n = 5$ for N328D mix, $n = 5$ for N328D, $n = 6$ for E357K mix and $n = 5$ for E357K. In B and C, $***P < 0.001$ versus wild-type, $^{\dagger\dagger}P < 0.01$ versus mixed in N328D or in E357K; $^{\S}P < 0.05$; $^{\S\S}P < 0.01$ versus mutant in E357K. One-way ANOVA and Newman-Keuls test was used to determine significance compared to the wild-type condition and between mutations.

mutant conditions was not different (Fig. 4B) (for $\beta 3^{\text{HA}}$ subunit: 0.64 ± 0.03 for N328D mixed versus 0.55 ± 0.05 for N328D mutant and 0.75 ± 0.04 for E357K mixed and 0.66 ± 0.05 for E357K mutant; for $\alpha 1$ subunit: 0.85 ± 0.08 for N328D mixed versus 0.82 ± 0.04 for N328D mutant and 0.71 ± 0.07 for E357K mixed and 0.60 ± 0.07 for E357K mutant; for $\gamma 2$ subunit: 0.73 ± 0.03 for N328D mixed versus 0.65 ± 0.07 for N328D mutant and 0.64 ± 0.03 for E357K mixed and 0.41 ± 0.04 for E357K mutant. The integrated protein density values (IDVs) of wild-type subunits were arbitrarily taken as 1 in all cases. A similar phenomenon of relatively increased mutant protein per gene dose in the homozygous compared to the heterozygous condition has also been reported for other GABA_A receptor mutations (Kang, *et al.*, 2009b; Todd *et al.*, 2014). This suggests that the mutant subunit is subject to more rapid disposal in the presence of the wild-type subunit as in the mixed condition compared with the mutant condition. Only the mutant $\beta 3$ (N328D) subunit had reduced total $\beta 3$

subunit expression but the $\beta 3$ (E357K) subunit had reduced total $\gamma 2$ subunits.

The reduced surface subunit expression with mutant $\alpha 1\beta 3$ (N328D) $\gamma 2$ and $\alpha 1\beta 3$ (E357K) $\gamma 2$ receptors could be due to reduced total subunit expression because the mutations may affect the biogenesis of GABA_A receptor subunits. We next determined total $\beta 3$ subunit expression by co-expressing the wild-type and mutant $\beta 3$ subunits with $\alpha 1$ and $\gamma 2$ subunits in HEK293T cells. HA-tagged $\beta 3$ subunits were used for immunohistochemistry while untagged $\beta 3$ subunits were used for western blot. With immunohistochemistry for total subunit expression, 0.5% TritonTM X-100 permeabilized cells were stained with anti-HA and anti- $\gamma 2$ subunit antibodies. Cells expressing the wild-type subunits displayed a primary localization to the plasma membrane for both $\beta 3^{\text{HA}}$ and $\gamma 2$ subunits. In contrast, $\beta 3^{\text{HA}}$ and $\gamma 2$ subunits in both mutant $\alpha 1\beta 3$ (N328D)^{HA} $\gamma 2$ and $\alpha 1\beta 3$ (E357K)^{HA} $\gamma 2$ receptors accumulated intracellularly and had impaired trafficking to the cell surface (Supplementary Fig. 2).

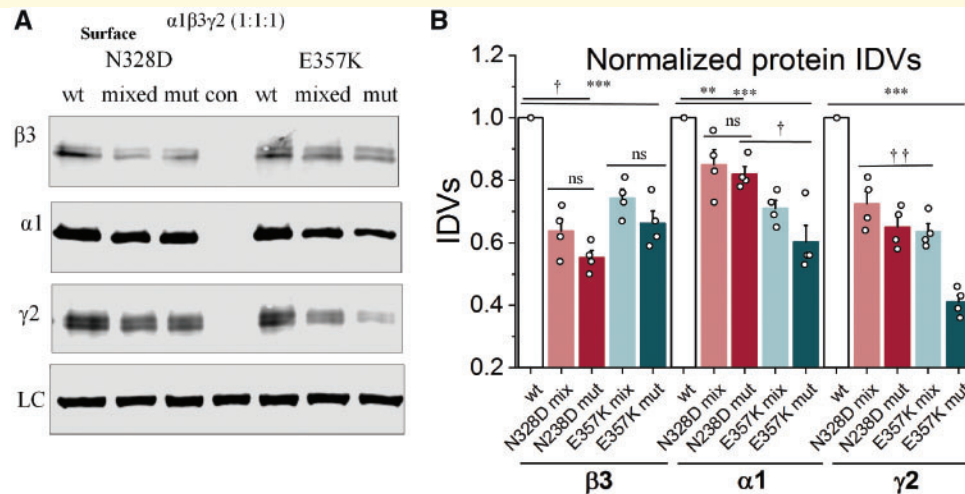


Figure 4 Both mutant $\alpha 1\beta 3(N328D)\gamma 2$ and $\alpha 1\beta 3(E357K)\gamma 2$ receptors had reduced subunit surface expression. (A) HEK293 T cells were transfected with human $\alpha 1$ and $\gamma 2$ subunits in combination with the wild-type $\beta 3$ subunit alone (WT), mixed wild-type and mutant $\beta 3$ subunit for mixed, or the mutant $\beta 3(N328D)^{HA}$ or $\beta 3(E357K)^{HA}$ subunits alone for mutant (mut) for 48 h. Surface protein samples were collected through biotinylation and probed by rabbit polyclonal anti- $\beta 3$ or anti- $\gamma 2$ subunit or mouse monoclonal anti- $\alpha 1$ subunit antibodies. LC = loading control (ATPase). (B) Surface expression of each subunit protein from western blot was quantified. The integrated protein density values (IDVs) were normalized to the loading control first and then to the wild-type which is arbitrarily taken as 1. $**P < 0.01$, $***P < 0.001$ versus wild-type, $†P < 0.05$, $††P < 0.01$, N328 heterozygous (het) versus E357K het, ns = non-significant, $n = 4$ different transfections, one-way ANOVA and Newman-Keuls test. Values are expressed as mean \pm SEM.

