Gain-of-function GABRB3 variants identified in vigabatrin-hypersensitive epileptic encephalopathies

Nathan L. Absalom,1 Vivian W. Y. Liao,1 Kavitha Kothur,2 Dinesh C. Indurthi,1,4 Bruce Bennetts,3,4 Christopher Troedson,5 Shekeeb S. Mohammad,2 Sachin Gupta,5 Iain S. McGregor,6 Michael T. Bowen,6 Damien Lederer,7 Sandrine Mary,7 Liesbeth De Waele,9 Katrien Jansen,9 Deepak Gill,2 Manju A. Kurian,9,10 Amy McTague,9,10 Rikke S. Møller,11,12 Philip K. Ahring,1 Russell C. Dale2 and Mary Chebib1

*Present address: Department of Physiology and Biophysics, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, NY 14203, USA.

Variants in the GABRB3 gene encoding the β3-subunit of the γ-aminobutyric acid type A (GABAa) receptor are associated with various developmental and epileptic encephalopathies. Typically, these variants cause a loss-of-function molecular phenotype whereby γ-aminobutyric acid has reduced inhibitory effectiveness leading to seizures. Drugs that potentiate inhibitory GABAergic activity, such as nitrazepam, phenobarbital or vigabatrin, are expected to compensate for this and thereby reduce seizure frequency. However, vigabatrin, a drug that inhibits γ-aminobutyric acid transaminase to increase tonic γ-aminobutyric acid currents, has mixed success in treating seizures in patients with GABRB3 variants: some patients experience seizure cessation, but there is hypersensitivity in some patients associated with hypotonia, sedation and respiratory suppression. A GABRB3 variant that responds well to vigabatrin involves a truncation variant (p.Arg194*) resulting in a clear loss-of-function. We hypothesized that patients with a hypersensitive response to vigabatrin may exhibit a different GABAergic phenotype. To test this hypothesis, we evaluated the phenotype of de novo variants in GABRB3 (p.Glu77Lys and p.Thr287Ile) associated with patients who are clinically hypersensitive to vigabatrin. We introduced the GABRB3 p.Glu77Lys and p.Thr287Ile variants into a concatenated synaptic and extrasynaptic GABAa receptor construct, to resemble the GABAa receptor expression by a patient heterozygous for the GABRB3 variant. The mRNA of these constructs was injected into Xenopus oocytes and activation properties of each receptor measured by two-electrode voltage clamp electrophysiology. Results showed an atypical gain-of-function molecular phenotype in the GABRB3 p.Glu77Lys and p.Thr287Ile variants characterized by increased potency of γ-aminobutyric acid A without change to the estimated maximum open channel probability, deactivation kinetics or absolute currents. Modelling of the activation properties of the receptors indicated that either variant caused increased chloride flux in response to low concentrations of γ-aminobutyric acid that mediate tonic currents. We therefore propose that the hypersensitivity reaction to vigabatrin is a result of GABRB3 variants that exacerbate GABAergic tonic currents and caution is required when prescribing vigabatrin. In contrast, drug strategies increasing tonic currents in loss-of-function variants are likely to be a safe and effective therapy. This study demonstrates that functional genomics can explain beneficial and adverse anti-epileptic drug effects, and propose that vigabatrin should be considered in patients with clear loss-of-function GABRB3 variants.

1 Faculty of Medicine and Health, School of Pharmacy, Brain and Mind Centre, The University of Sydney, Sydney, New South Wales 2006, Australia
2 Kids Neuroscience Centre at The Children’s Hospital at Westmead, Westmead, New South Wales 2145, Australia
3 Department of Molecular Genetics, The Children’s Hospital at Westmead, Westmead, New South Wales 2145, Australia

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Introduction

Developmental and epileptic encephalopathies (DEEs) are a group of severe childhood epilepsies often associated with co-morbidities including developmental delay (DD), intellectual disability, movement disorders and autistic features (French, 2006). Variants in the GABRB3 gene that code for β3 γ-aminobutyric acid type A (GABA₅) receptor subunits are associated with DEE (Johannesen et al., 2016; Möller et al., 2016; Absalom et al., 2019; Hernandez et al., 2019; Maljevic et al., 2019).

GABA₅ receptors are ligand-gated ion channels that regulate neurotransmission via the inhibitory neurotransmitter, GABA. Nineteen genes code for subunits that make up the various GABA₅ receptor subtypes. These subunits mix and match to form distinct receptor subtypes (Johannesen et al., 2016; Liao et al., 2019) that express at different cellular and brain regions to regulate neurophysiological responses such as movement, learning and memory.

The β3 subunit of the GABA₅ receptor is widely expressed across many regions of the brain (Fritschy et al., 1992; Persohn et al., 1992; Wisden and Seeburg, 1992; Pirker et al., 2000) often combining with various α subunits (such as α1) and γ2 subunits to form pentameric synaptic GABA₅ receptors with a 2α:2β:2γ stoichiometry (Fig. 1A). These receptors respond to high levels of synaptically released GABA to mediate a phasic response. In addition, the β3 subunit is found at extrasynaptic sites, combining with either α5 or δ subunits resulting in extrasynaptic receptors that respond to comparatively low levels of GABA to mediate a tonic GABAergic response.
As GABA<sub>A</sub> receptors work to maintain normal brain function, genetic variants in individual genes such as the GABRB3 can lead to neurophysiological dysfunction including seizure disorders such as febrile seizures, generalized epilepsy with febrile seizures+, myoclonic atonic epilepsy, West syndrome, Dravet syndrome and other severe unclassified DEEs (Johannesen et al., 2016; Møller et al., 2016; Absalom et al., 2019; Hernandez et al., 2019; Maljevic et al., 2019). An analysis of the known variants in GABA<sub>A</sub> receptors proposed that most variants lead to loss-of-function molecular phenotypes through impaired transcription or translation, misfolding and degradation, endoplasmic reticulum retention of truncated or functional receptors or impairments in receptor activation (Hernandez and Macdonald, 2019). However, increased GABAergic activity can also lead to seizure phenotypes, such as in loss-of-function SLC6A1 variants (Mattison et al., 2018), and loss-of-function GABA<sub>A</sub> receptor molecular phenotypes cannot simply be assumed. Nevertheless, with the prevalence of loss-of-function GABA<sub>A</sub> receptor variants, drugs that enhance GABAergic neurotransmission could be expected to help reduce seizures in these patients, yet many patients with DEE are either refractory to current treatments or they worsen symptoms or cause a severe adverse reaction.

Vigabatrin is an irreversible GABA transaminase inhibitor used as adjunctive therapy for focal seizures and monotherapy for infantile spasms. It is thought to alleviate seizures by increasing tonic, or persistent, GABAergic inhibitory currents (Wu et al., 2001, 2003). Therefore, it may be anticipated that vigabatrin would be useful in patients with GABRB3 variants that have a reduction in the number or a functional impairment of β3-containing GABA<sub>A</sub> receptors.

However, recent reports described two patients presenting with GABRB3 variants that responded adversely to vigabatrin. When administered vigabatrin, these patients responded with reduced seizures but suffered severe adverse effects, such as decreased alertness, extreme drowsiness, hypotonia, sedation and respiratory difficulties, that reversed upon treatment cessation. Next-generation sequencing identified the GABRB3 variants p.Glu77Lys and p.Thr287Ile (Papandreou et al., 2016; Kothur et al., 2018). Our hypothesis is that the adverse responses to vigabatrin are a consequence of the molecular phenotype of the individual GABRB3 variants.

In this study, we sought to determine whether the molecular phenotype of the variants could explain the patients hypersensitivity to vigabatrin. Our results demonstrated that patients who responded adversely to vigabatrin had atypical gain-of-function molecular phenotypes when compared to patients that responded well to vigabatrin who had typical loss-of-function phenotype. We propose that functionally analysing variants of patients is essential to avoid adverse effects when prescribing drug treatments.

