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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 β3 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 β3 subunits is variable. However, it was consistent that surface expression of partnering γ2 subunits was lower when co-expressed with mutant than with wild-type subunits. Because γ2 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 β3 (N328D) subunit was more reduced than the juvenile absence epilepsy mutant β3 (E357K) subunit. Interestingly, both mutant β3 subunits impaired postsynaptic clustering of wild-type $GABA_A$ receptor γ2 subunits and prevented γ2 subunits from incorporating into $GABA_A$ receptors at synapses, although by different cellular mechanisms. Importantly, wild-type γ2 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 β3 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 β3 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 (Alldred *et al.*, 2005). Unlike other $GABA_A$ receptor subunits that require assembly with partnering subunits for surface localization, the β3 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 β3 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 β3 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, β3 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 β3 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 β3 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/Shujia Huang/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 $\alpha1$, $\beta3$, $\gamma2$, and haemagglutinin (HA)-tagged $\beta3$ or $\gamma2$ 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 $\beta3$ and $\gamma2$ subunits. The protein expression pattern and biophysical properties of the HA-tagged constructs were unaltered compared with those of native $\beta3$ or $\gamma2$ 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 $\gamma2$ subunit ($\gamma2$ S) 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.*, 2009*b*). Briefly, HEK293T cells were transfected using polyethylenimine (PEI) reagent (40 kD, Polysciences) at a DNA:PEI ratio of 1 μg:2.5 μl and harvested 48 h after transfection. Total 3 μg of cDNA of $GABA_A$ receptor $\alpha1$, $\beta3$ and $\gamma2$ s subunits were transfected at a 1:1:1 cDNA ratio. For mixed condition, the wild-type and the mutant $\beta3$ subunits were mixed at a 1:1 cDNA ratio with 0.5 μg for each. The same transfection protocol was used for flow cytometry, immunocytochemistry, western blot and patch clamp recordings in HEK293 T cells. Measurement of surface expression of $GABA_A$ receptor subunits using flow cytometry has been described previously (Kang *et al.*, 2009*b*). 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-β2/3 antibody (1:200) or anti-HA for HA-tagged $\gamma2$ 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.*, 2009*b*, 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 $0.5–1 \times 10^5$ for immunohistochemistry and 2×10^5 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 Neurobasal™ 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 mm^2 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/6 J 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 $\alpha1\beta3^{HA}\gamma2$, mutant $\alpha1\beta3(N328D)^{HA}\gamma2$ s or mutant $\alpha1\beta3(E357K)^{HA}\gamma2$ s receptors were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton™ 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.*, 2009*a*, 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 1.0/1.2 M sucrose interface. To prepare postsynaptic densities, the synaptosome layer fraction was diluted to 0.32 M sucrose by adding 2.5 volumes of 4 mM HEPES (pH 7.4) and balanced with HEPES-buffered sucrose. The diluted synaptosome layer preparation was then centrifuged at 150 000*g* for 30 min (TH 641: 29 600 rpm). After centrifugation, the pellet was collected and suspended by adding 4 ml 0.5% Triton™ 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 amd 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 (Alldred *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,