Mutant $\beta 3$ subunits had reduced receptor puncta in synapses likely due to intracellular retention of mutant subunits

In the CNS, GABA_A receptors are normally expressed in neurons in which the cellular structure and intracellular machinery are more complex than in HEK293 T cells. Therefore, we expressed the wild-type and mutant $\beta 3^{HA}$ subunits in rat cortical neurons. We cultured the neurons from embryonic Day 18 pups for 5–7 days and then transfected the neurons using calcium precipitation. The immunostaining and western blots were carried out 8–10 days after transfection. The expression patterns of wild-type and mutant $\beta 3^{HA}$ subunits in neurons were quite different as determined by both immunohistochemistry (Fig. 5A) and western blot (Fig. 5B). The wild-type $\beta 3^{HA}$ subunits displayed a clear pattern of puncta on neuronal processes and the soma with minimal intracellular somatic distribution, while the mutant $\beta 3(N328D)^{HA}$ and $\beta 3(E357K)^{HA}$ subunits had increased intracellular somatic expression (5.2 ± 0.8 for wild-type versus 16.8 ± 3.1 for N328D and 24.5 ± 4.2 for E357K) (Fig. 5C) and reduced expression of puncta on the soma and neuronal processes (42.3 ± 4.8 for wild-type versus 4.6 ± 1.5 for N328D and 16.1 ± 2.5 E357K) (Fig. 5D). The total expression of $\beta 3^{HA}$ subunits in neurons was much lower than that in HEK293T cells, and the expression level of the mutant subunits in neurons was reduced compared to that of wild-type $\beta 3^{HA}$ subunits (0.61 ± 0.03 for N328D and 0.74 ± 0.02 for E357K (the IDVs of wild-

type $\beta 3^{HA}$ subunits were arbitrarily taken as 1) (Fig. 5E). This suggests that $\beta 3(N328D)^{HA}$ subunits are less stable than $\beta 3(E357K)^{HA}$ subunits in neurons, which was consistent across cell types.

GABRB3 mutations differentially decreased the synaptic clustering partnering $\gamma 2$ subunits

Multiple GABA_A receptor subunits are expressed in mammalian CNS neurons with varied spatial and temporal distributions as well as subunit compositions. The altered subcellular localization of mutant $\beta 3$ subunits may affect the subcellular distribution of partnering subunits. We determined the distribution of subunits that partner with $\beta 3$ subunits. It is known that $\gamma 2$ subunits co-assemble with $\beta 3$ subunits and are critical for GABA_A receptor synaptic clustering (Allred *et al.*, 2005). We co-stained $\gamma 2$ subunits with the major inhibitory neuronal presynaptic marker vGAT (Fig. 6A). The cell nuclei were marked with the nuclear marker TO-PRO-3. Immunostaining of vGAT and $\gamma 2$ subunits revealed multiple dendritic and somatic puncta. The number of vGAT puncta were not different between wild-type and mutant subunits (29.3 ± 2.9 for wild-type versus 28.5 ± 3.1 for N328D and 31 ± 5.2 E357K) (Fig. 6B); however, the number of $\gamma 2$ puncta were reduced in neurons expressing $\beta 3(N328D)^{HA}$ and $\beta 3(E357K)^{HA}$ subunits when compared with neurons expressing wild-type $\beta 3^{HA}$ subunits (39.4 ± 6.6 for wild-type versus 16.5 ± 4.2 for N328D