### Materials and methods

This study was performed in accordance with ethical principles for medical research outlined in the Declaration of Helsinki. All genetic studies were performed with informed consent of the patients or their responsible relatives and were approved by the local ethical committees. All procedures using *Xenopus laevis* frogs and harvesting of oocytes were approved by the animal ethics committee of the University of Sydney (animal ethics committee No. 2016/970) in accordance with the National Health and Medical Research Council of Australia code for the care and use of animals. Frogs were housed in custom-built tanks with a plastic shelter, with three frogs to a tank. Water was reticulated and water quality checked weekly. Frogs were fed twice weekly with Wardley’s reptile sticks.

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**Figure 1 Structural location of GABA<sub>A</sub> receptor variants.** (A) Pentameric structure from the extracellular side of the membrane of the α1/β2 synaptic GABA<sub>A</sub> receptor (Masiulis et al., 2019). α1 subunits are in blue, β3 in red and γ2 in green. (B) Schematic of a single β3 GABA<sub>A</sub> receptor showing the location of the β3<sup>E77K</sup>, β3<sup>R194I</sup> and β3<sup>T287I</sup> residues (red) in the extracellular and the four transmembrane domains M1–M4 (black). (C) Also shown is the structure of the interface between β3 and α1 subunits from side-on showing the truncation of the β3<sup>T194I</sup> subunit, the structure displaying the β3<sup>E77K</sup> residue (black sticks and spheres) in the coupling region connecting the extracellular domain and the transmembrane domains, and the β3<sup>T287I</sup> residue (black sticks and spheres) at the top of the pore lining M2 region. (D) The sequence alignment for selected subunits is shown below in the loop 2 and M2 regions that contain the β3<sup>E77K</sup> and β3<sup>T287I</sup> variants, respectively (red).
(Wardley, USA) and Irradiated Adult Xenopus Diet (Xenopus Express, Florida, USA).

**Patients**

We report the electrophysiological effects of variants previously reported and the detailed clinical case of two previously reported and one novel GABRB3 gene variant (maternally inherited with the affected relative included) (Papandreou et al., 2016; Kothur et al., 2018). We chose to analyse patients on the following criteria: one variant identified as loss-of-function (novel variant p. Arg194*) associated with a favourable response to vigabatrin; and two variants (p.Glu77Lys and p.Thr287Ile) associated with the adverse reaction of hypersensitivity to vigabatrin (Papandreou et al., 2016; Kothur et al., 2018).

Electrophysiological analyses were performed for two GABRB3 variants and compared to the wild-type (WT) to correlate their functional response to their pharmacoresponse to vigabatrin and nitrazepam where used.

**Molecular biology**

The human concatenated \(\gamma_2\)-\(\beta_3\)-\(\alpha_1\)-\(\beta_3\)-\(\alpha_1\) receptor construct has previously been described (Absalom et al., 2019; Liao et al., 2019). The \(\gamma_2\)-\(\beta_3\)-\(\alpha_5\)-\(\beta_3\)-\(\alpha_5\) subunit was created by subcloning \(\alpha_5\) subunits with flanking linker sequences into the \(\gamma_2\)-\(\beta_3\)-\(\alpha_1\)-\(\beta_3\)-\(\alpha_1\) construct. Briefly, vectors containing DNA encoding the \(\alpha_5\) subunits with linker sequences and unique restriction sites were purchased from Genscript (Singapore). The \(\alpha_5\) subunits were cut and \(\beta_3\) subunits were ligated with the vector containing the \(\gamma_2\) subunit to create concatenated receptors with linker sequences in the order of \(\gamma_2\)-\(\beta_3\)-\(\alpha_5\)-\(\beta_3\)-\(\alpha_5\)-ATGAGS-\(\alpha_5\).To create \(\beta_3^{3\text{T}287\text{I}}\) and \(\beta_3^{3\text{E}77\text{K}}\) sets of concatenated receptors, human cDNA for monomeric \(\beta_3\) GABA\(_{\alpha}\) receptor subunits were mutated using QuikChange II Site Directed Mutagenesis Kit (Agilent Technologies, Mulgrave, Australia) then subcloned into the \(\gamma_2\)-\(\beta_3\)-\(\alpha_1\)-\(\beta_3\)-\(\alpha_1\) or The \(\gamma_2\)-\(\beta_3\)-\(\alpha_5\)-\(\beta_3\)-\(\alpha_5\) construct using unique restriction sites. Fidelity of all coding sequences were verified by double stranded sequencing. DNA gel electrophoresis ensured incorporation of the five subunits. cRNA was produced from linearized cDNA using the mMessage mMachne T7 Transcription kit (Thermo Fisher, Scoresby, Australia).

**Xenopus surgery and oocyte preparation**

In brief, a section of ovarian lobe from *X. laevis* was surgically removed under anaesthesia induced by tricaine, cut and digested with 35 mg of collagenase-A in 15 ml OR2 (in mM: NaCl, 82.5; HEPES, 5; MgCl\(_2\), 2 and KCl, 2; pH 7.4) at 18°C for ~1 h until oocytes were fully detached. Oocytes were then injected with 2 ng of cRNA per cell encoding a concatenated receptor and incubated for 2–4 days at 18°C in ND96 solution (in mM: NaCl, 96; KCl, 2; MgCl\(_2\), 1; CaCl\(_2\), 1.8; HEPES, 5; pH 7.4) supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 \(\mu\)g/ml gentamicin.

**Two-electrode voltage-clamp recording**

Cell currents were recorded using the two-electrode voltage-clamp method as previously described (Absalom et al., 2019). Briefly, cells were impaled with microelectrodes filled with 3 M KCl then voltage clamped at \(-60\) mV. Currents were recorded using a GeneClamp 500B (Axon Instrument, Foster City, USA) or OC-725C amplifier Clamp (Warner Instrument Corp., Hamden, USA) and digitized with a Powerlab 8/36 and LabChart version 8.03 (ADInstruments, Sydney, Australia).

For concentration–response curves and estimated open probability (Est \(P_{O_{\text{max}}}\)), a 3 mM concentration of GABA was applied as a reference three times and peak currents were normalized to the mean current of the last two GABA applications. When estimating \(P_{O_{\text{max}}}\) after three consecutive applications of reference 3 mM GABA solution, 10 mM GABA, 1 \(\mu\)M diazepam and 3 \(\mu\)M etomidate was co-applied at \(\gamma_2\)-\(\beta_3\)-\(\alpha_1\)-\(\beta_3\)-\(\alpha_1\) and 10 mM GABA and 10 \(\mu\)M etomidate was co-applied at \(\gamma_2\)-\(\beta_3\)-\(\alpha_5\)-\(\beta_3\)-\(\alpha_5\) receptors. A washout period of 10–12 min was performed between GABA applications to prevent effects from desensitization. Experiments were performed over a minimum of two different batches of oocytes.

**Receptor desensitization assay**

To measure current decay rates, dead volume was reduced and single concentrations of GABA were applied for 120 s. Traces were fitted to an exponential decay in GraphPad Prism (v8).

**Data analysis and statistics**

Concentration–response curves were fitted using GraphPad Prism 8 to a monophasic Hill equation of the form:

\[
I = I_{\text{max}} \left( \frac{[A]^{n_H}}{[A]^{n_H} + EC_{50}^{n_H}} \right),
\]

where \(I_{\text{max}}\) is the maximum current, \(EC_{50}\) is the concentration eliciting half-maximum response, \([A]\) is the ligand concentration and \(n_H\) is the Hill slope. Individual oocytes where a complete concentration–response curve was taken are recorded as a single \(n\). Responses were normalized to the fitted maximum response of individual curves. The \(EC_{50}\) is from the fitting of Hill equations to all data, while the \(\log EC_{50}\), \(I_{\text{max}}\) and \(n_H\) values are the mean and
error derived from fitting curves to individual experiments.