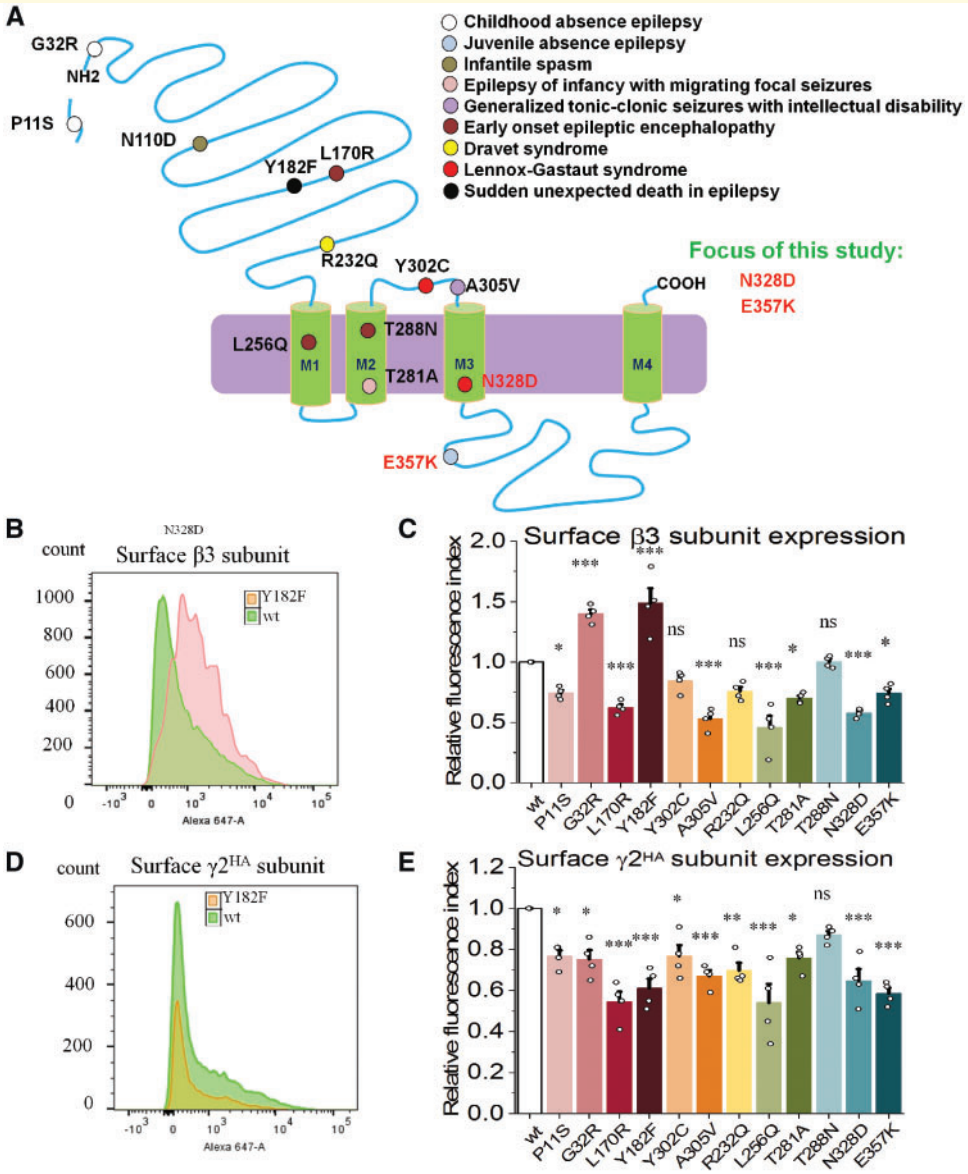

Figure 1 Reduced partnering $GABA_A$ receptor $\gamma 2$ subunit at cell surface is a common phenomenon across GABRB3 epilepsy mutations. (**A**) Schematic presentation of β3 subunit protein topology and locations of mutations in human *GABRB3* associated with various epilepsy syndromes and neurodevelopmental disorders. These mutations are distributed in various locations and domains of the β3 subunit protein peptide. The coloured dots represent the relative locations of the epilepsy related mutations. (**B**–**E**) The flow cytometry histograms depict surface β3 (**B**) or HA-tagged $\gamma 2$ subunit ($\gamma 2^{HA}$) (**D**) expression levels on HEK293T cells co-expressing the human wild-type or the mutant β3 subunit cDNAs with the $\alpha 1$ and HA-tagged $\gamma 2$ subunit cDNAs for 48 h. The expression levels of the β3 or $\gamma 2^{HA}$ subunits in the mutant $\alpha 1\beta 3(Y182F)\gamma 2^{HA}$ receptors was chosen as example. The HA-tagged $\gamma 2$ subunit protein was immunostained with a mouse anti-HA antibody while β3 subunits were immunostained with the mouse anti-β2/3 antibody (BD17). The relative surface levels of each mutant β3 subunit were normalized to the wild-type β3 subunit when co-expressed with $\alpha 1$ and $\gamma 2^{HA}$ cDNAs. The relative subunit expression level of β3 (**C**) or $\gamma 2^{HA}$ (**E**) subunits were normalized to those obtained with co-expression of $\gamma 2^{HA}$ subunit with $\alpha 1$ and the wild-type β3 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 β3 subunit surface expression was likely due to the ability of β3 subunits to form homo-pentamers and to traffic to the cell surface. However, the significance of β3 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 administrated 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 administrated 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 β3 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 β3 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 $\alpha1\beta3\gamma2$ receptors had decreased current amplitudes when evoked with a saturating concentration of GABA (1 mM). However, the $\alpha1\beta3$(N328D)$\gamma2$ receptor currents were more decreased than the $\alpha1\beta3$(E357K)$\gamma2$ 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 β3 subunits as reduced sensitivity to zinc would reflect the incorporation of $\gamma2$ subunits. The current loss of the $\alpha1\beta3$(E357K)$\gamma2$ channels during the 6 s co-application of zinc (10 μM) and GABA (1 mM) was greater relative to the wild-type receptor current and the mutant $\alpha1\beta3$(N328D)$\gamma2$ 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 $\alpha1\beta1$-3 receptors when $\gamma2$ 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 $\gamma2$ subunits.

Both GABRB3 mutations decreased surface levels of β3, α1, and γ2 subunits but to different extents

Reduced current amplitude of β3 (N328D) and β3 (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 $\beta3^{HA}$, mutant $\beta3(N328D)^{HA}$ or mutant $\beta3(E357K)^{HA}$ subunits with $\alpha1$ and $\gamma2$ 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 β3 subunit surface expression in mixed and