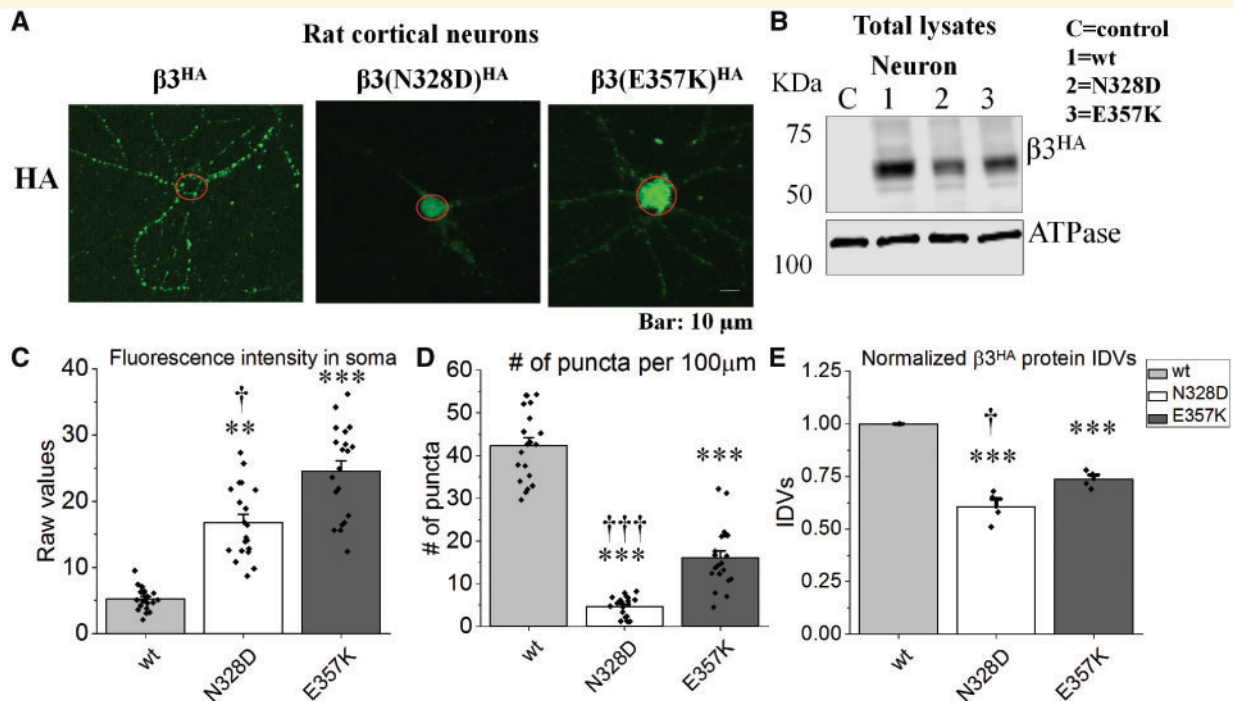


Figure 5 Both mutant $\beta 3(N328D)^{HA}$ and $\beta 3(E357K)^{HA}$ subunits had reduced distribution in dendrites in neurons. (A) Rat cortical neurons were transfected with wild-type ($\beta 3^{HA}$) or mutant [$\beta 3(N328D)^{HA}$ and $\beta 3(E357K)^{HA}$] subunits at Day 5–7 in culture. At 8–10 days after transfection, the neurons were permeabilized and stained with mouse monoclonal anti-HA antibody and visualized with fluorophore Alexa-488. (B) Total lysates of mouse cortical neurons expressing the empty vector pCDNA (control), the HA-tagged wild-type (WT) ($\beta 3^{HA}$) or the mutant $\beta 3$ subunits $\beta 3(N328D)^{HA}$ and $\beta 3(E357K)^{HA}$ in 100 mm² dishes were collected at Day 8–10 after transfection. The lysates were separated by SDS-PAGE and immunoblotted with mouse monoclonal anti-HA antibody. ATPase was used for loading control. (C) The raw fluorescence values in somata were measured. The identification of somata as red circled area for each neuron was arbitrary because no neuronal nuclei marker was applied in this experiment. (D) $\beta 3^{HA}$ subunit fluorescence puncta in neurons expressing the wild-type or the mutant $\beta 3^{HA}$ subunits were quantified. The total fluorescence puncta per 100 μ m were measured. In C and D, $n = 20$ neurons for each condition from four different transfections. At least three to five coverslips were surveyed from each transfection (E). The total expression of the wild-type or the mutant $\beta 3^{HA}$ subunit in neurons was quantified. The IDVs were normalized to the loading control first and then to the wild-type which is arbitrarily taken as 1 ($n = 4$ different transfections). $**P < 0.01$, $***P < 0.001$ versus wild-type, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$ versus E357K. For C and D, one-way ANOVA and Newman-Keuls test. For E, one sample t-test and unpaired t-test were used for N328D versus E357K. Values are expressed as mean \pm SEM.

and 19.7 ± 5.3 E357K) (Fig. 6C). The $\gamma 2$ subunit is critical for clustering GABA_A receptor in synapses (Alldred *et al.*, 2005), suggesting a reduced postsynaptic distribution of GABA_A receptors with both mutations due to reduced $\gamma 2$ subunits in synapses.

Mutant $\beta 3$ subunits are reduced in inhibitory synapses relative to wild-type $\beta 3$ subunits

Next, we determined the expression of mutant $\beta 3$ subunits in the inhibitory postsynaptic regions in cultured rat cortical neurons. Gephyrin is the established postsynaptic marker for inhibitory synapses and a scaffold protein for GABA_A receptors. GABA_A receptor $\beta 3$ subunits have been demonstrated to be important for receptor assembly (Connolly *et al.*, 1996) while $\gamma 2$ subunits are required for postsynaptic receptor clustering (Alldred *et al.*, 2005).

There was a reduced distribution of $\gamma 2$ subunit puncta with expression of either mutant subunit, suggesting a reduced synaptic distribution of GABA_A receptors. We then validated the effect of the mutations on receptor synaptic localization. We co-stained the $\beta 3^{HA}$ subunit with gephyrin in rat cortical neurons expressing wild-type or mutant $\beta 3^{HA}$ subunits. The cell nuclei were stained with the nuclear marker TO-PRO-3 (Fig. 7A). Consistent with the findings for $\beta 3^{HA}$ subunit expression (Fig. 5A, C and D), $\beta 3^{HA}$ subunit puncta co-stained with gephyrin were reduced (35.5 ± 4.6 for wild-type versus 7.8 ± 2.5 for N328D and 22.5 ± 5.1 for E357K) (Fig. 7B). Not surprisingly, the fluorescent signalling of $\beta 3^{HA}$ subunits overlapping gephyrin fluorescence was also reduced ($51.2\% \pm 8.3\%$ for wild-type versus $11.8\% \pm 3.7\%$ for N328D and $31.3\% \pm 6.2\%$ for E357K (Fig. 7C). Similar reduction was observed for $\gamma 2$ subunit gephyrin/overlapping (data not shown). Because gephyrin is the marker