For nitrazepam concentration–response curves, the percent modulation was derived by the equation:

\[
\text{Per cent Modulation} = 100 \times \frac{I_{\text{Nitrazepam}} - I_{\text{GABA EC10}}}{I_{\text{GABA EC10}}}
\]

and fitted to the Hill equation for parameters of the curves.

The Estimated \( P_{\text{Omax}} \) for individual experiments was derived by the equation:

\[
\text{Est. } P_{\text{Omax}} = \frac{I_{3 \text{mM GABA}}}{I_{3 \text{mM GABA}} + I_{1 \text{mM Diazepam}} + I_{3 \text{mM Etomidate}}}
\]

To determine the decay rates, individual traces from the peak current were fitted to the equation:

\[
I = (I_0 - \text{Plateau})e^{-kt} + \text{Plateau},
\]

where \( I \) is the current at time \( t \), \( I_0 \) is the current at time point 0, Plateau is the current at steady-state desensitization and \( k \) is the decay constant.

The modelled response was determined by assuming an equal expression ratio (1:1:1:1) of WT, \( \gamma\beta3^-\alpha\beta3^-\alpha \), \( \gamma\beta3^-\alpha\beta3^+\alpha \) and \( \gamma\beta3^-\alpha\beta3^+\alpha \) receptors and given by the equation:

\[
\text{Modelled Response} = 0.25 \times (\text{Est Po(wt)} \times \frac{[A]^{\text{H(wt)}} + \text{Est Po(1)} \times \frac{I_{1 \text{mM GABA(1)}}}{I_{1 \text{mM GABA(1)}} + I_{1 \text{mM GABA(2)}}} \times \frac{[A]^{\text{H(1)}}}{[A]^{\text{H(1)}} + I_{3 \text{mM GABA(1)}}} + \text{Est Po(2)} \times \frac{I_{1 \text{mM GABA(2)}}}{I_{1 \text{mM GABA(2)}} + I_{1 \text{mM GABA(3)}}} \times \frac{[A]^{\text{H(2)}}}{[A]^{\text{H(2)}} + I_{3 \text{mM GABA(2)}}} + \text{Est Po(3)} \times \frac{I_{1 \text{mM GABA(3)}}}{I_{1 \text{mM GABA(3)}} + I_{1 \text{mM GABA(4)}}} \times \frac{[A]^{\text{H(3)}}}{[A]^{\text{H(3)}} + I_{3 \text{mM GABA(4)}}})
\]

where the Est Po(wt), \( n_H(wt) \) and \( EC_{50(wt)} \) are the Est Po\(_{\text{max}}\), Hill co-efficient and \( EC_{50} \) of the wild-type receptor, Est Po(1), \( n_H(1) \) and \( EC_{50(1)} \) are the respective parameters of the \( \gamma\beta3^-\alpha\beta3^-\alpha \) receptor, Est Po(2), \( n_H(2) \) and \( EC_{50(2)} \) are respective parameters of the \( \gamma\beta3^-\alpha\beta3^+\alpha \) receptor Est Po (3), \( n_H(3) \) and \( EC_{50(3)} \) are the respective of the \( \gamma\beta3^-\alpha\beta3^+\alpha \) receptors.

The change in response was determined by subtracting the WT concentration–response curve. The GABA concentration that vigabatrin induces was increased by 30-fold to compensate for the higher \( EC_{50} \) values determined using concatenated receptors expressed in oocytes compared to free subunits expressed in recombinant mammalian cells (Mortensen et al., 2011).

For statistical analysis, Est \( P_{\text{Omax}} \) values and parameters derived from concentration–response curves were compared with a one-way ANOVA with Tukey’s post hoc test. Significance values of \( P < 0.05 \), \( P < 0.01 \) and \( P < 0.001 \) are shown in the results section. For desensitization, the decay rates were plotted against log\(_{10}\) GABA concentrations and fitted to a linear curve. An F-test was performed to determine if the relationship between the decay constants and GABA concentration for each variant and the WT could be described by a single curve. If not, a Mann–Whitney test was then performed to determine if the data were significantly different between the WT and variant receptors. Power calculations were performed for comparisons between maximum currents, log\( EC_{50} \) and Est \( P_{\text{Omax}} \) with a one-way ANOVA with an estimated effect size of 0.5, power of 0.85 and a significance level of 0.05 for a minimum number of experiments of 9.

**Blinding and randomization**

Experiments were not blinded or randomized, but performed on a semi-automated recording apparatus that enabled four experiments to be performed at any one time.

**Data availability**

Raw data are available on request.

**Results**

**Patient that responds to vigabatrin without hypersensitivity**

The vigabatrin-responsive patient chosen represented a typical loss-of-function \( GABRB3 \) variants that was prescribed vigabatrin as monotherapy. This variant is a novel case of a maternally-inherited truncation variant p.Arg194* (cases 1 and 2). This variant leads to an aberrant protein sequence of the affected \( \beta3 \) subunit, and the resultant truncated protein cannot express the critical extracellular and pore-forming transmembrane motifs. Hence, these patients would only have functional \( \beta3 \) subunits from the unaffected allele.

**Clinical details of patient and affected mother that responds to vigabatrin without hypersensitivity**

Cases 1 and 2 (p.Arg194*) are a 29 month old girl and her affected mother. The girl presented with infantile spasms at the age of 7 months. EEG at onset showed hysparrhythmia, which later evolved into multifocal discharges. Introduction of vigabatrin was tolerated without side effect but was not sufficient to achieve seizure control and prednisolone was added with good effect. In the following period she was seizure free on monotherapy with 130 mg/kg vigabatrin, which was later switched to valproic acid. At last reported follow-up at 21 months of
age she had global DD and a right-sided hemiplegia. She could sit unsupported in a kyphotic position and was able to say a few words. The mother of the patient presented with atonic and generalized tonic clonic seizures at the age of two years. She was treated with valproic acid with good effect and has been seizure free since the age of 18 years. She has never tried vigabatrin. She has mild learning difficulties.

**Molecular phenotype of vigabatrin-responsive patients without hypersensitivity**

The $\beta_3^{R194*}$ truncation variant can be safely predicted to have a loss-of-function molecular phenotype. The $\beta_3^{R194*}$ variant introduces a stop codon within the extracellular domain leading to a truncation of the protein upstream of the transmembrane domain (Fig. 1B and C). This truncated subunit would not form receptor subunits as no transmembrane regions or pore region would be translated.

**Vigabatrin-hypersensitive patients**

The vigabatrin-hypersensitive patients were chosen for their clinical response to vigabatrin but had exacerbated hypotonia sedation and/or respiratory difficulties that reverted upon cessation of therapy. These included a patient (case 3) with de novo GABRB3 variant p.Glu77Lys (Kothur et al., 2018), that was previously reported as part of 105 patients investigated using a gene panel where the detailed case was not previously reported, and a previously reported de novo GABRB3 variant p.Thr287Ile (Papandreou et al., 2016) with an updated case report (case 4). The p.Glu77Lys and p.Thr287Ile variants were located at the conserved extracellular loop 2 and M2 domains (Fig. 1B–D), respectively that play key roles in channel activation.