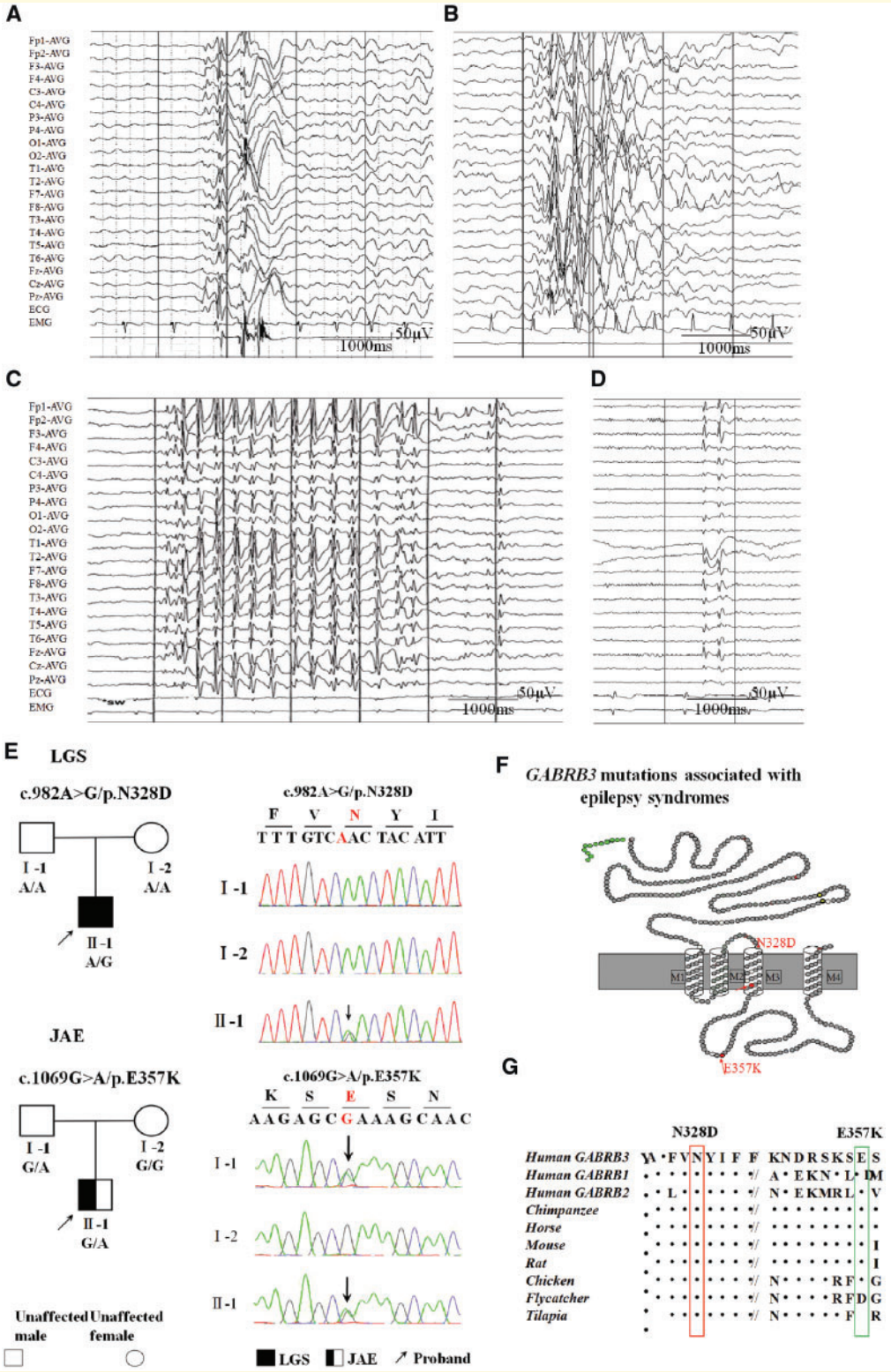

Figure 2 Clinical and molecular genetic findings in GABRB3 in patients with Lennox-Gastaut syndrome (LGS) or juvenile absence epilepsy (JAE). (**A** and **B**) Representative EEGs from each patient are presented. The *top* traces show EEG recordings from the patient carrying the *GABRB3* (N328D) variation, 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.

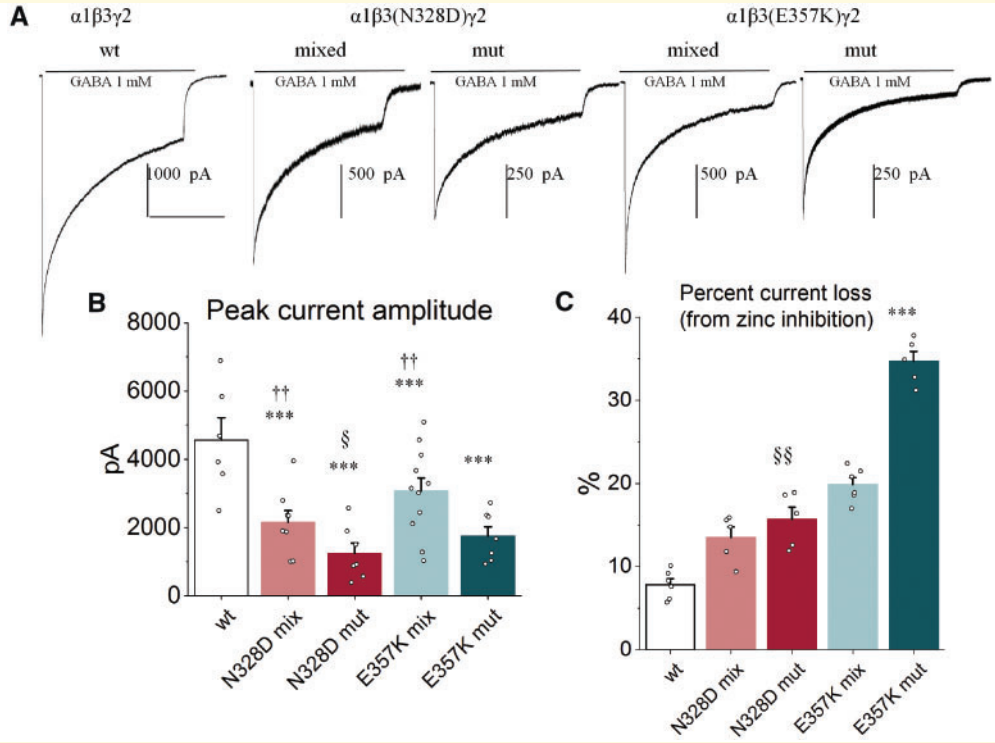

Figure 3 Mutant α1β3γ2 receptors showed decreased GABA-evoked whole-cell currents and altered zinc inhibition. (**A**–**C**) HEK 293 T cells were transfected with human α1 and γ2 subunits in combination with the wild-type or mutant β3(N328D) or β3(E357K) subunits for 48 h. *EGFP* cDNA was included as 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 α1β3γ2 receptors. Values are expressed as mean ± 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 $\beta3^{HA}$ 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 α1 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 γ2 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.*, 2009 *b*; 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 β3(N328D) subunit had reduced total β3 subunit expression but the β3(E357K) subunit had reduced total γ2 subunits.