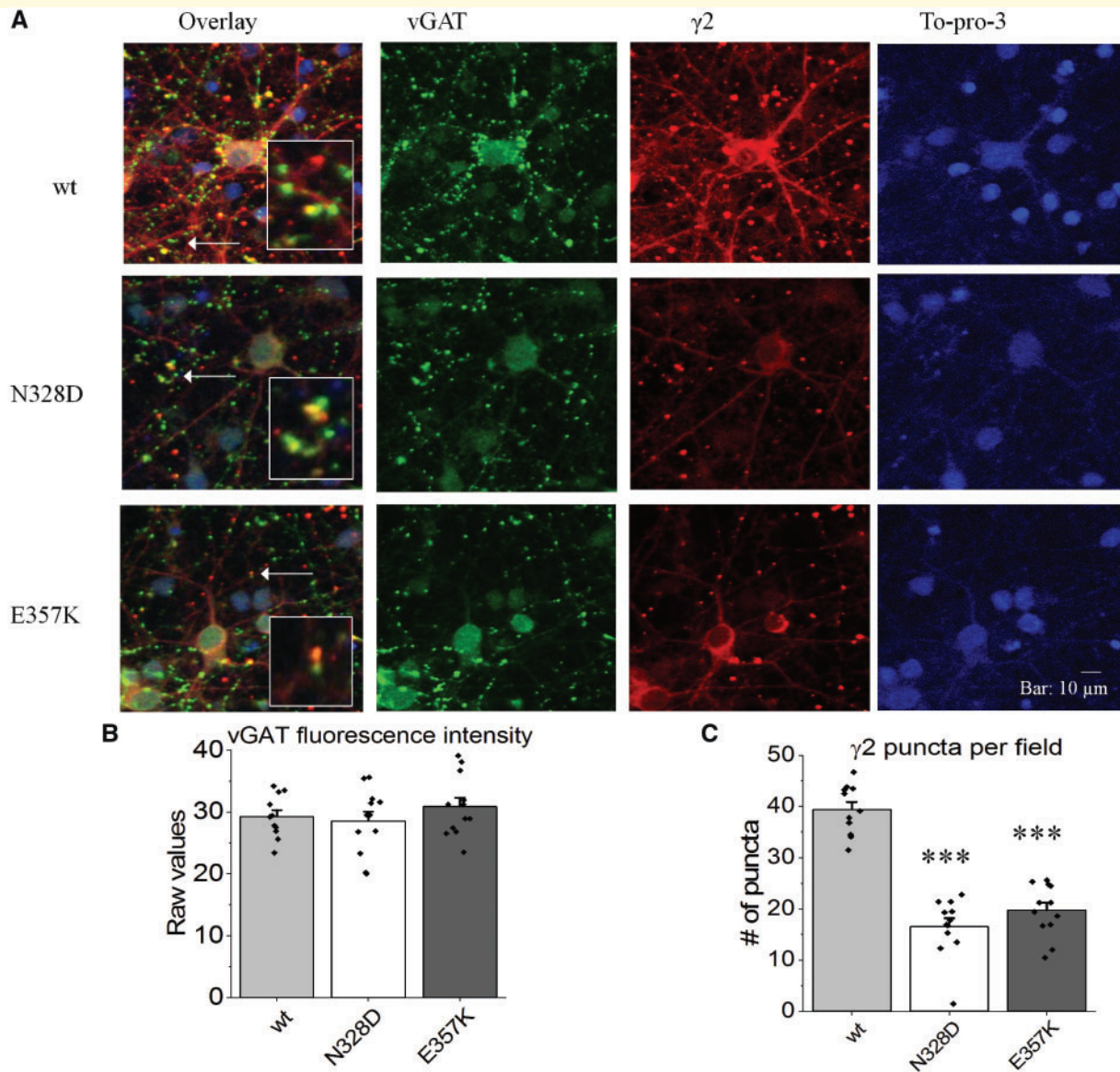


Figure 6 Rat cortical neurons expressing the mutant $\beta 3$ (N328D)^{HA} or $\beta 3$ (E357K)^{HA} subunit had reduced $\gamma 2$ subunits in synapses. **(A)** Rat cortical neurons were transfected with wild-type or mutant $\beta 3$ subunits at Day 5 in culture. At 8 days after transfection, the neurons were permeabilized and co-stained with rabbit polyclonal anti- $\gamma 2$ and mouse monoclonal anti-vGAT (vesicular GABA transporter) antibodies. Mouse polyclonal IgGs were visualized with the fluorophore Alexa-488 while the rabbit IgGs were visualized with rhodamine. Cell nuclei were stained with TO-PRO-3. White arrow indicates area enlarged in insets. **(B)** Total fluorescence intensity of vGAT in rat cortical neurons expressing the wild-type or the mutant $\beta 3$ subunits was quantified by ImageJ. **(C)** Total $\gamma 2$ subunit fluorescence puncta in neurons expressing the wild-type or the mutant $\beta 3$ subunits in each visualized whole field were quantified. In **B** and **C**, $n = 11$ for wild-type, 12 for N328D and 12 for E357K non-overlapping visualized fields from four different transfections. *** $P < 0.001$ versus wild-type, One-way ANOVA and Newman-Keuls test. Values are expressed as mean \pm SEM.

for inhibitory synapses, this suggests a reduced presence of mutant $\beta 3^{\text{HA}}$ subunits in inhibitory synapses.