**Clinical details of vigabatrin-hypersensitive patients**

Case 3 (p.Glu77Lys) is a 4-year-old girl who presented with spasms since the age of 5 months in the form of sudden jerking of arms and legs occurring in clusters of 5–10 spasms, with 4–5 clusters per day (Table 1). Her early development prior to spasms was normal, although there was loss of social smile and reduced cooing for 1 month before spasm onset. On examination her head circumference was at 90th centile, weight was 90th centile and length was 75th centile. She was hypotonic with poor neck control, delayed visual maturation and responsiveness. Her EEG showed high amplitude spike and wave and polyspike and wave in the posterior region, and MRI brain showed the prominence of CSF spaces and mild ventriculomegaly. Her CSF studies including neurotransmitters were normal. She was treated with corticosteroid according to UKISS protocol (10 mg prednisolone four times per day) but continued to have spasms up to 60 spasms/day despite treatment with high dose steroids, plus nitrazepam, sodium valproate, zonisamide and pyridoxine. Due to failure to respond, vigabatrin was introduced, but was associated with extreme drowsiness and exacerbation of hypotonia on only 70 mg/kg/day (800 mg BD), and was therefore weaned off over the next few weeks. She was subsequently stabilized and spasms ceased on a combination of nitrazepam and the ketogenic diet. At 18 months of age, she became seizure free except for infrequent myoclonic seizures. Her ongoing EEG showed frequent slowing over the bilateral frontal region but no epileptiform discharges were noted. Following the cessation of spasms, she showed significant improvement in gross motor development. However, she continued to have persistent language delay with limited social interaction suggestive of autism spectrum disorder.

Case 4 (p.Thr287Ile) is a male infant who presented with neonatal hypotonia, then focal tonic seizures from 3 months of age. EEG showed multifocal discharges but not hypersprhythmia. Introduction of vigabatrin resulted in seizure cessation but caused severe exacerbation of hypotonia, sedation and respiratory difficulties necessitating cessation of vigabatrin. Unfortunately seizures recurred from 5 months of age, and were refractory to carbamazepine, levetiracetam, topiramate, sodium valproate and the ketogenic diet. At last reported follow-up at 3 years and 2 months, he continues to have refractory epilepsy with 10–20 seizures per day of multiple seizure types with behavioural arrest, focal motor, myoclonic jerks and brief tonic seizures. There is global DD with paucity of movements yet anti-gravity movements, some pyramidal signs with brisk reflexes and upgoing plantars, and some vocalizations. There is subtle dysmorphic features and microcephaly (0.4th centile).

**Assessing the molecular phenotype of GABRB3 missense variants**

Assessing the function of GABA$_A$ variants is complicated by the fact that variants are typically dominant and synaptic receptors form with a 2α:2β:1γ stoichiometry. As patients have one normal and one mutated allele, a mixture of receptors with WT and variant subunits will form. For patients carrying a variant in the $\beta_3$-subunit ($\beta_3^{E77K}$ and $\beta_3^{T287I}$), two simple receptor populations express with all WT subunits or with two $\beta_3$ variant subunits. These receptors can easily be expressed in vitro with free γ2 and α1 subunits, but would be predicted to each only comprise of 25% of the receptor population within the patient (Fig. 2A). The two other receptors expressed contain one WT $\beta_3$ and one variant $\beta_3$ subunit. For a random distribution of subunits, these receptors will comprise ~50% of the receptor population and cannot easily
be evaluated by the heterologous expression of free subunits (Fig. 2A).

We recently developed an approach to determine the function of each of these subunit combinations (Absalom et al., 2019) by engineering all five GABA_A receptor subunits into the native receptor arrangement using linkers with unique restriction sites (c2-b3-a1-b3-a1) (Fig. 2A and B). This approach enables us to generate a library of receptors containing one or two copies of the mutation by a ‘cut and paste’ approach and then express them individually, recapitulating the full molecular phenotype of the variant. We analyse the function of all possible receptors by performing two-electrode voltage-clamp electrophysiology on concatenated WT, one copy or two copies of the β3E77K and β3T287I mutations expressed in Xenopus oocytes.

Table 1 Clinical phenotypes

| Case 1          | Case 2          | Case 3 (Kothur et al., 2018) | Case 4 (Papandreou et al., 2016) |
|-----------------|-----------------|-----------------------------|----------------------------------|
| Current age (year), sex | 2, female | 29, female | 4, female | 3.2, male |
| Detection method | Multigene epilepsy panel, Sanger confirmation | Sanger | Multigene epilepsy panel, Sanger confirmation | Multigene epilepsy panel, Sanger confirmation |
| Gene            | GABRB3 | GABRB3 | GABRB3 | GABRB3 |
| Nucleotide change | c.580C>T | c.580C>T | c.229G>A | c.860C>T |
| Protein change  | p.Arg194* | p.Arg194* | p.Glu77Lys | p.Thr287Ile |
| Type of variant | Stop | Stop | Missense | Missense |
| Inheritance     | Maternal | N/A | De novo | De novo |
| Age of onset epilepsy | 7 months | 2 years | 5 months | 3 months |
| Epilepsy syndrome | Infantile spasms | Infantile spasms | Infantile spasms | Infantile spasms |
| Seizure type    | Infantile spasms | Infantile spasms | Infantile spasms | Infantile spasms |
| Development     | DD | Learning difficulties | Hypotonia, delayed visual maturation | Hypotonia, severe global DD |
| Other features  | Right sided hemiplegia, plagiocephaly | Hypsarrhythmia | High amplitude Spike and Wave and poly-spike | Microcephaly, mild dysmorphism |
| Intercitial EEG findings | Porencephalic cyst left ACM area with secondary Wallerian degeneration tractus corticospinalis | NA | Prominence of CSF spaces and mild ventriculomegaly (5 months) | High amplitude multifocal discharges |
| MRI findings    | NA | NA | Normal (14 months) | Normal (14 months) |
| Medication tried | Prednisolone, vigabatrin | Valproic acid | Steroids, vigabatrin, valproate, zonisamide, pyridoxine, ketogenic diet, nitrazepam | Vigabatrin, carbamazepine, topiramate, sodium valproate |
| Reaction to vigabatrin | Seizure reduction, no side effects | NA | Severe drowsiness and hypotonia | Severe hypotonia, sedation, respiratory difficulties |
| Ongoing treatment | Valproic acid | None | Nil | Ketogenic diet, levetiracetam |
| Seizure outcomes | Seizure free by 10 months | Seizure free from 18 years | Infrequent myoclonic seizures, cessation of spasms | Ongoing focal motor, tonic, myoclonic seizures |
| Developmental outcome | Global DD, speech delay | Seizure free by 10 months | Seizure free by 10 months | Seizure free by 10 months |

Did not include GABA peak as this is filtered in routine reporting.

β3E77K and β3T287I variants do not alter maximal GABA-evoked current amplitudes

Using this approach we developed single and double mutated receptor constructs from the original WT construct (γ2-β3-x1-β3-x1) for each variant. This totalled six unique receptor constructs and mRNA was synthesized for each construct and injected into Xenopus oocytes. All three receptor constructs for each variants expressed robustly with 3 mM GABA-evoked currents in the 0.9–2.2 μA range (Fig. 2C). No significant difference in the maximum currents elicited by 3 mM GABA were observed between all six receptor constructs [one-way ANOVA, F(6,111) = 2.062, P = 0.0633; Table 2]. While not significantly different, the γ2-β3E77K-x1-β3E77K-x1 receptor
had approximately a 2-fold reduction in maximum current that may arguably be a result of a reduced maximum open probability, reduced cell surface expression or impaired protein folding. However, as the single subunit $\beta_3^{E77K}$ variant receptors exhibited similar absolute currents to the WT, it is unlikely that such a minor change in a 25% receptor subpopulation has much overall effect in a patient.