The reduced surface subunit expression with mutant α1β3(N328D)γ2 and α1β3(E357K)γ2 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 β3 subunit expression by co-expressing the wild-type and mutant β3 subunits with α1 and γ2 subunits in HEK293T cells. HA-tagged β3 subunits were used for immunohistochemistry while untagged β3 subunits were used for western blot. With immunohistochemistry for total subunit expression, 0.5% Triton^{TM} X-100 permeabilized cells were stained with anti-HA and anti-γ2 subunit antibodies. Cells expressing the wild-type subunits displayed a primary localization to the plasma membrane for both $\beta3^{HA}$ and γ2 subunits. In contrast, $\beta3^{HA}$ and γ2 subunits in both mutant $\alpha1\beta3(N328D)^{HA}\gamma2$ and $\alpha1\beta3\ (E357K)^{HA}\gamma2$ receptors accumulated intracellularly and had impaired trafficking to the cell surface (Supplementary Fig. 2).

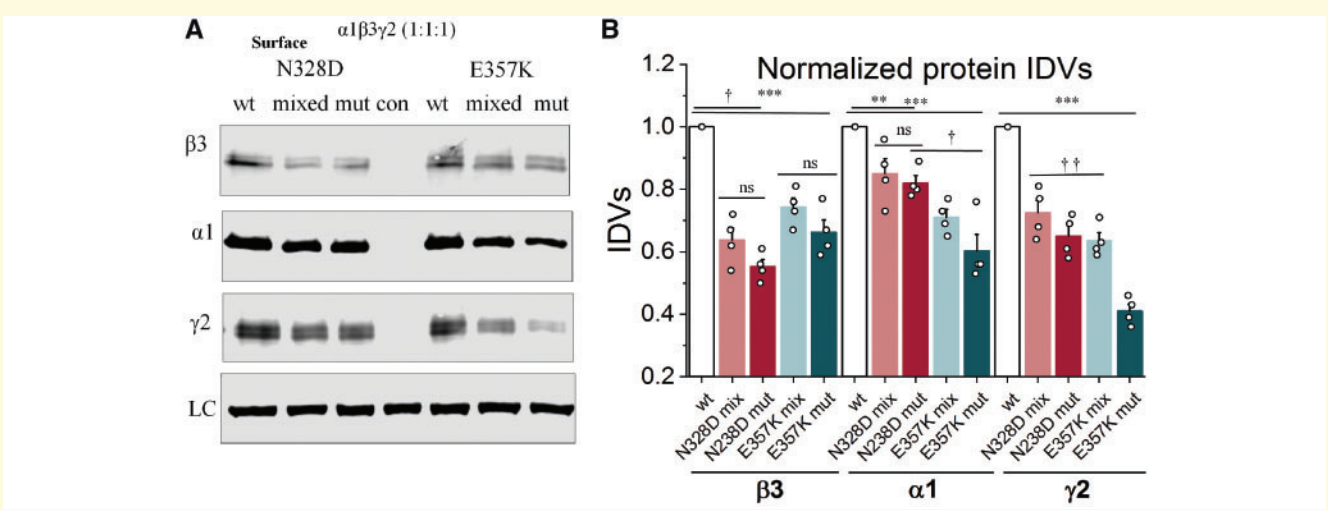

Figure 4 Both mutant α1β3(N328D)γ2 and α1β3(E357K)γ2 receptors had reduced subunit surface expression. (**A**) HEK293 T cells were transfected with human α1 and γ2 subunits in combination with the wild-type β3 subunit alone (WT), mixed wild-type and mutant β3 subunit for mixed, or the mutant β3(N328D)^{HA} or β3(E357K)^{HA} subunits alone for mutant (mut) for 48 h. Surface protein samples were collected through biotinylation and probed by rabbit polyclonal anti-β3 or anti-γ2 subunit or mouse monoclonal anti-α1 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, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, 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 ± SEM.

Mutant β3 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 β3^{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 β3^{HA} subunits in neurons were quite different as determined by both immunohistochemistry (Fig. 5A) and western blot (Fig. 5B). The wild-type β3^{HA} subunits displayed a clear pattern of puncta on neuronal processes and the soma with minimal intracellular somatic distribution, while the mutant β3(N328D)^{HA} and β3(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 β3^{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 β3^{HA} subunits (0.61 ± 0.03 for N328D and 0.74 ± 0.02 for E357K (the IDVs of wild-type β3^{HA} subunits were arbitrarily taken as 1) (Fig. 5E). This suggests that β3(N328D)^{HA} subunits are less stable than β3(E357K)^{HA} subunits in neurons, which was consistent across cell types.

