Functional genomics of epilepsy-associated mutations in the $\text{GABA}_\alpha$ receptor subunits reveal that one mutation impairs function and two are catastrophic

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Running Title: Concatenated $\text{GABA}_\alpha$ receptor epilepsy mutations

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ABSTRACT

A number of epilepsy-causing mutations have recently been identified in the genes of the $\alpha_1$, $\beta_3$, and $\gamma_2$ subunits comprising the $\gamma$-aminobutyric acid type A (GABA$_\alpha$) receptor. These mutations are typically dominant, and in certain cases such as the $\alpha_1$ and $\beta_3$ subunits, they may lead to a mix of receptors at the cell surface that contain no mutant subunits, a single mutated subunit, or two mutated subunits. To determine the effects of mutations in a single subunit or in two subunits on receptor activation, we created a concatenated protein assembly that links all five subunits of the $\alpha_1\beta_3\gamma_2$ receptor and expresses them in the correct orientation. We created nine separate receptor variants with a single-mutant subunit and four receptors containing two subunits of the $\gamma_2^{R323Q}$, $\beta_3^{D120N}$, $\beta_3^{T157M}$, $\beta_3^{Y302C}$ and $\beta_3^{S254F}$ epilepsy-causing mutations. We found that the single-mutated $\gamma_2^{R323Q}$ subunit impairs GABA activation of the receptor by reducing GABA potency. A single $\beta_3^{D120N}$, $\beta_3^{T157M}$, or $\beta_3^{Y302C}$ mutation also substantially impaired receptor activation, and two copies of these mutants within a receptor were catastrophic. Of note, an effect of the $\beta_3^{S254F}$ mutation on GABA potency was dependent on the location of this mutant subunit within the receptor, possibly because of the membrane environment surrounding the transmembrane region of the receptor. Our results highlight that precise functional genomic analyses of GABA$_\alpha$ receptor mutations using concatenated constructs can identify receptors with an intermediate phenotype that contribute to epileptic phenotypes and that are potential drug targets for precision medicine approaches.

Epileptic encephalopathies are a devastating group of severe childhood epilepsies with poor developmental outcomes that are often resistant to pharmacological treatment (1). In many cases, the causes are genetic, and recent advances in whole-genome sequencing have identified a series of de novo and inherited mutations in various genes. Several mutations in genes that encode for the $\alpha_1$, $\beta_3$ (2-9), $\beta_3$ (4,7,8,10-12) and $\gamma_2$ subunits (4,7,9,13-16) of the gamma-aminobutyric acid type
A (GABA<sub>A</sub>) receptor (GABRA1, GABRB3 and GABRG2, respectively) have been identified that result in epileptic encephalopathies.

GABA<sub>A</sub> receptors are essential mediators of neurotransmission in both the developing and adult brain (17). These receptors are ion channels composed of five subunits that arrange around a central ion pore (18). When GABA is released from the synapse it binds to these receptors anchored at the postsynaptic membrane to open an ion channel, allowing chloride ions to pass, hyperpolarising and inhibiting the cell (19).

Many genes encoding for different subunits of the GABA<sub>A</sub> receptor are present in the mammalian brain including six α (α1-6), three β (β1-3), three γ (γ1-3), a δ, ε and π and the majority of receptors are thought to contain two α, two β and a γ subunit where they are anchored at the synapse, responding to high concentrations of GABA (20). Other combinations of receptors, often containing δ-subunits, are found extrasynaptically where they respond to low concentrations of GABA or spillover from the synapse (21). Each individual subunit consists of a large extracellular domain, four transmembrane domains where the second (M2) lines the channel pore, two short and one large loop linking transmembrane domains and a short carboxy terminus. At synaptic receptors, GABA binds within the β-α interface located between adjacent extracellular domains to trigger an activation pathway through a series of conformational changes that ultimately open the channel pore. These conformational changes are transmitted through interactions at the coupling region, where loops in the extracellular domain in close proximity to the membrane interact with the pre-M1 and M2-M3 loops that connect transmembrane domains (22). This results in tilt of the M2 domain to open the pore. Epilepsy-causing mutations identified in the α1, β3 and γ2 subunits are located at different regions throughout the protein, including amino acids throughout the activation pathway from the ligand-binding pocket and extracellular structural β-sheets, through to the coupling and transmembrane M1 and M2 regions.

Mutations in the GABA<sub>A</sub> receptor that cause epilepsy typically impair this process, either through misfolding of protein to reduce the number of receptors at the cell surface or disturbing the ability of the receptor to open in response to GABA (8). In all cases, the mutations that cause epileptic encephalopathies are dominant, with patients carrying one copy of the wild-type allele and one copy of the mutant allele (2-16). For mutations in the γ2 subunit, the resultant receptors will either be a wild-type or contain a single mutation. However, for β3 mutations a more complicated mixture of receptors will be expressed. A wild-type containing two normal β3 subunits, two hetero-mutant receptors containing a single mutation at either of the two β3 subunit locations within the pentamer, and a homo-mutant receptor containing the mutation at both β3 subunit locations within the pentamer can be formed. If the surface expression is unaffected and the distribution of mutant receptors into the complex is random, some 50 % of the resultant receptors will contain mutations with just a single copy of the mutation. To date there is a lack of research that assesses the effect of single copy of the mutation on GABA<sub>A</sub> receptor function; an important component that contributes to understanding the epilepsy phenotype of individuals. Therefore, it is vital to determine how single copies of the mutation, as well as two copies, alter the function of the receptor to properly characterize the molecular phenotype of the mutation.