**$\beta_3^{E77K}$ and $\beta_3^{T287I}$ variants increases the potency of GABA**

GABA$_A$ receptors are activated by GABA in a concentration-dependent manner, where a reduction in GABA potency has commonly been attributed to loss-of-function GABRB3 variants (Janve et al., 2016; Moller et al., 2017). The $\beta_3^{E77}$ residue is located in loop 2, part of the ‘coupling domain’ incorporating loops two, seven and nine, the pre-M1 helix and the M2 and M3 loop, that alters conformation during channel activation (Fig. 3A) (Kash et al., 2004; Laverty et al., 2019). Therefore, to determine if the GABA-activation properties of synaptic receptors were altered by the $\beta_3^{E77K}$ variant, we estimated the maximal open probability and constructed concentration–response curves of receptors to GABA.

The potency of GABA at the WT $\gamma$-$\beta_3$-$\alpha_1$-$\beta_3$-$\alpha_1$ receptor construct was 69 m$\mu$M and the maximum estimated open probability 0.94 (Fig. 3B–D). With observed values in the 0.93-1.0 range, none of the receptors containing the $\beta_3^{E77K}$ variant altered the maximum estimated open probability [one-way ANOVA, $F(6,62) = 1.17, P = 0.34$; Table 2]. In contrast, there was a significant difference in the GABA potency at the three different receptors [one-way ANOVA, $F(6,67) = 6.208 P < 0.0001 \log \text{EC}_{50}$].

Receptors with one copy of the $\beta_3^{E77K}$ variant significantly increased the GABA potency with EC$_{50}$’s ranging between 20-34 m$\mu$M for $\gamma$-$\beta_3^{E77K}$-$\alpha_1$-$\beta_3$-$\alpha_1$ and $\gamma$-$\beta_3$-$\alpha_1$-$\beta_3^{E77K}$-$\alpha_1$ ($< 0.01$ Dunnett’s post hoc test; logEC$_{50}$ c.f. WT; Table 2). Receptors with two copies of the $\beta_3^{E77K}$ variant also displayed a significant increase in the GABA potency compared to WT, reducing the EC$_{50}$ to 21 m$\mu$M (Table 2).

The $\beta_3^{T287}$ residue is located in the M2 region that lines the channel pore, lying one turn of the helix above a key leucine conserved across ligand-gated ion channels that forms a narrow diameter within the pore (Phulera et al., 2018; Laverty et al., 2019) (Fig. 4A). The M2 helix undergoes a tilt when the receptor transitions to an open state, and as such we estimated the maximal open probability and constructed concentration–response curves of $\beta_3^{T287I}$ concatenated receptors to GABA.

None of the receptors containing $\beta_3^{T287I}$ variants altered the Est Po$_\text{max}$ with values ranging between 0.93 and 1.0 [one-way ANOVA, $F(6,62) = 1.17, P = 0.34$; Fig. 4B; Table 2]. Receptors with one copy of the variant either significantly increased the GABA potency, reducing the EC$_{50}$ to 23 m$\mu$M for $\gamma$-$\beta_3$-$\alpha_1$-$\beta_3^{T287I}$-$\alpha_1$, $< 0.01$ Dunnett’s post hoc test; logEC$_{50}$ c.f. WT or the increase was not significant (EC$_{50}$ = 43 m$\mu$M for $\gamma$-$\beta_3^{T287I}$-$\alpha_1$-$\beta_3$-$\alpha_1$, $P > 0.05$; Dunnett’s post hoc test; Fig. 4C and D; Table 2). Receptors with two copies of the $\beta_3^{T287I}$ variants displayed significant increase in the GABA potency compared to WT, reducing the EC$_{50}$ to 22 m$\mu$M ($< 0.01$, Dunnett’s post hoc test; Fig. 4C and D, Table 2).

Thus, the common molecular phenotype for the vigabatrin-hypersensitive variants that we identified in receptors containing the $\beta_3^{E77K}$ or the $\beta_3^{T287I}$ variant is an increase in the GABA potency, and therefore an increase in...
Upon activation by GABA, the channel pore of the GABA$_A$ receptor opens, then shifts to an equilibrium that includes a desensitized state that is unable to conduct ions. In the case of GABA$_A$ receptors, chloride passes from the outside to the inside of the cell to hyperpolarize the cell, acting as a ‘brake’ for neuronal firing. If the receptor is highly desensitized the required chloride is unable to pass through the receptor and neuronal firing continues. Conversely, if the receptor does not desensitize then chloride will continue to permeate for extended times and neuronal firing could be excessively reduced. Variants in GABA$_A$ receptor subunits have previously been identified that increase the desensitization properties of the receptor (Shen et al., 2017; Butler et al., 2018) leading to an overall loss-of-function profile.

To quantify the macroscopic desensitization, we applied a single concentration of GABA, fitted the current decay to an exponential curve and determined the rate constant at different concentrations of GABA (Fig. 5A–C). As the potency of receptors with two copies of the variant was altered the most, we performed these experiments only at receptors containing two copies of the variants where any change in desensitization would be the largest. For WT receptors, the rate constants at concentrations of 0.1, 0.3, 1 and 3 mM GABA were 11 ± 2 ms, 22 ± 2 ms, 28 ± 2 ms and 31 ± 2 ms, for the $\beta_3^{E77K}$ were 30 ± 4 ms, 42 ± 3 ms, 56 ± 5 ms and 47 ± 6 ms and for the $\beta_3^{T287I}$ receptors were 20 ± 3 ms, 30 ± 6 ms, 28 ± 6 ms and 36 ± 6 ms ($n = 6$).

To compare the rate constants between receptors, we plotted decay constants as a function of the log$_{10}$ GABA concentration. Empirically, the data fitted well to a linear curve (Fig. 5D and E). An $F$-test demonstrated that the WT and $\beta_3^{T287I}$ linear functions could be described by the same curve [$F(2,43) = 2.34, P = 0.0108$], but the WT and $\beta_3^{E77K}$ linear functions could be described by different curves [$F$-test, $F(2,51) = 28.09, P < 0.001$]. A further Mann–Whitney test demonstrated that the WT and $\beta_3^{E77K}$ data were not significantly different ($P = 0.111$). This demonstrates that the variants do not lead to a loss-of-function molecular phenotypes through alterations in the desensitization profiles.

Table 2: Functional parameters of receptors containing one, or two, copies of each variant

| $\beta_3$ Variants | Construct | Activation properties |
|--------------------|-----------|----------------------|
| $\beta_3^{E77K}$   | $\gamma_2$-$\beta_3$-$\gamma_1$-$\beta_3$-$\gamma_1$ | $I_{\text{im}}$ GABA = SEM (nA) | $EC_{50}$ (µM) – logEC$_{50}$ = SEM | Est Po |
|                   | $\gamma_2$-$\beta_3$-$\gamma_5$-$\beta_3$-$\gamma_5$ | 2095 ± 126 (13)$^b$ | 69 (14) | 4.11 ± 0.06 | 0.94 ± 0.03 (10) |
|                   | $\gamma_2$-$\beta_3^{E77K}$-$\gamma_1$-$\beta_3$-$\gamma_1$ | 808 ± 90 (20) | 108 (10) | 3.93 ± 0.05 | 0.90 ± 0.01 (10) |
|                   | $\gamma_2$-$\beta_3$-$\gamma_7$-$\beta_3$-$\gamma_7$ | 1731 ± 241 (21) | 20 (10) | 4.62 ± 0.07 | 0.98 ± 0.04 (10) |
|                   | $\gamma_2$-$\beta_3^{E77K}$-$\gamma_1$-$\beta_3^{E77K}$-$\gamma_1$ | 1764 ± 245 (13) | 27 (10) | 4.63 ± 0.06 | 1.02 ± 0.04 (10) |
|                   | $\gamma_2$-$\beta_3^{E77K}$-$\gamma_1$-$\beta_3^{E77K}$-$\gamma_1$ | 946 ± 140 (16) | 21 (10) | 4.69 ± 0.06 | 0.94 ± 0.04 (8) |
|                   | $\gamma_2$-$\beta_3^{E77K}$-$\gamma_5$-$\beta_3^{E77K}$-$\gamma_5$ | 465 ± 66 (20) | 49 (10) | 4.31 ± 0.04 | 0.95 ± 0.05 (10) |
| $\beta_3^{T287I}$ | $\gamma_2$-$\beta_3^{T287I}$-$\gamma_1$-$\beta_3$-$\gamma_1$ | 2181 ± 223 (14) | 22 (10) | 4.65 ± 0.06 | 1.00 ± 0.02 (10) |
|                   | $\gamma_2$-$\beta_3^{T287I}$-$\gamma_5$-$\beta_3^{T287I}$-$\gamma_5$ | 712 ± 13 (20) | 45 (10) | 4.30 ± 0.08 | 1.01 ± 0.03 (10) |

$^a$WT values except rate constants are also reported in Absalom et al. (2019).