GABRB3 mutations differentially decreased the synaptic clustering partnering γ2 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 β3 subunits may affect the subcellular distribution of partnering subunits. We determined the distribution of subunits that partner with β3 subunits. It is known that γ2 subunits co-assemble with β3 subunits and are critical for $GABA_A$ receptor synaptic clustering (Alldred *et al.*, 2005). We co-stained γ2 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 γ2 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 γ2 puncta were reduced in neurons expressing β3(N328D)^{HA} and β3(E357K)^{HA} subunits when compared with neurons expressing wild-type β3^{HA} subunits (39.4 ± 6.6 for wild-type versus 16.5 ± 4.2 for N328D

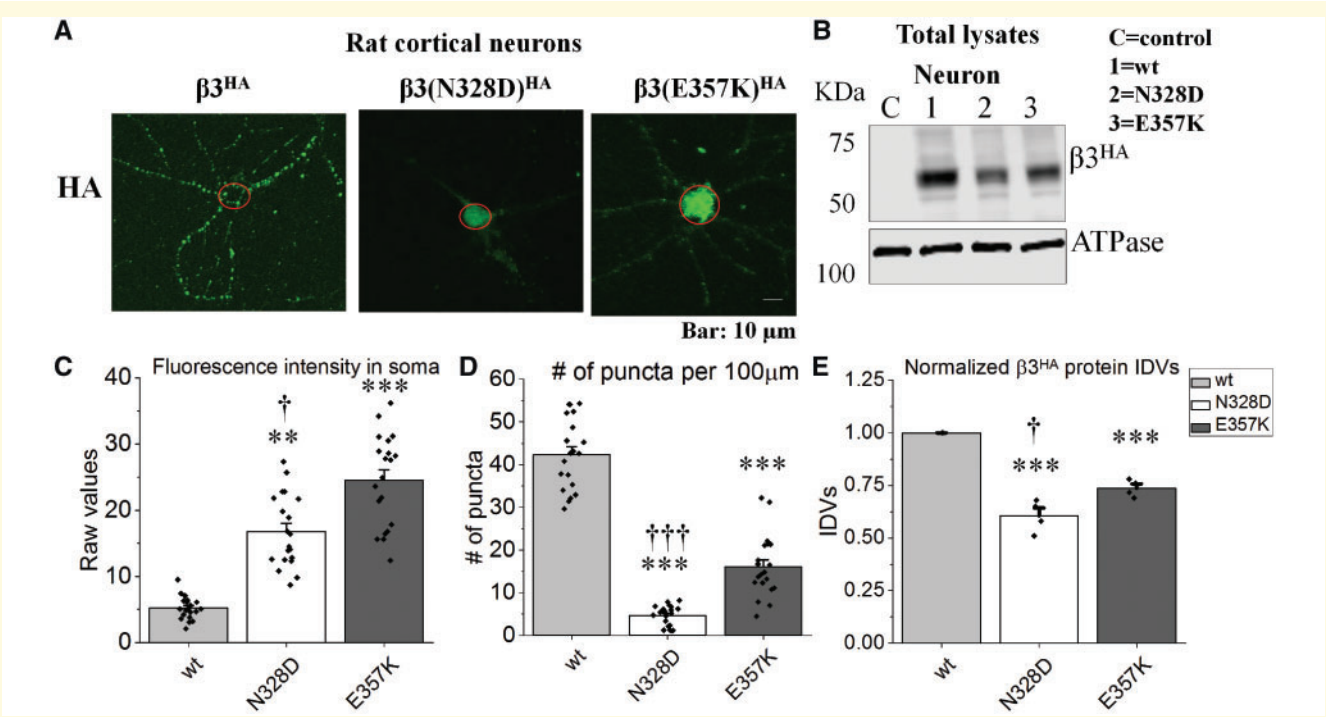

Figure 5 Both mutant β3(N328D)^{HA} and β3(E357K)^{HA} subunits had reduced distribution in dendrites in neurons. (**A**) Rat cortical neurons were transfected with wild-type (β3^{HA}) or mutant [β3(N328D)^{HA} and β3(E357K)^{HA}] subunits at Day 5–7 in culture. At 8–10 days after transfection, the neurons 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) (β3^{HA}) or the mutant β3 subunits β3 (N328D)^{HA} and β3 (E357K)^{HA}) in 100 mm^2 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**) β3^{HA} subunit fluorescence puncta in neurons expressing the wild-type or the mutant β3^{HA} subunits were quantified. The total fluorescence puncta per 100 μm 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 β3^{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, $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ 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 ± SEM.

and 19.7 ± 5.3 E357K) (Fig. 6C). The γ2 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 γ2 subunits in synapses.

Mutant β3 subunits are reduced in inhibitory synapses relative to wild-type β3 subunits

Next, we determined the expression of mutant β3 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 β3 subunits have been demonstrated to be important for receptor assembly (Connolly *et al.*, 1996) while γ2 subunits are required for postsynaptic receptor clustering (Alldred *et al.*, 2005). There was a reduced distribution of γ2 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 β3^{HA} subunit with gephyrin in rat cortical neurons expressing wild-type or mutant β3^{HA} subunits. The cell nuclei were stained with the nuclear marker TO-PRO-3 (Fig. 7A). Consistent with the findings for β3^{HA} subunit expression (Fig. 5A, C and D), β3^{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 β3^{HA} subunits overlapping gephyrin fluorescence was also reduced (51.2% ± 8.3% for wild-type versus 11.8% ± 3.7% for N328D and 31.3% ± 6.2% for E357K (Fig. 7C). Similar reduction was observed for γ2 subunit gephyrin/overlapping (data not shown). Because gephyrin is the marker