We chose five mutations to investigate using the concatenated construct including one in the γ2 subunit (γ2<sup>R323Q</sup>) and four in the β3 subunit (β3<sup>D120N</sup>, β3<sup>T157M</sup>, β3<sup>S254F</sup> and β3<sup>Y302C</sup>) (Figure 1A). These mutations were chosen as they were located in different regions along the activation pathway of the receptor, including the M2-M3 coupling loop of the γ2 subunit (γ2<sup>R323Q</sup>). The β3 mutations were located in the area surrounding the ligand-binding site (β3<sup>D120N</sup>), a β-sheet within the extracellular domain (β3<sup>T157M</sup>), the M2-M3 coupling loop (β3<sup>Y302C</sup>) and the M1 transmembrane region (β3<sup>S254F</sup>) (Figure 1B,C). Previous functional genomic analysis of these mutations in Xenopus oocytes and HEK-293 cells have demonstrated that the γ2<sup>R323Q</sup>, β3<sup>D120N</sup> and β3<sup>Y302C</sup> mutations substantially reduce either the potency of GABA, or the magnitude of GABA-activated currents when expressed in α1β3γ2 or αβ3γ3 receptors (8,11,15) while the β3<sup>T157M</sup> mutation caused only subtle changes at αβ3γ3 receptors (8). However, these experiments did not fully describe the molecular phenotype of the mutations, as they were unable to distinguish how receptors that contain one or two
copies of the mutation differ from the wild-type receptor, or from each other.

To resolve this question, we created a concatenated α1β3γ2 GABA<sub>A</sub> receptor construct with five linked subunits in the sequence γ2-β3-α1-β3-α1. For each mutation, we then created a set of receptor constructs that resembled the expressed receptors from an individual with a dominant mutation. Typically, but not always, a single copy of the mutation impaired the activation properties of the receptor, while a second copy intensified the effect of the mutation to be catastrophic.

We propose that precise functional genomic analysis using concatenated receptors can identify the phenotype of the individual receptors that are expressed by patients with dominant mutations. This information may ultimately assist in precision medicine approaches, where mutant GABA<sub>A</sub> receptors containing a single mutation can be targeted to treat individual patients.

**Results**

**Activation Properties of Wild-type Concatenated Receptor**

To determine how receptors with either a single or two copies of an epilepsy-causing mutation differ from wild-type receptors, we created a concatenated receptor construct with five subunits linked by AGS repeats (Figure 2A). Although concatenated ligand-gated ion channels have previously been created, many of these do not reliably form in the standard orientation (23), or are mixtures of dimeric and trimeric concatenated constructs (24). When injected alone, these dimeric and trimeric constructs can result in low currents that could potentially confound the analysis of mutations that impair receptor function (25). However, constructs of concatenated pentameric GABA<sub>A</sub> receptors have also been described that contain β2 subunits (26). Therefore, the new construct was designed with five subunits in the same order so that the subunits would arrange themselves primarily counter-clockwise when viewed from the extracellular side of the membrane, forming two GABA-binding sites at the β3-α1 interfaces and a benzodiazepine binding site at the α1-γ2 interface (Figure 2B). Sequence encoding for the signal peptide of the α1 and β3 subunits were removed and four different linkers were incorporated with lengths calculated with the same methodology as previously (23). To ensure predominately counter-clockwise expression, the first linker was designed to be relatively short and subsequent linkers were longer with similar lengths when N- and C-terminals were taken into account. These linkers contained peptide sequences of (AGS)<sub>5</sub> between the γ2 and β3 subunits, (AGS)<sub>5</sub>LGS(AGS)<sub>5</sub> between the first β3 and α1 subunits, AGT(AGS)<sub>5</sub> between the α1 and β3 subunits and (AGS)<sub>5</sub>ATG(AGS)<sub>5</sub> between the final β3 and α1 subunits, to form the DNA construct encoding the five subunits in the order of γ2-β3-α1-β3-α1 (Figure 2A,B).

cRNA (2 ng) of the wild-type concatenated construct was injected into *Xenopus* oocytes and the oocytes were incubated for 2–4 days. Denaturing agarose gel electrophoresis was performed on the RNA to ensure a single band at the correct size only was transcribed, and a western blot was performed to ensure that the protein was properly translated and degradation products were not observed (Supporting Information S1). Peak currents were measured using two-electrode voltage clamp electrophysiology upon application of a range of GABA solutions, and the measured responses were used to construct the concentration-response curve (Figure 2C). Injection of the wild-type cRNA resulted in robust GABA-activated currents, with 3 mM GABA eliciting an average current of 2 μA (Figure 2C, Table 1). GABA activated the wild-type concatemer with an EC<sub>50</sub> of 69 μM, similar to our previously published value of 53 μM and other previously published reports (e.g 74 μM (27)) where unlinked α1, β3 and γ2 subunits were injected into *Xenopus* oocytes (28).