$^b$Number of individual oocytes (n) are in parentheses.

$^cP < 0.05$.

$^{**P < 0.01}$, one-way ANOVA, Dunnet’s t-test c.f. WT.

The activation of the receptors at lower GABA concentrations. This contrasts from previous findings that variants in $\beta_3$ subunits typically result in loss-of-function receptors. However, variants may have other characteristics that would lead to loss in inhibitory GABAergic transmission and thus we next investigated desensitization profiles that have been reported to have changed in other GABA$_A$ receptor subunit variants (Butler et al., 2018).

$\beta_3^{E77K}$ and $\beta_3^{T287I}$ variants do not alter macroscopic desensitization characteristics of the receptors

Upon activation by GABA, the channel pore of the GABA$_A$ receptor opens, then shifts to an equilibrium that includes a desensitized state that is unable to conduct ions. In the case of GABA$_A$ receptors, chloride passes from the outside to the inside of the cell to hyperpolarize the cell, acting as a ‘brake’ for neuronal firing. If the receptor is highly desensitized the required chloride is unable to pass through the receptor and neuronal firing continues. Conversely, if the receptor does not desensitize then chloride will continue to permeate for extended times and neuronal firing could be excessively reduced. Variants in GABA$_A$ receptor subunits have previously been identified that increase the desensitization properties of the receptor (Shen et al., 2017; Butler et al., 2018) leading to an overall loss-of-function profile.

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To compare the rate constants between receptors, we plotted decay constants as a function of the log$_{10}$ GABA concentration. Empirically, the data fitted well to a linear curve (Fig. 5D and E). An $F$-test demonstrated that the WT and $\beta_3^{T287I}$ linear functions could be described by the same curve [$F(2,43) = 2.34, P = 0.0108$], but the WT and $\beta_3^{E77K}$ linear functions could be described by different curves [$F$-test, $F(2,51) = 28.09, P < 0.001$]. A further Mann–Whitney test demonstrated that the WT and $\beta_3^{E77K}$ data were not significantly different ($P = 0.111$). This demonstrates that the variants do not lead to a loss-of-function molecular phenotypes through alterations in the desensitization profiles.

Taken together, these data demonstrate that both the $\beta_3^{E77K}$ and $\beta_3^{T287I}$ variants result in a significant increase
**Figure 3** Gain-of-function molecular phenotype of β3E77K variant. (A) The location of the β3E77K variant in a schematic of the β3 subunit and in the protein structure displaying a close-up of the coupling interface between the extracellular and transmembrane regions. (B) Bar graphs representing individual experiments (dots) and the mean ± SD for the estimated open probability of WT (black) and β3E77K constructs containing one copy of the variant in the 2nd subunit (red), one copy in the 4th subunit (purple) and two copies in the 2nd and 4th subunit (blue). The estimated open probability was calculated by comparing the response at 3 mM GABA to the response at 10 mM GABA, 3 μM etomidate and 1 μM diazepam. (C) Representative traces of currents recorded from Xenopus oocytes injected with WT (black) and β3E77K constructs containing one copy of the variant in the 2nd subunit (red), one copy in the 4th subunit (purple) and two copies in the 2nd and 4th subunit (blue). GABA concentrations were applied as indicated by the black bars to construct the concentration–response curves. Scale bars indicate 200 nA and 30 s, except for the blue bar that represents 50 nA and 30 s. (D) Concentration–response curves of WT and (i) concatenated constructs containing the β3E77K variant in the 2nd (red) or 4th subunit and (ii) the concatenated construct containing a β3E77K variant in the 2nd and 4th subunits. Symbols represent mean ± SD and data were fitted to the Hill equation.

**Figure 4** Gain-of-function molecular phenotype of β3T287I variant. (A) The location of the β3T287I variant in a schematic of the β3 subunit and in the protein structure displaying a close-up of the M2 domains of the β3 (red) and α1 (blue) subunits. (B) Bar graphs representing individual experiments (dots) and the mean ± SD for the estimated open probability of WT (black) and β3T287I constructs containing one copy of the variant in the 2nd subunit (red), one copy in the 4th subunit (purple) and two copies in the 2nd and 4th subunit (blue). The estimated open probability was calculated by comparing the response at 3 mM GABA to the response at 10 mM GABA, 3 μM etomidate and 1 μM diazepam. (C) Representative traces of currents recorded from Xenopus oocytes injected with WT (black) and β3T287I constructs containing one copy of the variant in the 2nd subunit (red), one copy in the 4th subunit (purple) and two copies in the 2nd and 4th subunit (blue). GABA concentrations were applied as indicated by the black bars to construct the concentration–response curves. Scale bars indicate 200 nA and 30 s, except for the purple bar that represents 2 000 nA and 30 s. (D) Concentration–response curves of WT and (i) concatenated constructs containing the β3T287I variant in the 2nd (red) or 4th subunit and (ii) the concatenated construct containing a β3T287I variant in the 2nd and 4th subunits. Symbols represent mean ± SD and data were fitted to the Hill equation.
in the sensitivity of the receptor to GABA without increased desensitization, consistent with a gain-of-function molecular phenotype.

**Response of $\beta^3_{E77K}$ and $\beta^3_{T287I}$ variants to nitrazepam**

Benzodiazepines, including nitrazepam, are positive allosteric modulators of GABA$_A$ receptors that predominantly affect phasic currents, usually through prolonging inhibitory postsynaptic currents (Otis and Mody, 1992). As nitrazepam reduced seizures without adverse effects for the patient with the $\beta^3_{E77K}$ variant, we evaluated the sensitivity of receptors to nitrazepam and compared the potency and efficacy of nitrazepam modulation of GABA (EC$_{10}$)-elicited currents (1 μM) at WT and variant receptors.

We constructed concentration–response curves to nitrazepam at WT receptors and receptors containing two copies of the $\beta^3_{E77K}$ or $\beta^3_{T287I}$ variant. The WT receptor had an EC$_{50}$ of 122 μM ($-\log$EC$_{50} = 6.96 \pm 0.03$) to nitrazepam and a maximum modulation ($E_{\text{max}}$) of 186 ± 4.6%. ($n = 5$) (Fig. 6A). Neither the $\beta^3_{E77K}$ and $\beta^3_{T287I}$ variant significantly altered the potency of nitrazepam with EC$_{50}$'s of 99 and 89 nM, respectively ($-\log$EC$_{50} = 7.04 \pm 0.03$ and 7.05 ± 0.02, $n = 5$, one-way ANOVA, F(3,17) = 3.258, $P > 0.05$). The efficacy of nitrazepam was significantly reduced to 122% at $\beta^3_{E77K}$ [one-way ANOVA, F(3,16) = 61.9, $P < 0.0001$] ($E_{\text{max}} = 122 \pm 3\%$, $n = 5$, Dunnett’s post hoc test, $P < 0.0001$) but not at $\beta^3_{T287I}$ receptors ($E_{\text{max}} = 174 \pm 4.7\%$, $n = 5$, Dunnett’s post hoc test, $P > 0.05$). These data suggest that nitrazepam is less efficacious at modulating receptors containing a $\beta^3_{E77K}$ variant, likely exerting greater effects in the patient via WT receptor subtypes.