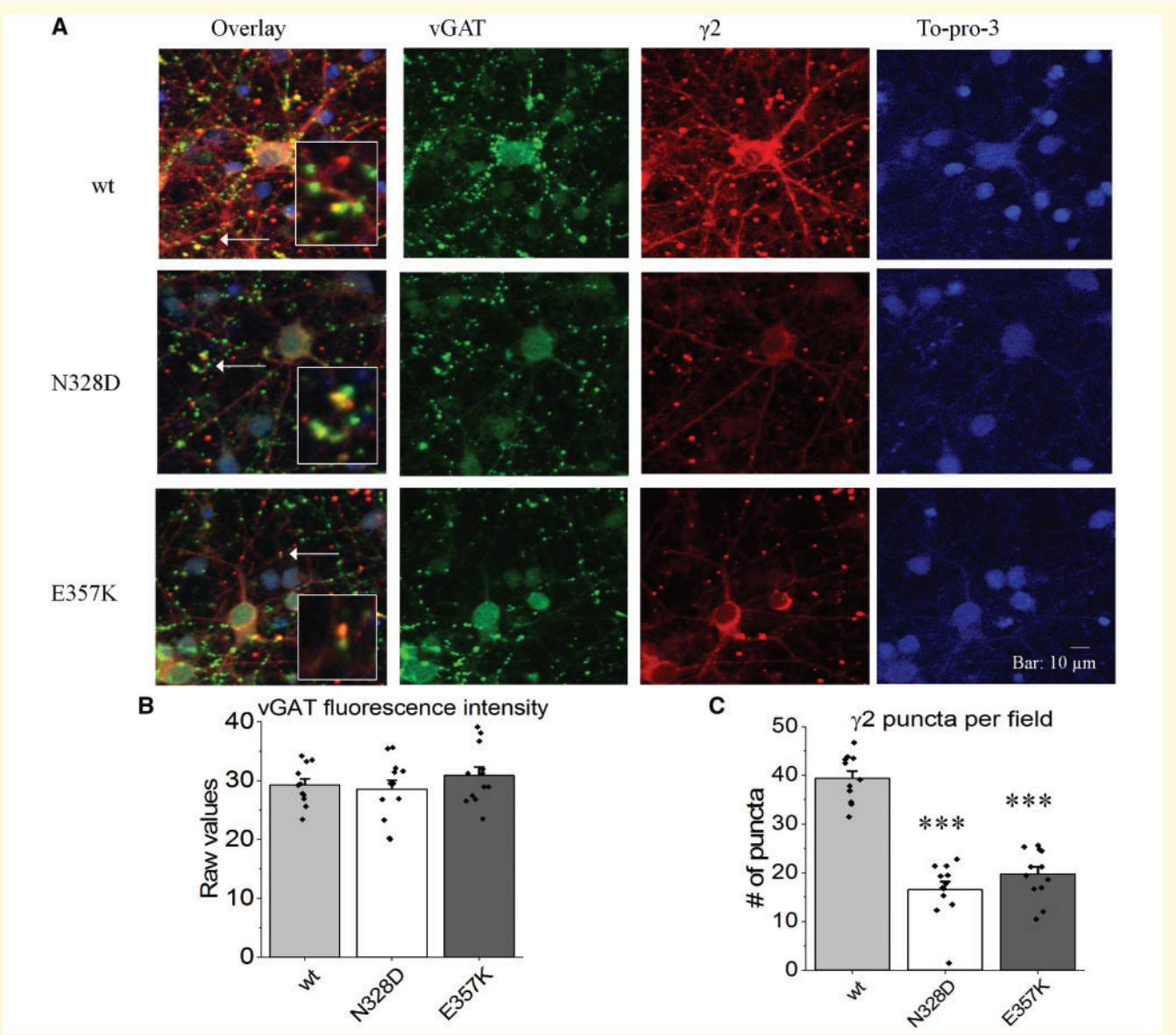

Figure 6 Rat cortical neurons expressing the mutant β3 (N328D)^{HA} or β3 (E357K)^{HA} subunit had reduced γ2 subunits in synapses. (**A**) Rat cortical neurons were transfected with wild-type or mutant β3 subunits at Day 5 in culture. At 8 days after transfection, the neurons were permeabilized and co-stained with rabbit polyclonal anti-γ2 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 β3 subunits was quantified by ImageJ. (**C**) Total γ2 subunit fluorescence puncta in neurons expressing the wild-type or the mutant β3 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 ± SEM.

for inhibitory synapses, this suggests a reduced presence of mutant β3^{HA} subunits in inhibitory synapses.

Impaired synaptic incorporation of γ2 subunits in *Gabrb3*$^{+/-}$ mice

Both *GABRB3* (N328D) and *GABRB3* (E357K) mutations reduced the function of the mutant β3 subunits. Therefore, a similar impairment of γ2 subunits at synapses should be identified in the *GABRB3* loss-of-function condition. We determined total expression of β3 and γ2 subunits in *Gabrb3*$^{+/-}$ mice. First, we determined the γ2 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