To ensure that the receptors were arranging in the correct orientation, the modulation of GABA-elicited currents of our concatenated receptors were measured using a benzodiazepine, clobazam that binds selectively to the α1-γ2 interfaces. When clobazam was co-applied with 10 μM GABA, the response of the activated receptors was increased with increasing clobazam concentrations (Figure 2D). We constructed a concentration-response curve of clobazam modulation of 10 μM GABA-activated currents. The maximum modulation by clobazam was 306 % and the EC<sub>50</sub> value of clobazam was 86 nM, similar to previously published values of 256 % and 132 nM at non-concatenated α1β2γ2 receptors (29) (Figure 2D). Taken together, the GABA and clobazam
concentration-response curves demonstrate that the concatenated receptor reliably replicates the activation properties of its respective unlinked receptor. We then used this construct as a backbone so that mutant β3 and or γ2 subunit(s) can be inserted at specific regions of the pentameric construct to analyse the effects of epilepsy-causing mutations. For γ2 mutations a single copy of the mutation was inserted into the receptor and for β3 mutations, either a single copy of the mutation was inserted within different subunits, or two copies of the mutation were inserted into the receptor.

**Absolute Expression Levels of Mutant Receptors**

We chose five mutations to investigate using concatemers including one in the γ2 subunit (γ2R323Q) and four in the β3 subunit (β3D120N, β3T157M, β3S254F and β3Y302C). These mutations were chosen as they are located in different regions along the activation pathway of the receptor. In order from the extracellular to transmembrane domains, the amino acids included the area surrounding the ligand-binding site (β3D120N), a β-sheet located in the extracellular domain of the β3 subunit (β3T157M), the M2-M3 coupling loop of both the γ2 (γ2 R323Q) and β3 subunit (β3 Y302C) and the M1 region (β3 S254F). The β3 D120 and β3 Y302 residues are located at the interface of the α1 and β3 subunits, the β3 T157M residue is located within the β-sheet of the β3 subunits, and the β3 S254 residue is located at the interface of a β3 and α1 subunit, or the interface of a β3 and γ2 subunit.

For the γ2 mutation, we introduced a single copy of the γ2R323Q mutation into the 1st subunit of the concatenated construct. For each of the β3 mutations, we created a set of three constructs with a mutation in either the 2nd or 4th β3 subunit and a construct with a mutation in both 2nd and 4th β3 subunits (Figure 3A). We injected 2 ng of cRNA encoding for each of the constructs and then compared the absolute currents elicited by 3 mM GABA at the mutant receptors to the wild-type (Figure 3B).

Strikingly, the incorporation of two mutations into the receptor was catastrophic for three of the β3 mutations. When two copies of the β3 D120N or β3 T157M mutations were incorporated into the concatemer, the GABA-elicted currents were too small to be measured, while the incorporation of two β3 Y302C mutations significantly reduced the GABA-elicted currents (I3mM_GABA = 77 nA γ2-

β3 Y302C-α1-β3 Y302C-α1, I3mM_GABA = 2.1 µA, wild-type). In contrast, there was no significant difference in the current amplitudes compared to wild-type when two copies of the β3 S254F mutation was incorporated into the receptor (I3mM_GABA = 1.5 µA, γ2-β3 S254F-α1-β3 S254F-α1) (Figure 3B, C).

In contrast, the incorporation of a single mutation into the receptor did not cause the same marked effects on the magnitude of absolute currents, with no more than a 3-fold reduction at any mutated concatemer. A single γ2 R323Q mutation in the 1st subunit of the concatemer had similar GABA-activated currents (I3mM_GABA = 1.4 µA, γ2 R323Q-β3-α1-β3-α1) to wild-type, while the introduction of a single β3 D120N mutation at either subunit location significantly reduced current amplitudes (I3mM_GABA = 700 and 970 nA γ2-β3 D120N-α1-β3-α1 or γ2-β3-α1-β3 D120N-α1, respectively). When introduced at the first β3 subunit, a single β3 T157M or β3 S254F mutation significantly reduced the current amplitudes (I3mM_GABA = 1.1 and 1.2 µA, γ2-β3 T157M-α1-β3-α1 and γ2-β3 S254F-α1-β3-α1 respectively), but not when introduced in the second (I3mM_GABA = 2.0 and 2.5 µA, γ2-β3-α1-β3 T157M-α1 and γ2-β3-α1-β3 S254F-α1 cotemers, respectively). A single β3 Y302C mutation significantly reduced GABA-activated currents when introduced in the second β3 subunit, but not the first (I3mM_GABA = 1.3 µA and 1.8 µA, γ2-β3-α1-β3 Y302C-α1 and γ2-β3 Y302C-α1-β3-α1, respectively) (Figure 3B, C).