Impaired synaptic incorporation of $\gamma 2$ subunits in *Gabrb3*^{+/-} mice

Both *GABRB3* (N328D) and *GABRB3* (E357K) mutations reduced the function of the mutant $\beta 3$ subunits. Therefore, a similar impairment of $\gamma 2$ subunits at synapses should be

identified in the *GABRB3* loss-of-function condition. We determined total expression of $\beta 3$ and $\gamma 2$ subunits in *Gabrb3*^{+/-} mice. First, we determined the $\gamma 2$ subunit expression in the deep layers of somatosensory region in the *Gabrb3*^{+/-} mice. We surveyed layers 5–6 in somatosensory cortex from both wild-type and *Gabrb3*^{+/-} mice. The choice of these layers was based on the involvement of thalamocortical circuitry in seizure generation and neurons in layers 5–6 receive direct projection of ventrobasal

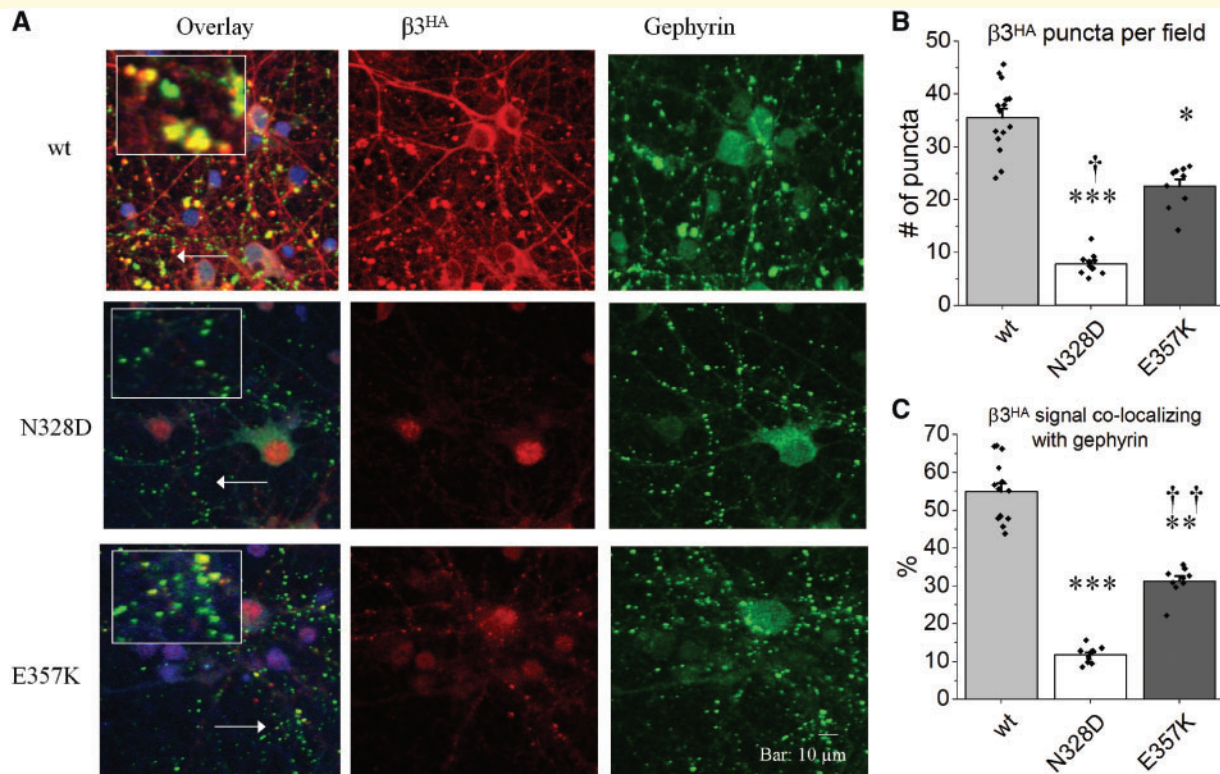


Figure 7 The mutant $\beta 3(\text{N328D})^{\text{HA}}$ and $\beta 3(\text{E357K})^{\text{HA}}$ subunits have reduced co-localization with the inhibitory postsynaptic marker gephyrin. **(A)** Rat cortical neurons were transfected with wild-type or mutant $\beta 3$ subunits at Days 5–7 in culture. At 8–10 days after transfection, the neurons were permeabilized and co-stained with rabbit polyclonal anti-HA and mouse monoclonal anti-gephyrin antibodies. Mouse polyclonal IgGs were visualized with the fluorophore Alexa-488 while the rabbit IgGs were visualized with rhodamine. Cell nuclei were stained with TO-PRO-3. White arrow indicates area enlarged in insets. **(B)** Total fluorescence puncta of $\beta 3^{\text{HA}}$ subunits in rat cortical neurons expressing wild-type or mutant $\beta 3$ subunits in each whole visualized field were quantified. **(C)** The per cent $\beta 3^{\text{HA}}$ subunit fluorescence signal overlapping gephyrin fluorescence signal was quantified in neurons expressing the wild-type or the mutant $\beta 3$ subunits in each whole field. In **B** and **C**, $n = 15$ for wild-type, 11 for N328D, and 9 for E357K; non-overlapping visualized fields from four different transfections. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus wild-type, † $P < 0.05$, †† $P < 0.01$ versus E357K, one-way ANOVA and Newman-Keuls test). Values are expressed as mean \pm SEM.