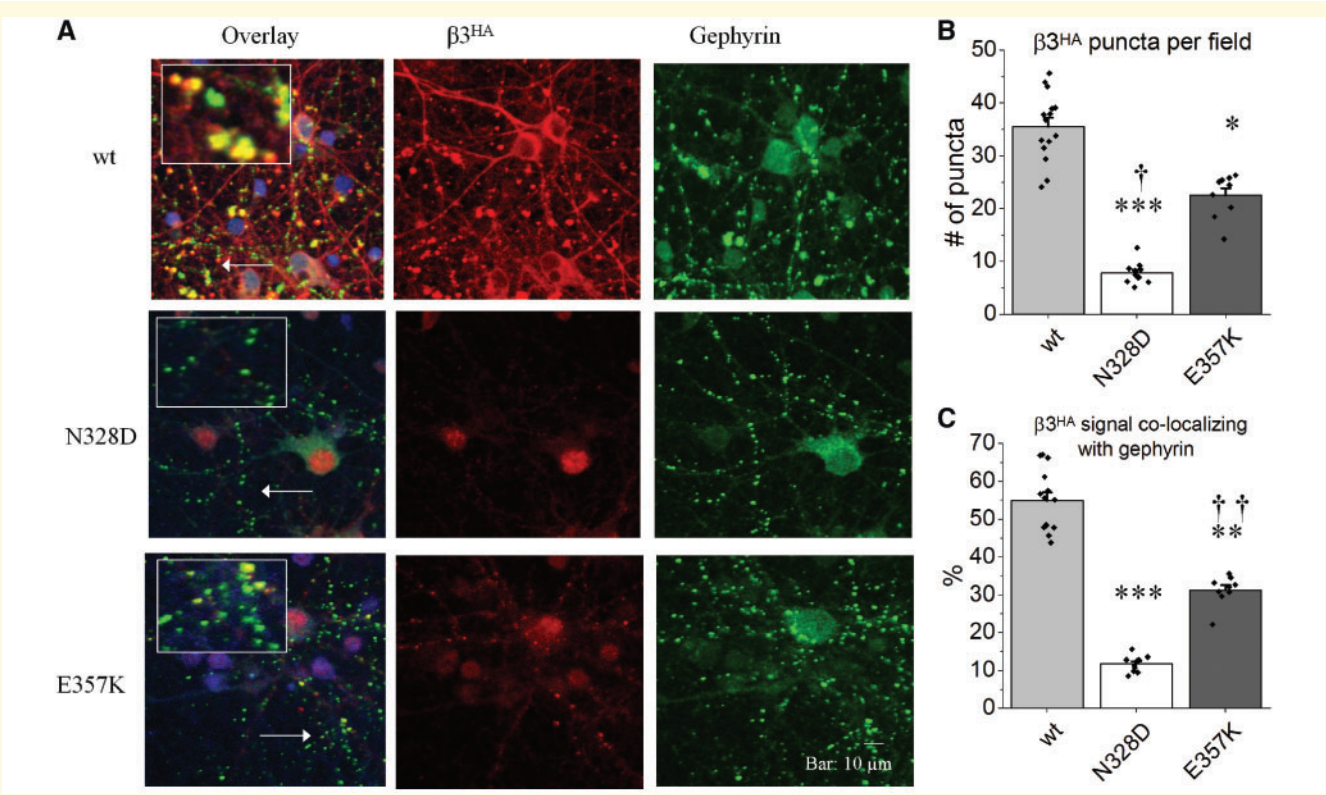

Figure 7 The mutant β3(N328D)^{HA} and β3(E357K)^{HA} subunits have reduced co-localization with the inhibitory postsynaptic marker gephyrin. (**A**) Rat cortical neurons were transfected with wild-type or mutant β3 subunits at Days 5–7 in culture. At 8–10 days after transfection, the neurons were permeabilized 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 β3^{HA} subunits in rat cortical neurons expressing wild-type or mutant β3 subunits in each whole visualized field were quantified. (**C**) The per cent β3^{HA} subunit fluorescence signal overlapping gephyrin fluorescence signal was quantified in neurons expressing the wild-type or the mutant β3 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, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ versus E357K, one-way ANOVA and Newman-Keuls test). Values are expressed as mean ± 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 γ2 subunits at synapses by co-staining γ2 subunits and gephyrin (Fig. 8A). We also isolated synaptosomes from the forebrain by subcellular fractionation and determined the protein levels of β3 and γ2 subunits and gephyrin in synaptosomes (Fig. 8B). Similar to the findings from cultured neurons, the fluorescence values of both γ2 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, β3 (0.51 ± 0.05 for heterozygous versus wild-type ± 1) and γ2 (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 β3 subunits. Further study is required to elucidate the interaction of γ2 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