Although several concatemers containing single mutations had significant reductions in the maximum absolute currents elicited by 3 mM GABA, this crude approach is a poor measure of how mutations alter receptor properties. Variation in the maximum absolute current can be introduced in several ways that are a consequence of experimental conditions. These include large rightward shifts in the EC50, small changes in the RNA concentration, the incubation time and the rate at which the individual oocytes form protein and express receptors at the cell surface. However, mutations may also cause changes in the intrinsic activation properties of the receptor that reduce the current passing across the synapse. This can occur through changes in the potency of GABA or changes in the efficacy, where GABA reverts to a more partial agonist, or both. To determine whether the mutations changed either the potency or efficacy of GABA activation, we next constructed
concentration-response curves to GABA and estimated the maximum open probability of GABA to determine whether the mutations changed these intrinsic activation properties of the receptor, when either one or two copies of the mutation were present.

\( \gamma^{R323Q} \) Impairs GABA-Activation Properties of the Receptor

We therefore constructed concentration-response curves to GABA and standardised the response against an estimated maximum open probability of the receptor for the wild-type and each mutation. We initially compared the \( \gamma^{R323Q} \), \( \beta3-\alpha1-\beta3-\alpha1 \) mutant receptor to the wild-type, as this mutation is incorporated into the receptor within the only \( \gamma2 \)-subunit (Figure 4A). The potency of GABA has previously been shown to be reduced at \( \alpha1\beta2\gamma2 \) receptors when expressed in HEK293 cells, and we would expect comparable results similar effects in our concatenated construct (15).

We first tested whether the \( \gamma^{R323Q} \) mutation altered the potency of GABA. We constructed concentration-response curves to GABA at \( \gamma^{R323Q} \)-\( \beta3-\alpha1-\beta3-\alpha1 \) receptors to determine the EC\(_{50}\) of the mutant receptors. These experiments were run on an automated protocol, where 3 mM GABA was applied as a reference and internal standard three times during the experiment for all receptors (Figure 4A). Similar to the results reported using this mutation with free \( \alpha1 \) and \( \beta3 \) subunits in HEK293 cells (15), there was a decrease in the potency of GABA with a significant 4.5-fold decrease in the potency of GABA (EC\(_{50}\) = 315 \( \mu \)M) (Figure 4A, Table 1), demonstrating that the activation of receptors by GABA is impaired by the \( \gamma^{R323Q} \) mutation.

We then determined whether the maximal efficacy of GABA was impaired by the \( \gamma^{R323Q} \) mutation by estimating the maximum Po (Est. Po\(_{\text{max}}\)) at wild-type and \( \gamma^{R323Q} \)-\( \beta3-\alpha1-\beta3-\alpha1 \) receptors using a pharmacological technique similar to Shin et al (30). At oocytes expressing either wild-type or mutant receptors, we applied the 3 mM GABA reference and then co-applied 10 mM GABA with 1 \( \mu \)M etomidate and 3 \( \mu \)M diazepam to shift as many receptors as possible to the open state (Figure 4B). We assumed that the combination of GABA with etomidate and diazepam opened the receptors with a probability approaching one. The Est. Po\(_{\text{max}}\) of GABA for each receptor was then calculated by dividing the current elicited by 3 mM GABA by the current elicited by GABA, etomidate and diazepam, and corrected to account for shifts in the concentration-response curves (Figure 4B, Table 1). We refer to Est Po\(_{\text{max}}\) as an estimated maximum open probability as the true current amplitude may be underestimated by mutations that greatly impair receptor activation, modulation, or change desensitization kinetics. As expected, GABA elicited a very high Est. Po\(_{\text{max}}\) of 0.95 at wild-type receptors, consistent with single-channel recordings where the channel enters a long-lived open state (31). The \( \gamma^{R323Q} \) mutation did not significantly alter the Est. Po\(_{\text{max}}\) with a value of 0.85 (Figure 4B, Table 1).

When incorporated into the 1st subunit of the concatemer, the \( \gamma^{R323Q} \) mutation reduced the potency of GABA without causing a significant reduction in the efficacy. These changes in the activation properties of the receptor caused by the \( \gamma^{R323Q} \) mutation in the concatenated construct were similar to the reported effects of receptors comprised of unlinked subunits. Therefore, the concatenated construct is a suitable method of analysing the effect of mutations on the activation properties of the receptor.

A Single \( \beta3^{D120N} \) or \( \beta3^{T157M} \) Mutation Impairs GABA Potency, two Mutations are Catastrophic