**$\beta^3_{E77K}$ and $\beta^3_{T287I}$ variants increase GABA potency of extrasynaptic $\alpha_5$-containing receptors**

$\beta 3$ subunits are incorporated into a variety of GABA$_A$ receptors, including extrasynaptic $\alpha_5\beta_3\gamma$ receptors that mediate tonic currents. To determine how the $\beta^3_{E77K}$ and $\beta^3_{T287I}$ variants affected these receptors, we created a γ2-β3-α5-β3-α5 WT, γ2-β3$_{E77K}$-α5-β3$_{E77K}$-α5 and γ2-β3$_{T287I}$-α5-β3$_{T287I}$-α5 constructs. mRNA was synthesized for each construct and injected into Xenopus oocytes. All three receptor constructs expressed robustly with I$_{3mM\text{GABA}}$-evoked currents in the 0.5–0.8 μA range (Fig. 6B). No significant difference in the maximum currents elicited by 3 mM GABA were observed for the variants [one-way ANOVA, F(2,59) = 3.004, $P = 0.0571$; Table 2], although similar to the x1 constructs the γ2-β3$_{E77K}$-α5-β3$_{E77K}$-α5 receptor had the lowest absolute currents.

The potency of GABA at the WT γ-β3-α5-β3-α5 receptor construct was 108 μM and an Est P$_{O_{\text{max}}}$ value of 0.90 (Fig. 6C and D). The Est P$_{O_{\text{max}}}$ values were in a range of 0.90–1.01 and no significant difference was observed [one-way ANOVA, F(2,27) = 2.867, $P = 0.077$; Table 2]. However, both variants significantly reduced the GABA potency by ~2-fold compared to WT [one-way ANOVA, F(2,27) = 15.37, $P < 0.0001$ logEC$_{50}$, $P < 0.0001$]. Taken together, these data demonstrate that both the $\beta^3_{E77K}$ and $\beta^3_{T287I}$ variants result in a significant increase in the sensitivity of the receptor to GABA without increased desensitization, consistent with a gain-of-function molecular phenotype. This is the same functional change occurring in α5β3γ2 extrasynaptic receptor as is occurring when $\beta^3_{E77K}$ and $\beta^3_{T287I}$ variants are incorporated into the x1β3γ2 synaptic receptor.

**Why would gain of function variants lead to vigabatrin hypersensitivity?**

Vigabatrin is an irreversible inhibitor of GABA transaminase that blocks intracellular GABA degradation at the presynaptic terminal. The increased intracellular GABA concentrations reverse the direction of GABA transport, leading to non-vesicular release of GABA into the extracellular space (Wu et al., 2001, 2003) (Fig. 6E). In hippocampal cultures, 4 days of vigabatrin exposure...
caused an average increase in neuronal inhibition of 9 nS, with an implied \([\text{GABA}]_0\) of 0.5 \(\mu\)M. This results in large increases in tonic currents of the postsynaptic cell with little to no decrease in phasic inhibitory currents (Wu et al., 2003).

The \(\beta^3_{3R194}\) reduces the chloride flux at all GABA concentrations and, due to the reduction in functional receptors, the tonic current induced by vigabatrin likely would be lower than the WT. In contrast, the population of receptors expressed by both the \(\beta^3_{3E77K}\) and \(\beta^3_{3T287I}\) increased the chloride flux at lower GABA concentrations. The expression of concatenated GABA\(_A\) receptors in oocytes leads to a 30-fold higher GABA \(EC_{50}\) than in recombinant mammalian cells, where the \(EC_{50}\) \(\sim 2 \mu\)M (Mortensen et al., 2011). Therefore, at GABA concentrations in the oocytes equivalent to the \(\text{GABA}_0\) (\(\sim 15 \mu\)M), we record the highest increases in current elicited at receptors containing the \(\beta^3_{3E77K}\) and \(\beta^3_{3T287I}\) variants compared to WT, with \(\sim 20\%\) increase in currents. Vigabatrin, through the reversal of GABA transport in the presynaptic neuron, would therefore likely lead to considerably increased tonic inhibition in these variants compared to the WT receptor. Benzodiazepines, that do not elicit phasic currents from synaptic receptors, are unlikely to lead to the same adverse effect.
Discussion

GABA_A receptors are the primary mediators of inhibitory neurotransmission in the developing and adult brain. Variants in GABA_A receptors that alter the function of the receptor can modify neuronal excitability, leading to neurodevelopment disorders including epilepsy. Pathogenic variants in GABRB3 are commonly identified in individuals with mild to severe forms of epilepsy (Janve et al., 2016; Möller et al., 2017), signifying the importance of this gene in brain development and function. Located within an imprinting region that spans the 15q11-13 chromosome, variants in the GABRB3 gene are often clustered around central functional domains such as the extracellular GABA binding site, transmembrane helixes supporting or lining the pore (M1 and M2) or loop regions that couple ligand binding to channel gating (Niturad et al., 2017). We, and others, have shown that variants in GABA_A receptors often result in loss of receptor function predominantly manifesting as mutations that reduce receptor function by impairing channel gating or as a result of reduced subunit expression due to impaired transcription, impaired translation, misfolding and degradation, truncation, endoplasmic reticulum retention (Janve et al., 2016; Möller et al., 2017; Absalom et al., 2019; Hernandez and Macdonald, 2019; Hernandez et al., 2019).

Both the β3E77 and β3T287 residues are located in key activation regions of the receptor within loop 2 and the pore-lining transmembrane domain (M2) of the subunit, respectively. Loop 2 alters conformation during receptor activation, whereby the M2 region tilts to convert the channel from closed to open state (Laverty et al., 2019; Masiulis et al., 2019). Variants in these structural regions have been identified in GABA_A receptor subunits, and other ligand-gated ion channels, that lead to genetic disorders with both loss- and gain-of-function variants being reported for the different ion channels (Fig. 6E) (Plested et al., 2007). The β3T287I variant at the 13’ position faces the adjacent M2 domain of the neighbouring subunit, as opposed to the ion pore itself (Fig. 6E), and with a homologous variant identified in the GABRB1 gene (p.Thr287Ile), albeit with no functional data (Lien et al., 2016), the location appears to be a hotspot for spontaneous mutations. Alternatively, an evaluation of the β2 glutamate to lysine variant homologous to β3E77K showed no change in either the maximum absolute currents of GABA or concentration–response curve (Kash et al., 2004). For variants in these regions, it appears essential to perform functional genomics to ascertain the molecular phenotype of the variant.

The structural location of the variants may also underlie the differences in the sensitivities to nitrazepam. The β3E77 residue within loop 2 couples to the M2-M3 loop to mediate transitions earlier in the activation process than the M2 region where the β3T287 residue is located. The difference between nitrazepam efficacy in these two variants is most likely due to β3E77K altering the equilibrium between pre-activated conformational states of the receptor that benzodiazepines also alter (Gielen et al., 2012). In contrast, β3T287I, within the M2 region, is more likely to alter the equilibrium between conformational states later in the activation process, perhaps even between closed and open states, leading to little to no change in the efficacy of nitrazepam.