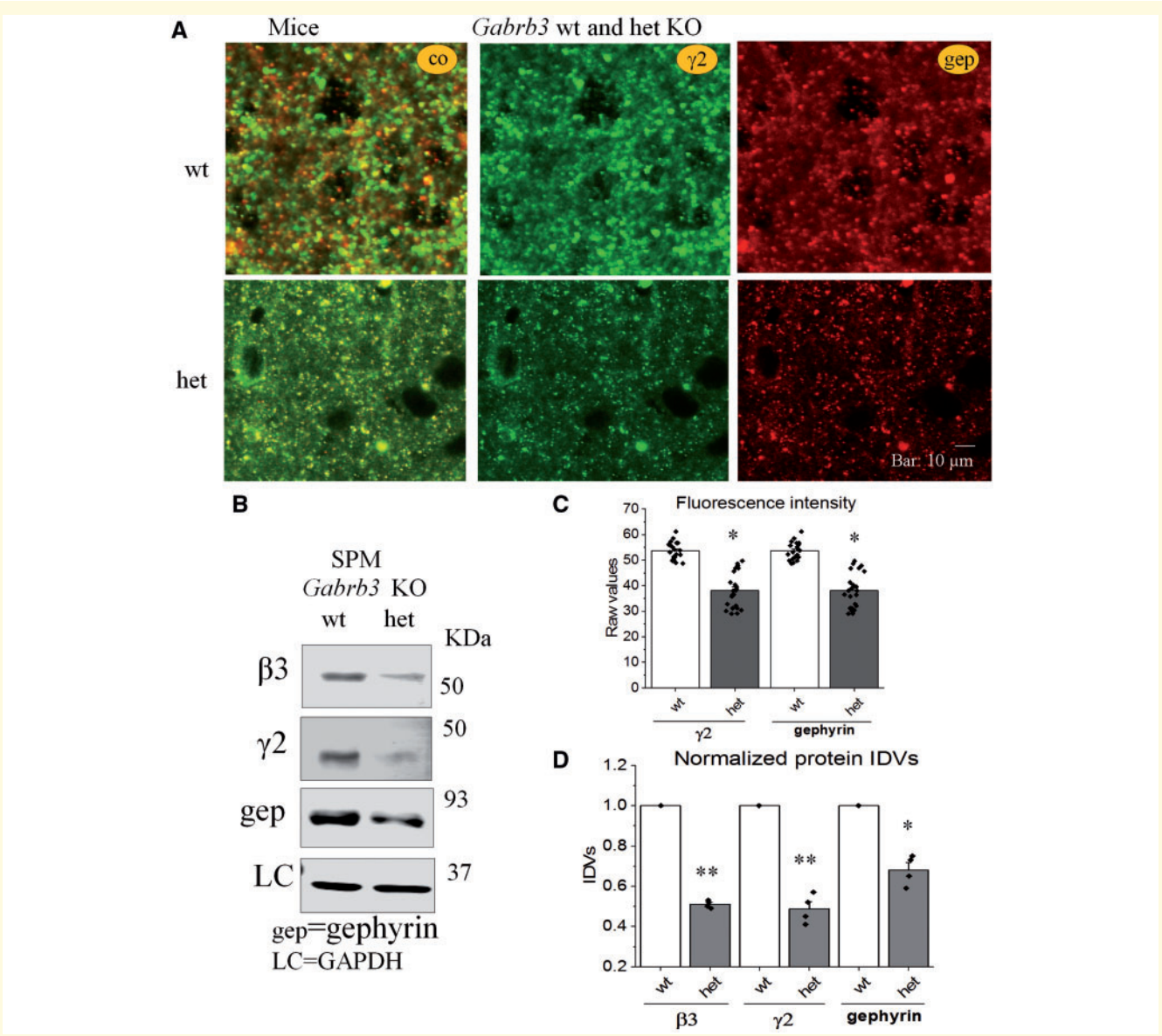

Figure 8 Loss of β3 subunit function impaired γ2 subunit clustering at inhibitory synapses in *Gabrb3*$^{+/-}$ mice. (**A**) Brain tissue from wild-type (wt) or heterozygous (het) *Gabrb3*$^{+/-}$ mice were briefly fixed, permeabilized and co-stained with rabbit polyclonal anti-γ2 and mouse monoclonal 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-β3 or anti-γ2 subunit or mouse monoclonal gephyrin antibodies. LC = control (GAPDH). (**C**) Fluorescence values of γ2 subunits or gephyrin were quantified (*$P < 0.05$ versus wild-type, unpaired *t*-test). Values were expressed as mean ± SEM, $n = 12$ sections for each genotype from four different pairs of mice for γ2, 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 γ2 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 αβ binary and αβγ tertiary receptors can be assembled (Connolly *et al.*, 1996). Although γ2 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 β3 subunit. It is worth noting that the variable surface expression with mutant β3 subunit expression could be because β3 subunits alone can form homopentamers and traffic to the cell surface; however, the physiological significance of β3 homopentamers is unclear. For the *GABRB3* (N328D) mutation, the impaired synaptic clustering was likely due to reduced total β3 (N328D) subunits, thus leading to reduced oligomerization and assembly of αβ3γ2 receptors. For the *GABRB3* (E357K) mutation, there were relatively more β3 subunits available that could oligomerize and assemble with γ2 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 γ2 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 β3 and γ2 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 β3 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 γ2 subunits may be related to different mutant β3 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 β3 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 β3(N328D) subunits are less stable than wild-type subunits while β3(E357K) subunits may have impaired oligomerization with γ2 subunits. It is likely that β3(E357K) subunits were more stable than β3(N328D) subunits, although both were less stable when compared with wild-type subunits. It is interesting that γ2 subunits were more reduced in α1β3(E357K)γ2 receptors than in α1β3(N328D)γ2 receptors. This could be due to differential binding affinity at the γ2/β3 interface or stability of γ2β3 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 γ2-β3(E357K) oligomers compared with wild-type and β3(N328D) subunits. It is also possible that there are some β3(E357K) subunit homopentamers but no β3(N328D) subunit homopentamers.

Inactivation of synaptic clustering via the γ2 subunit is a novel mechanism underlying the pathology of *GABRB3* mutations. This is consistent with the critical role of γ2 subunits for postsynaptic $GABA_A$ receptor clustering (Alldred *et al.*, 2005) and also suggests a close interaction of β3 and γ2 subunits, which needs further investigation. Unlike previous *GABRG2* mutations associated with severe epilepsy syndromes that suppress γ2 subunit partnering subunits by enhanced degradation (Kang *et al.*, 2009*a*), the *GABRB3* (N328D) mutation did not change the total

expression of the partnering subunits such as α1 subunits. However, the synaptic localization of γ2 subunits as well as of mutant β3 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 β3 subunits had reduced surface expression. In addition, the partnering α1 and γ2 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 γ2 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 γ2 subunits in neurons harbouring mutant β3 subunits, and this was validated in the *Gabrb3*$^{+/-}$ mouse by isolating the synaptosomes in the mouse forebrain. The β3(N328D) subunits were more impaired than the β3(E357K) subunits. It is likely that there are no or minimal mutant β3(N328D) subunits in synapses while there are some mutant β3(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 β3(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 β3 subunits disable the function of partnering γ2 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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