We next assessed the effects of two \( \beta3 \) mutations, \( \beta3^{D120N} \) and \( \beta3^{T157M} \) that are both located in the extracellular domain at the earlier stages of the activation pathway. The \( \beta3^{D120N} \) mutation has previously been expressed in combination with either \( \alpha1 \), \( \beta3 \) and \( \gamma2 \), or \( \alpha1 \) and \( \gamma2 \) free subunits to determine the effects of heterozygous or homozygous expression, respectively on receptor function (11). Both the gating properties and the absolute expression levels of the receptor were reduced in both cases, while in a separate study the incorporation of the \( \beta3^{T157M} \) mutation with \( \alpha5 \) and \( \gamma2 \) subunits only made subtle changes to the activation properties of the receptor (8). To determine the effect of the mutations when they were expressed in a single subunit within the receptor, we constructed concentration-response curves to GABA and measured the Est Po\(_{\text{max}}\) at concatenated receptors containing a single copy of a mutation.
We created concatenated constructs by introducing β3^{D120N} or β3^{T157M} mutations in the 2nd, 4th, or both 2nd and 4th subunits in the concatemer and constructed concentration-response curves to GABA (Figure 5A). A single copy of the β3^{D120N} mutation significantly reduced the potency of GABA by 16-20-fold, regardless of whether it was introduced at the 2nd or 4th subunit (EC_{50} = 1.14 and 1.47 mM, γ2-β3^{D120N}-α1-β3-α1 and γ2-β3-α1-β3^{D120N}-α1 respectively) (Figure 5B, Table 1). These EC_{50} values were not significantly different to each other, strongly suggesting that the subunit location of the β3^{D120N} mutation within the pentameric structure did not affect how the mutation altered receptor activation properties.

Similarly, the β3^{T157M} mutations significantly reduced the potency of GABA by 4-6-fold when introduced at either the second or fourth subunit (EC_{50} = 422 and 279 μM, γ2-β3^{T157M}-α1-β3-α1 and γ2-β3-α1-β3^{T157M}-α1 respectively) (Figure 5C, Table 1). Again, the EC_{50} values were not significantly different to each other, demonstrating that the subunit location of the β3^{T157M} did not affect how the mutation altered the activation properties of GABA. Neither receptor expressed measurable currents when a mutation was introduced in both β3 subunits, and as such concentration-response curves could not be constructed.

We next measured the Est. P_{max} of two receptors with a single copy of the mutation in the 2nd or 4th subunit respectively, to determine if the efficacy of GABA had been altered (Figure 5D). Despite the absolute current levels being reduced by the β3^{D120N} mutation, the combination of etomidate and diazepam failed to appreciably increase the maximal response to GABA at either receptor containing a single mutation (Figure 5D,E). This is likely due in part to the large rightward shift of the concentration-response curve where 3 mM GABA no longer elicits the maximum response. Similarly, the combination of etomidate and diazepam had little effect on the maximal GABA current elicited at the two receptors containing a single β3^{T157M} mutation (Figure 5D,E). Consequently, there was no significant difference in the Est. P_{max} at the four receptors (Est. P_{max} = 1.12 and 1.00, 0.9 and 0.96, γ2-β3^{D120N}-α1-β3-α1, γ2-β3-α1-β3^{D120N}-α1, γ2-β3^{T157M}-α1-β3-α1 and γ2-β3-α1-β3^{T157M}-α1 respectively) (Figure 5E, Table 1).

Taken together, single copies of the β3^{D120N} and β3^{T157M} mutations at the earlier stages of the activation pathway both impair GABA activation of the receptor by reducing the potency of GABA by approximately 20-fold and 5-fold, respectively, without altering the maximal efficacy of GABA. There was little difference in the effect of either single mutation when located at different subunits β3 within a pentamer. A second copy of the β3^{D120N} or β3^{T157M} mutation intensifies the effect of the mutation and appears to be catastrophic, leading to little to no functional receptor expression.

**β3^{S254F} and β3^{Y302C} Mutation Effects Are Dependent on Location and Number of Mutations**

We next assessed the effects of two other β3 mutations, β3^{Y302C} and β3^{S254F}. The β3^{Y302C} residue is located in the M2-M3 coupling loop, a key motif in the activation pathway that links extracellular and transmembrane domains. The β3^{S254} residue is located in the transmembrane regions within the M1 transmembrane helix that moves late in the activation process of ligand-gated ion channels (32). The β3^{Y302C} mutation has been shown to impair receptor activation when expressed with either α1 and γ2 subunits or α5 and γ2 receptors (8,11), while there are no functional data on how the β3^{S254F} mutation affects receptor activation.

A single copy of the β3^{Y302C} mutation, introduced at either the 2nd or the 4th subunit, significantly reduced the potency of GABA between 2-7-fold (EC_{50} = 167 and 471 μM, γ2-β3^{Y302C}-α1-β3-α1 and γ2-β3-α1-β3^{Y302C}-α1 respectively), and these EC_{50} values differed significantly to each other (Figure 6A, Table 1). Two copies of the β3^{Y302C} mutation were catastrophic, further reducing the potency of GABA by nearly 100-fold, an order of magnitude greater than either of the single mutations (EC_{50} = 6.81 mM, γ2-β3^{Y302C}-α1-β3^{Y302C}-α1). This EC_{50} value was significantly greater than the EC_{50} value of the wild-type or the two concatemers containing a single β3^{Y302C} mutation (Figure 6A, Table 1).