complex neurons (Donoghue and Ebner, 1981) and our previous study on *GABRG2* (Q390X) mutation associated with Dravet syndrome (Kang *et al.*, 2015). We determined the expression of $\gamma 2$ subunits at synapses by co-staining $\gamma 2$ subunits and gephyrin (Fig. 8A). We also isolated synaptosomes from the forebrain by subcellular fractionation and determined the protein levels of $\beta 3$ and $\gamma 2$ subunits and gephyrin in synaptosomes (Fig. 8B). Similar to the findings from cultured neurons, the fluorescence values of both $\gamma 2$ subunits (58 ± 7.9 for wild-type and 31.2 ± 7.8 for heterozygous) and the inhibitory synaptic marker, gephyrin (53.6 ± 3.1 for wild-type and 35.1 ± 5.3 for heterozygous) were reduced in the cortical somatosensory region of *Gabrb3*^{+/-} mice (Fig. 8C). Consistent with findings from the immunohistochemistry study, in the synaptosomes isolated from forebrain, $\beta 3$ (0.51 ± 0.05 for heterozygous versus wild-type ± 1) and $\gamma 2$ (0.49 ± 0.09 for heterozygous versus wild-type ± 1) subunits as well as gephyrin (0.68 ± 0.12 for heterozygous versus wild-type ± 1) were reduced in the *Gabrb3*^{+/-} mice (Fig. 8D). It is worth

noting that the expression of gephyrin was not reduced in neurons transfected with the mutant $\beta 3$ subunits. Further study is required to elucidate the interaction of $\gamma 2$ subunits and gephyrin in *Gabrb3*-deficient mice.

Discussion

GABRB3 mutations are associated with a variety of epilepsy syndromes, autism and intellectual disabilities

Mutations in *GABRB3* have been recognized as a major cause for severe epilepsy syndromes with intellectual disability (Le *et al.*, 2017) in addition to previous association with childhood absence epilepsy and autism (Delahanty *et al.*, 2011). Recent genetic sequencing has associated *GABRB3* with several epileptic encephalopathies including Lennox-Gastaut syndrome, infantile spasms, early onset