In this study, we describe four individuals with epilepsy and DD, three of which were prescribed and had different reactions to the anti-epileptic drug vigabatrin. One patient had a truncation within the GABRB3 gene that introduces a stop codon in the DNA coding sequence upstream of the transmembrane regions (β3R194* truncation). This truncation would abolish the functional expression of the β3 subunit, a classical loss-of-function variant. Interestingly this patient had worsening seizures in response to oxcarbazepine, a sodium channel blocker. Oxcarbazepine inhibits GABA release from the presynaptic terminal initiated via presynaptic NaV1.1 channels. As this patient has a typical loss-of-function GABRB3 variant, the addition of oxcarbazepine would further reduce the GABA-mediated inhibitory currents that are already lower than normal. This adverse response could have been avoided if the genetic information was known prior to treatment. In contrast this patient responded well to vigabatrin with reduced seizure frequency, becoming seizure free at 12 months as vigabatrin enhances GABA-mediated inhibitory currents.

In this patient, vigabatrin would be expected to increase the levels of tonic inhibition through the non-vesicular release of GABA mediated via the reversal of GABA transport induced by the blockade of GABA transporters. GABA_A receptors at the synapse are now persistently activated by the increased GABA concentrations, whereby these receptors will be continually activated leading to excess tonic currents. As a means to alleviate seizures that are ultimately caused by reduced GABA_A receptor expression, increased tonic inhibition is already known to be a key compensatory mechanism responsible for the relatively mild phenotype of mice with homozygous z1 GABA_A receptor deletions. Apart from a slight handling tremor, these mice have no pronounced phenotype, and analysis of tonic currents at cerebral granular cells identified ~60% increases in tonic current, identifying a plausible compensatory mechanism (Ortinski et al., 2006). Utilizing vigabatrin to increase the tonic current in patients with GABRB3 truncation variants would thus represent a rational approach to therapy.

The two patients carrying the heterozygous missense GABRB3 variants (p.Glu77Lys and p.Thr287Ile) initially showed a promising reduction in seizures to vigabatrin, but subsequently deteriorated with decreased alertness, extreme drowsiness, hypotonia, sedation and respiratory difficulties. These patients exhibited an atypical gain-of-function molecular phenotype as evidenced by increased GABA sensitivity, no alterations to receptor
desensitization characteristics, and little change to the Est open probability or maximum GABA currents. The enhanced sensitivity to GABA at $\beta^3_{E77K}$ and $\beta^3_{T287I}$ containing receptors is likely to underlie the hypersensitive clinical reaction to vigabatrin. The increase in non-vesicular GABA release that is converted to a tonic current will be exacerbated at receptors containing variants that increase the potency of GABA. Furthermore, when the $\beta^3_{E77K}$ or $\beta^3_{T287I}$ variants are incorporated into α5β3γ2 receptors that normally mediate tonic currents, the resulting receptors also display increased GABA sensitivity. Under vigabatrin treatment, any compensation mechanisms appear unable to prevent the hypersensitive reactions in these two patients with $\textit{GABRB3}$ variants that result in gain-of-function molecular phenotypes. Despite good efficacy for treating infantile spasms, including our case 1, ~5% of patients exhibit symptoms of vigabatrin hypersensitivity manifesting as significant drowsiness, hypotonia and respiratory difficulties (Lux et al., 2005), and similarly to what was observed with our patients, the adverse response was reversible (Lux et al., 2005; Hernández Vega et al., 2014).

Benzodiazepines, however, did not exacerbate symptoms or lead to severe adverse reactions, despite having a mechanism of action that increases GABAergic inhibitory currents. Our patient that carried the $\beta^3_{E77K}$ (case 3) variant had a significant reduction in seizures over a 6-month period when treated with nitrazepam combined with a ketogenic diet with no overt adverse response. When nitrazepam was analysed, it had reduced efficacy at this variant but maintains normal efficacy at WT. Furthermore, benzodiazepines act differently to vigabatrin. They are positive allosteric modulators of synaptic GABA$_A$ receptors that selectively bind to $\alpha$-2 subunit interfaces, mainly prolonging phasic currents rather than increasing tonic currents (Sigel and Ernst, 2018). These results indicate that functional assessment of direct acting GABA$_A$ receptor antiepileptics is important in understanding the mechanism for how patients with distinct GABA$_A$ variants may respond to certain drugs or drug classes.

Gain-of-function variants in $\textit{GABRB3}$ gene have never been previously reported, although a report of gain-of-function variants causing spontaneous GABA ion channel opening and increased GABA potency of recombinant receptors containing the $\textit{GABRB1}$ (p.Leu285Arg) variant located within the M2 domain were associated with alcohol dependence in mice (Anstee et al., 2013). Furthermore enhanced GABA potency has been reported for a variant in the $\textit{GABRA5}$ variant (p.Val294Leu), three in $\textit{GABRA3}$ and one in $\textit{GABRA1}$. However unlike the $\beta^3_{E77K}$ and $\beta^3_{T287I}$ variants, these variants apparently either resulted in greater receptor desensitization (Butler et al., 2018; Steudle et al., 2020) or exhibited significantly reduced current amplitudes (Niturad et al., 2017), indicating a possible net loss of GABAergic neurotransmission. The peak concentration of GABA in the synaptic cleft will reach ~1mM, a saturating GABA concentration. However, this concentration only lasts for less than a millisecond before the GABA is taken up by presynaptic transporters whereby the binding occupancy of GABA$_A$ receptors are not always saturated (Nusser et al., 1997). It would therefore be predicted that in the patients there will be either an increase in the peak amplitude of IPSCs, or a prolonged response of IPSCs, depending on the cell type and synaptic properties in different regions of the brain. Regardless, we would expect a generalized increase in inhibitory GABAergic neurotransmission within these patients.

The idea that increased GABAergic function causes DEE may seem counterintuitive, but extracellular GABA per se can have a pro-convulsive action on the epileptic network through disinhibition and alterations in neuronal development (Pavlov and Walker, 2013). Epilepsy-causing loss-of-function variants in the gene encoding the GABA uptake transporter $\textit{SLC6A1}$ result in an accumulation of GABA in the synapses that leads to over activation of synaptic and extrasynaptic GABA$_A$ receptors (Mattison et al., 2018). Furthermore, the $\textit{GABRB3}$ variants described here (p.Glu77Lys and p.Thr287Ile) are de novo and manifest during embryonic development. During development, GABA is excitatory and depolarizing, controlling cell proliferation, growth, migration and differentiation via extrasynaptic GABAergic signalling. Thus increased GABA potency at extrasynaptic receptors will affect development and the formation of normal neuronal networks. As the $\textit{GABRB3}$ gene has been associated with epilepsy, development, motor, learning and memory (Tanaka et al., 2012) and autism (Delahanty et al., 2011) and given its location throughout the brain, the gain-of-function molecular phenotype is likely contributing to the clinical presentations of seizures, head lag, autism and DD.

Treatments for patients with gain-of-function mutations must differ to those with loss-of-function mutations since further enhancing ‘hyperactive’ GABA$_A$ receptors could potentially worsen symptoms. Indeed a patient carrying the $\textit{GABRA5}$ variant that had a gain-of-function phenotype experienced increased seizure frequency while on phenobarbital, and extreme sedation while on clonazepam, albeit this could also be the natural course of the patient’s epilepsy (Butler et al., 2018). Evidently, caution must be taken when interpreting why a patient with a specific variant responds differently to one drug than another. Even without background genetic effects, the pharmacodynamics will be complex where both variant and WT receptors will be affected by the drug.

In conclusion, this is the first study that describes GABA$_A$ receptor variants that exhibit a gain-of-function molecular phenotype and may be a hitherto under-acknowledged cause of DEEs. The gain-of-function molecular phenotype can explain the hypersensitive reaction to vigabatrin seen in our patients and caution is required when prescribing vigabatrin to patients with GABA$_A$
receptor variants. We propose that different molecular phenotypes will require different drug treatments targeted at the functional effect of the epilepsy-causing variant, paving the way for a precision medicine approach to the treatment of some forms of DEE.

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Competing interests

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