Additionally, the introduction of the β3^{Y302C} mutation significantly reduced the Est. P_{max} compared to wild-type, regardless of the subunit location of the mutation or whether one, or two copies of the mutation were introduced. The efficacy of GABA was least affected by one copy of the β3^{Y302C} mutation at the 4th subunit (Est. P_{max}
of the $\beta_3^{Y302C}$ mutation at the 2nd subunit significantly reduced the efficacy of GABA compared to the wild-type or the receptor with a single mutation at the 4th subunit (Est. $P_{\text{max}} = 0.4$, $\gamma_2$-$\beta_3^{Y302C}$-$\alpha_1$-$\beta_3$-$\alpha_1$). Two copies of the $\beta_3^{Y302C}$ mutation resulted in the lowest efficacy of GABA ($P_{\text{max}} = 0.24$, $\gamma_2$-$\beta_3^{Y302C}$-$\alpha_1$-$\beta_3^{Y302C}$-$\alpha_1$) (Figure 6B,C, Table 1). This demonstrates that, unique amongst the mutations that we have investigated, the single $\beta_3^{Y302C}$ mutation impairs activation of the receptor to decrease both the potency and maximum efficacy of GABA activation. Differences in the magnitude of the reduction in the maximal efficacy suggests these residues may not be equivalent when located in different subunits.

The introduction of the $\beta_3^{S254F}$ mutation did not follow the same pattern as the other mutations, which reduced the potency of GABA regardless of the subunit location of the mutation. Instead, when the mutation was introduced at the 2nd subunit, the $EC_{50}$ value of GABA was significantly increased nearly 3-fold compared to the wild-type ($EC_{50} = 181 \mu M$, $\gamma_2$-$\beta_3^{S254F}$-$\alpha_1$-$\beta_3$-$\alpha_1$) demonstrating that the activation properties of this concatemer were impaired (Figure 6D, Table 1). However, when the mutation was introduced at the 4th subunit, the $EC_{50}$ value of GABA significantly decreased 2-fold compared to the wild-type, as did the $EC_{50}$ when the $\beta_3^{S254F}$ mutation was introduced at both the 2nd and 4th subunits ($EC_{50} = 34.2$ and $29.6 \mu M$, $\gamma_2$-$\beta_3$-$\alpha_1$-$\beta_3^{S254F}$-$\alpha_1$ and $\gamma_2$-$\beta_3^{S254F}$-$\alpha_1$-$\beta_3^{S254F}$-$\alpha_1$ respectively) (Figure 6D, Table 1). This demonstrates that the subunit location of the $\beta_3^{S254F}$ mutation defines the functional effect of the receptor, determining whether the potency of GABA has increased or decreased. The maximal efficacy of GABA was not changed by the $\beta_3^{S254F}$, regardless whether one or two copies of the mutation were incorporated into the concatemer. (Est. $P_{\text{max}} = 1.00$, 0.95 and 0.89, $\gamma_2$-$\beta_3^{S254F}$-$\alpha_1$-$\beta_3$-$\alpha_1$, $\gamma_2$-$\beta_3$-$\alpha_1$-$\beta_3^{S254F}$-$\alpha_1$ and $\gamma_2$-$\beta_3^{S254F}$-$\alpha_1$-$\beta_3^{S254F}$-$\alpha_1$ respectively) (Figure 6E,F, Table 1). The differences in the $EC_{50}$ value at receptors with a single $\beta_3^{S254F}$ mutation at different subunit locations suggests that these locations are not equivalent in the activation pathway.

**Discussion**

Recent advances in whole-genome sequencing have enabled the identification of a large number of *de novo* mutations that cause a range of severe childhood epilepsies. In all of these cases, the mutations are dominant (2-16), whereby patients will contain one wild-type and one mutant copy of the gene. In cases where the mutations are in the $\beta_3$ subunit of the GABA$_A$ receptor, this will lead to several potential receptors being expressed in each subtype, with hetero- and homo-mutant receptors containing single mutations at either of the two $\beta_3$ subunits and a homo-mutant receptor containing mutations at both $\beta_3$ subunits. Precise functional genomic analysis requires the understanding of how each of these individual receptors are affected by the mutation, as these receptors could be expressed and contribute to the pathology of the disorder, or even be targeted by GABAergic drugs to treat the seizures.

The *in vitro* analysis of these mutations has, to date, relied solely on injection or transfection of wild-type and/or mutant subunits in heterologous systems, and quantification of receptor expression levels complimented with whole-cell recording and, at times, single-channel analysis (8,11,15). However, this approach is inadequate to describe the entire molecular phenotype, as mixed populations of receptors with one or two mutations will form at a ratio of 2:1. This will be particularly problematic when the maximal efficacy is reduced by the mutation, as the higher efficacy of the wild-type receptor will dominate the signal in whole-cell recordings. Therefore, by using a concatenated receptor construct, we have derived results from hetero-mutant $\beta_3$ receptors that provide significant insights into the molecular phenotypes of epilepsies caused by GABA$_A$ receptor mutations, as well as knowledge about the activation mechanisms of these receptors.