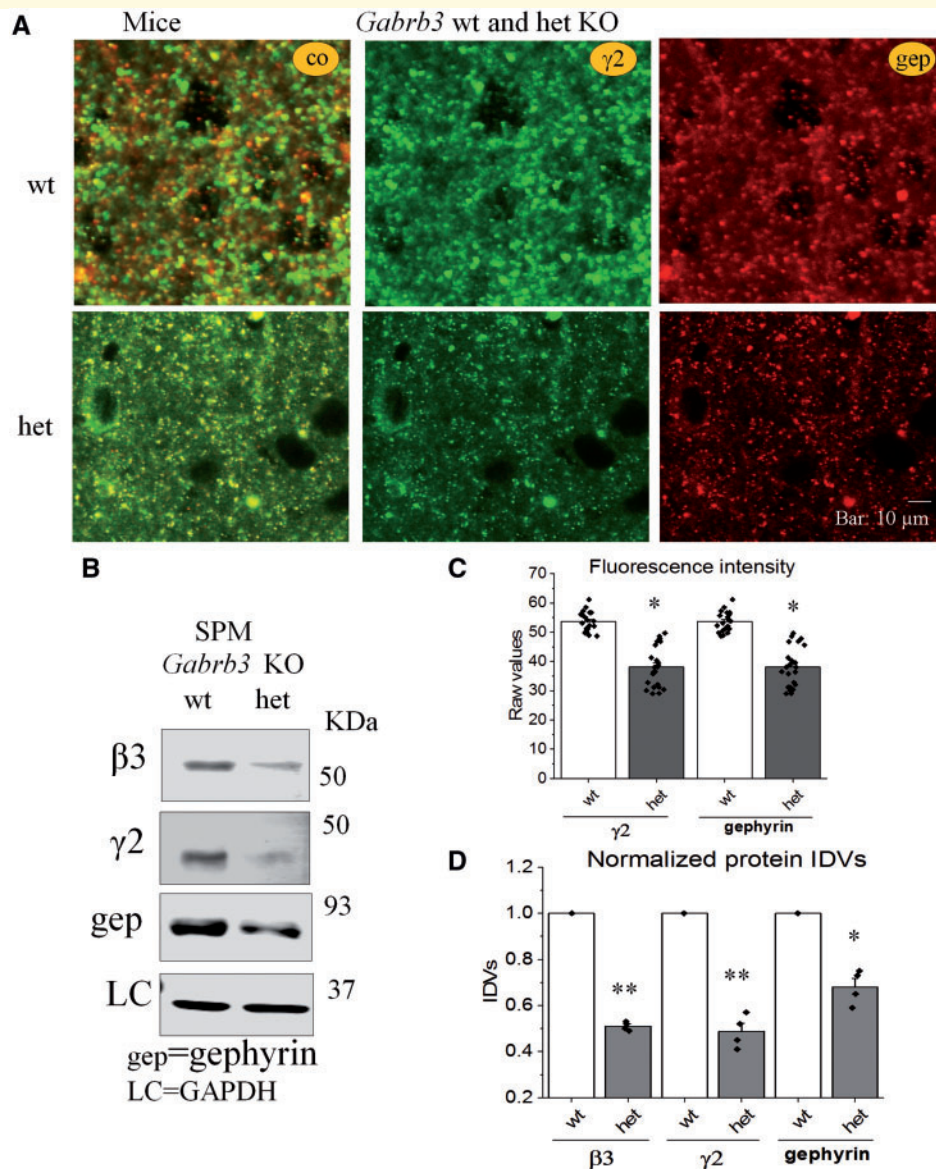


Figure 8 Loss of $\beta 3$ subunit function impaired $\gamma 2$ subunit clustering at inhibitory synapses in *Gabrb3*^{+/-} mice. **(A)** Brain tissue from wild-type (wt) or heterozygous (het) *Gabrb3*^{+/-} mice were briefly fixed, permeabilized and co-stained with rabbit polyclonal anti- $\gamma 2$ and mouse monoclonal anti-gephyrin (gep) antibodies. Rabbit polyclonal IgGs were visualized with the fluorophore Alexa-488 while the mouse IgGs were visualized with rhodamine. Co = co-localization; KO = knockout. **(B)** Synaptosome protein samples were collected through subcellular fractionation and probed by rabbit polyclonal anti- $\beta 3$ or anti- $\gamma 2$ subunit or mouse monoclonal gephyrin antibodies. LC = control (GAPDH). **(C)** Fluorescence values of $\gamma 2$ subunits or gephyrin were quantified (**P* < 0.05 versus wild-type, unpaired t-test). Values were expressed as mean \pm SEM, *n* = 12 sections for each genotype from four different pairs of mice for $\gamma 2$, and *n* = 20 sections for wild-type and 23 sections for heterozygous from seven different pairs of mice for gephyrin. For each section, three representative regions were chosen, and the average value of the three regions was taken as *n* = 1). **(D)** The integrated protein density values (IDVs) from synaptosome preparations were normalized to the loading control first and then to the wild-type, which was arbitrarily taken as 1 (*n* = 4 pairs of 2–4-month-old mice, one-sample t-test).

epileptic encephalopathy, and Dravet syndrome (Le *et al.*, 2017). We have also shown that a disruption in the promoter region of *GABRB3* causes ocular hypopigmentation and visual defects, suggesting complex genetic regulation of *GABRB3* and adjacent genes (Delahanty *et al.*, 2016) and a role in brain development. However, the role of *GABRB3* in neurodevelopmental disorders such as severe epilepsy

syndromes as well as the pathophysiology underlying the phenotypic heterogeneity are far from clear (He *et al.*, 2019). For example, among the reported mutations, *GABRB3* (N110D) is associated with infantile spasms while *GABRB3* (R232Q) is associated with Dravet syndrome. This study has focused on comparison between two novel mutations in *GABRB3* associated with

Lennox-Gastaut syndrome and juvenile absence epilepsy; it would be of importance to elucidate the difference between mutations associated with other epileptic encephalopathies such as infantile spasms and Dravet syndrome with in-depth study in neuronal conditions and *in vivo* mouse models.

Impaired $\gamma 2$ subunit synaptic clustering is a shared pathophysiology among GABRB3 epilepsy mutations

GABA_A receptor assembly has been extensively studied, and it is known that both $\alpha\beta$ binary and $\alpha\beta\gamma$ tertiary receptors can be assembled (Connolly *et al.*, 1996). Although $\gamma 2$ subunits are not required for receptor assembly, they are required for clustering of receptors at synapses (Alldred *et al.*, 2005). We propose that impaired presence of receptors at synapses is a common mechanism for GABRB3 mutations associated with genetic epilepsies. Previous work on GABRB3 mutations associated with Lennox-Gastaut syndrome and infantile spasms indicated that the mutations altered channel gating by decreasing single channel open probability or altered channel deactivation, without loss of surface receptors (Janve *et al.*, 2016). In the current study we compared multiple GABRB3 epilepsy mutations and identified a common pathophysiology regardless of the trafficking capacity of each mutant $\beta 3$ subunit. It is worth noting that the variable surface expression with mutant $\beta 3$ subunit expression could be because $\beta 3$ subunits alone can form homopentamers and traffic to the cell surface; however, the physiological significance of $\beta 3$ homopentamers is unclear. For the GABRB3 (N328D) mutation, the impaired synaptic clustering was likely due to reduced total $\beta 3$ (N328D) subunits, thus leading to reduced oligomerization and assembly of $\alpha\beta 3\gamma 2$ receptors. For the GABRB3 (E357K) mutation, there were relatively more $\beta 3$ subunits available that could oligomerize and assemble with $\gamma 2$ subunits, but surface receptors were still reduced compared with wild-type surface receptors. The GABRB3 (N328D) mutation resulted in a less stable protein than the GABRB3 (E357K) mutation. However, other disease modifying factor such as genetic background may also play a role but this merits more investigation.

GABRB3 mutations associated with different epilepsies had variable synaptic receptor expression

The GABRB3 (N328D) and GABRB3 (E357K) mutations are associated with epilepsy syndromes with different severities. GABRB3 (N328D) is associated with a severe epilepsy syndrome, Lennox-Gastaut syndrome, while GABRB3 (E357K) is associated with a mild epilepsy syndrome, juvenile absence epilepsy. The patient carrying the GABRB3 (N328D) mutation had developmental delay with intellectual disability while the patient carrying the

GABRB3 (E357K) mutation was developmentally normal. This is possibly because the GABRB3 (N328D) mutation produced a subunit with a more severely reduced synaptic distribution. We have demonstrated that a moderate upregulation of $\gamma 2$ subunits would significantly attenuate the epilepsy phenotype in epilepsies with GABRG2 mutations (Kang *et al.*, 2015; Warner *et al.*, 2016; Xia *et al.*, 2016; Huang *et al.*, 2017). It is worth noting that the loss of $\beta 3$ and $\gamma 2$ subunit-containing synaptic receptors is tolerated to a certain extent, as the father carrying the GABRB3 (E357K) mutation has no seizures. However, it is unknown if this is relevant for $\beta 3$ subunits and if there is any compensation with other GABA_A receptor subunits in the same stoichiometric position in patients carrying GABRB3 mutations. Furthermore, there may be other non GABR genetic modifiers involved in the clinical phenotype presentation (Mistry *et al.*, 2014; Hawkins *et al.*, 2016).