**Mutations Impair Synaptic Transmission Through Efficacy or Potency of GABA Activation**

There is an enormous amount of understanding of how GABA$_A$R opening is triggered through an activation pathway initiated by GABA binding that then opens the intrinsic ion channel to mediate neuronal inhibition (22). Briefly, the agonist binds at the interface between a $\beta_3$ and an $\alpha_1$ subunit at the extracellular domain causing a series of conformational changes within the receptor that lead to the transmembrane domains. At the interface of the extracellular and transmembrane domains, a set of interacting loops
including the β1-β2, β6-β7 and β8-β9 in the extracellular domain, and pre-M1 and M2-M3 loops connecting the transmembrane domain, alter their conformation during receptor activation (33,34). This leads to a tilting of the M2 helices and ultimately channel opening. Additionally, the number of molecules bound are important, where two molecules of agonist are required to be bound to fully activate the receptor (35).

Synaptic receptors that contain a γ2-subunit typically have distinct intrinsic activation properties from extrasynaptic receptors that contain a δ-subunit, including a high efficacy where the maximal open probability elicited by GABA approaches one and lower potency (31,36). The maximal efficacy of an agonist, or whether the agonist is partial or full, is largely defined by transitional conformational states, known as pre-activated or “flip” states, that precede the final conformational change that opens the channel gate (37). Residues at the earlier stage of the activation pathway (11) appear to have no influence on the maximal efficacy of GABA, with β3D120N and β3T157 single mutations having a similar efficacy to the wild-type. At these mutations, located near the ligand-binding domain and within an extracellular structural β-sheet, respectively, GABA remains acting as essentially a “full” agonist, activating the receptor with a very high maximal open probability. However, the β3Y302C mutations in the M2-M3 loop change the intrinsic property of receptor activation such that the maximal efficacy of GABA is significantly reduced. Essentially, GABA has become a “partial” agonist at these receptors, where the mutation in the coupling region may be destabilizing transitional conformational states. Further, the efficacy of GABA was different depending on the subunit location of the β3Y302C mutation, suggesting that the two mutations are not entirely equivalent.

### Asymmetrical Effects of the Same Mutation at Different Subunit Locations

A notable feature of these mutations is the differential effect of the β3S254F mutation when located at different subunit locations, and slightly different levels of reductions in the efficacy of GABA at receptors with a β3Y302C mutation in different locations. This is in contrast with the β3D120N and β3T157M mutations where the location of the individual mutations had no significant effect. It is known that the pseudosymmetry of the pentameric receptor can cause positional effects of mutations (38) and there are two possible reasons for these differences. First, the local environment surrounding mutated residues may be identical at the two subunit locations for some mutations but not others. Second, the conformational changes during the activation process may not be symmetrical from ligand binding to channel opening. Furthermore, in epilepsy-causing mutations that impair surface expression, the location of the mutation can also determine the severity of the effect (39).

Recent advances in cryogenic electron microscopy (Cryo-EM) has enabled the solving of many membrane-bound proteins to a very fine resolution. During the preparation of this manuscript, a Cryo-EM structure of the α1β3γ2 receptor with and without GABA and diazepam bound, and a structure of the α1β1γ2 receptor was published (40,41). The sequences between the β2 and β3 subunits are highly homologous and identical within the regions containing the mutations studied. Hence, we can utilize the Cryo-EM structure to explain the positional effects, or lack thereof, of our mutations.

Within the GABA and diazepam-bound α1β3γ2 structure, the β3120 residue was located within the general vicinity of the ligand-binding site at the interface of the β3 and α1 subunits (Figure 7A) (11,41). The Cryo-EM structure indicated that the local environment surrounding the β3120 residues is identical, making contacts with the adjacent α1M141 and α1P142 residues regardless of the subunit the mutation is in (Figure 7A). Similarly, the β3T157 residue is located within a β-sheet within the interior of the β3 subunit where the amino acid sidechains interact entirely with residues within the β3 subunit, and the local environment that surrounds the amino acid residues is identical (Figure 7B). Therefore, the similarity of the functional changes caused by mutations at these residues suggests that both the local environments are identical and that the conformational changes at these initial stages of the activation pathway are symmetrical.

The β3Y302C residue is located in the M2-M3 loop, a key motif in the coupling of ligand binding to channel gating that moves considerably during the channel activation process. Although there are differences in the activation processes of single
β3Y302C mutant receptors depending on the subunit that the mutation is located in, the local environment surrounding the β3Y302 residue is similar at the two subunits (Figure 7C). There are slightly different poses for the β3Y302 residue in each of the different locations, but it is known that the M2-M3 region alters conformation during the gating process (42), and it may be differences in the conformational changes that cause subtle differences in the effect of the mutation when it is present at the different locations.

The Cryo-EM structure of the α1β1γ2 GABA_A receptor identified asymmetry within the transmembrane regions of the receptor (40). While the distance between subunits was the same, the angles in the pentamer substantially differed along with the tilt of the transmembrane helices when compared to the β3 subunit. This asymmetry of key secondary structures may contribute to the β3Y302C mutation having different effects when introduced in different regions within the transmembrane regions of GABA_A receptors, where the transitional or pre-activated states are subtly different at the coupling regions depending on where the first GABA molecule is bound.

The β3S254F mutation caused markedly different effects depending on the subunit the mutation was introduced. The β3S254 residue is located deep within the transmembrane region of the M1 helix, where it can interact with other transmembrane helices including the M2 helix of the adjacent subunit.