The difference in reducing synaptic $\gamma 2$ subunits may be related to different mutant $\beta 3$ subunit stability

Contrary to GABRG2 nonsense mutations, in which increased mutant subunit amount is associated with more severe epilepsy phenotype (Kang *et al.*, 2013), less stable mutant $\beta 3$ subunits are associated with a more severe epilepsy phenotype. This echoes the finding from mouse models with loss of function mutations in *Gabrg2* (Crestani *et al.*, 1999; Kang *et al.*, 2015) and *Gabrb3* (Homanics *et al.*, 1997). Our findings suggest that $\beta 3$ (N328D) subunits are less stable than wild-type subunits while $\beta 3$ (E357K) subunits may have impaired oligomerization with $\gamma 2$ subunits. It is likely that $\beta 3$ (E357K) subunits were more stable than $\beta 3$ (N328D) subunits, although both were less stable when compared with wild-type subunits. It is interesting that $\gamma 2$ subunits were more reduced in $\alpha 1\beta 3$ (E357K) $\gamma 2$ receptors than in $\alpha 1\beta 3$ (N328D) $\gamma 2$ receptors. This could be due to differential binding affinity at the $\gamma 2/\beta 3$ interface or stability of $\gamma 2\beta 3$ oligomers. It has been demonstrated that GABA_A receptor subunits form dimers or oligomers first and then form pentamers (Klausberger *et al.*, 2001). It is possible that there is impaired binding between $\gamma 2$ - $\beta 3$ (E357K) oligomers compared with wild-type and $\beta 3$ (N328D) subunits. It is also possible that there are some $\beta 3$ (E357K) subunit homopentamers but no $\beta 3$ (N328D) subunit homopentamers.

Inactivation of synaptic clustering via the $\gamma 2$ subunit is a novel mechanism underlying the pathology of GABRB3 mutations. This is consistent with the critical role of $\gamma 2$ subunits for postsynaptic GABA_A receptor clustering (Alldred *et al.*, 2005) and also suggests a close interaction of $\beta 3$ and $\gamma 2$ subunits, which needs further investigation. Unlike previous GABRG2 mutations associated with severe epilepsy syndromes that suppress $\gamma 2$ subunit partnering subunits by enhanced degradation (Kang *et al.*, 2009a), the GABRB3 (N328D) mutation did not change the total

expression of the partnering subunits such as $\alpha 1$ subunits. However, the synaptic localization of $\gamma 2$ subunits as well as of mutant $\beta 3$ subunits was reduced. We found that receptors containing either *GABRB3* (N328D) or *GABRB3* (E357K) mutant subunits had reduced GABA-evoked peak current amplitude. This is likely due to a reduced number of functional receptors at the cell surface. Indeed, for both mutations, the mutant $\beta 3$ subunits had reduced surface expression. In addition, the partnering $\alpha 1$ and $\gamma 2$ subunits also had reduced surface expression but not always reduced total protein expression. This thus suggests intracellular retention of mutant as well as partnering wild-type subunits. This phenomenon is consistent with what has been observed for the $\gamma 2$ subunit in which mutations have been frequently associated with febrile seizures (Baulac *et al.*, 2001; Wallace *et al.*, 2001; Tan *et al.*, 2007), GTCS, and Dravet syndrome (Ishii *et al.*, 2014; Kang *et al.*, 2015), as well as recently reported missense mutations associated with unspecified epileptic encephalopathies (Shen *et al.*, 2017).

A subtle difference in mutant receptor trafficking and function may have a profound impact on early brain development and disease phenotype

This study identified impaired synaptic clustering of wild-type $\gamma 2$ subunits in neurons harbouring mutant $\beta 3$ subunits, and this was validated in the *Gabrb3*^{+/-} mouse by isolating the synaptosomes in the mouse forebrain. The $\beta 3$ (N328D) subunits were more impaired than the $\beta 3$ (E357K) subunits. It is likely that there are no or minimal mutant $\beta 3$ (N328D) subunits in synapses while there are some mutant $\beta 3$ (E357K) subunits in synapses (Fig. 7D). Additionally, there may be differentially altered receptor assembly in each mutant condition, but this needs further study. For example, it is not entirely clear how mutant $\beta 3$ (E357K) subunits are assembled in pentamers either as homopentamers or as heteropentamers. Nevertheless, we made a step forward in understanding the pathophysiology of *GABRB3* mutations in a physiologically relevant system and identified a common mechanism: the mutant $\beta 3$ subunits disable the function of partnering $\gamma 2$ subunits, which are critical for synaptic clustering (Supplementary Fig. 3).

Future work is needed to understand the impact of the mutations in the context of brain development. Impaired GABA_A receptor activation may contribute to abnormal synaptogenesis and brain development, which may result in a phenotype beyond neuronal network inhibition and seizures and explains those non-seizure phenotypes in *GABRB3* mutations. It has been demonstrated that in the developing mouse cortex, local GABA release on dendrites induces gephyrin puncta and dendritic spine formation via GABA_A receptor activation (Oh *et al.*, 2016). Consequently, impaired GABA_A receptor function would

compromise the process of synaptogenesis. Indeed, the GABA_A receptor blocker gabazine significantly reduced gephyrin clustering while a GABA_B receptor blocker did not alter gephyrin puncta (Oh *et al.*, 2016). Compromise of the early depolarizing neurons due to the impaired GABA_A receptor function caused by the mutations could impair synaptogenesis and circuit formation, and thus result in complex neurodevelopmental disorders including epilepsy, autism, and intellectual disability. This thus suggests that in addition to impairing GABAergic synaptic neurotransmission, which may give rise to seizures, *GABRB3* plays a critical role in the pathogenesis of the phenotype for neurodevelopmental disorders such as autism and impaired cognition, which are commonly seen in severe epilepsy syndromes as comorbidities (Brooks-Kayal, 2010; Kang and Barnes, 2013).

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Competing interests

The authors declare no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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