The M1 helix of one β3 subunit is adjacent to the M3 of an α1 subunit, while the M1 helix of the second β3 subunit is adjacent to the M3 of the γ2 subunit. This difference may underlie the different functional changes when the mutation is introduced at different locations. The sidechains of the β3S254 residues themselves make intra-subunit interactions within the β3 subunit, and the introduction of the phenylalanine residue is unlikely to make different sidechain interactions when introduced at the two locations (Figure 8). However, the bulky sidechain is likely to cause structural rearrangements when occupying a larger volume, where the backbone of the M1 helix backs onto the M3 helix of either the γ2-or α1-subunit. At a critical part of the M3 helix, the two subunits have different amino acid sequences, and the M3 helices are in markedly different conformations in different Cryo-EM structures.

The M3 helix of the α1 subunit is parallel to the M1 helix of the β3 subunit in the apo and GABA and diazepam-bound α1β3γ2 structures (Figure 8A,B). The M3 helix of the γ2 subunit is similarly parallel in the GABA and diazepam-bound α1β3γ2 structure, but in the apo-structure the M3 helix of the γ2 subunit is tilted, angling away from the M1 helix of the β3 subunit at the extracellular end. Further, the γ2F343 residue has an altered sidechain conformation. A similar change in the M3 conformation of the M3 helix is also seen in the α1β3γ2 Cryo-EM structure (40)(Figure 8C). A plausible reason for the different activation properties of the two single β3S254F mutant receptors is that when the residue is mutated adjacent to the γ2-subunit, the subsequent rearrangements introduce twisting or tilting of the α-helices to favour a closed conformation, but when adjacent to an α1-subunit the interactions stabilize an intermediate or open state.

**Molecular Phenotype of the Mutations**

There is a wealth of information that suggests impairment of GABA_A-receptor mediated inhibition can lead to seizures. These include pharmacological evidence where antagonists of the GABA_A receptor such as bicuculline induce seizures (43), and genetic evidence where mutations in GABA_A receptor subunits are known to reduce receptor translocation to the cell surface (44) or impair the activation properties of the receptor (8,11,13,15). In this study, we focussed on five distinct mutations, four of which clearly impaired the activation by GABA when only a single copy of the mutation is present. Our approach using the concatenated receptor enabled us to assess the complexity of receptors that contain mutations in one, or both β3 subunits.

For three of the mutations, the resulting receptors followed a predictable pattern. A single copy of the mutation, introduced at either β3 subunit, caused a substantial impairment of the activation of the receptor by GABA. When a second mutation is introduced to the receptor, there is a catastrophic change to the receptor such that either the magnitude of currents are too low to be measured or markedly reduced. Regardless of whether the expression of these receptors is affected by the mutation, we would expect synaptic transmission of GABA-elicited currents to be impaired in the inhibitory pathways within these.
patients, with receptors containing single mutations mediating reduced neurotransmission, and receptors with two copies of the mutation mediating little, if any, chloride currents.

The effects of the β3S254F mutation were very different, having markedly different shifts in the potency of GABA depending on the location of the mutation. Notably, mutations in the proline residue that precedes the same serine in the β1 subunit also causes the potency of GABA to increase, but the same mutation in an α1 subunit failed to assemble in HEK293 cells (45). Although we found robust activation of the receptors in our oocyte expression system, it is possible that the mutations also impair assembly or trafficking to the cell surface in the mammalian cell. We also cannot rule out that the mutant subunit preferentially arranges itself in the location where the potency of GABA is reduced, leading to impaired GABA activation. Importantly, we have only considered the effect of the mutation on the synaptic α1β3γ2, and the effect of the mutation in other GABA receptor subtypes, including α5β3γ2 or α4β3δ receptors, may contribute to the overall phenotype, including seizures. Indeed, all β3 mutations would be expressed in these subtypes and as such, the effects of the β3D120N, β3T157M and β3T302C on extrasynaptic receptors also needs to be considered, particularly when the β3T157M mutation has been reported to have little effect when expressed in the α5β3γ2 subtype (8). It is also possible that the β3S254F mutation is not truly pathogenic in itself, however this mutation has been subsequently identified de novo in another patient and is therefore very unlikely not to be pathogenic (46).

Conclusions and Future Directions

Genetic epileptic encephalopathies are a devastating group of severe childhood epilepsies that are often resistant to pharmacological treatment and include patients with mutations in genes that encode for the GABA_A receptor. Introducing mutations to concatenated receptors demonstrate that the number of mutations within the receptor matter. Typically, the incorporation of one mutation impairs the activation properties of the receptor, reducing the GABA potency, efficacy or both and the incorporation of two mutations is often catastrophic to receptor function. However, the mutations are complex, where individual mutations can also increase the potency of GABA and the subunit location of the mutation can also determine the functional change in the mutation. The resultant molecular phenotype is likely a complex mixture of receptors with a mix of wild-type receptors, receptors containing a single mutation and receptors containing two mutations. Furthermore, receptors containing one mutation that have an intermediate effect on the activation process may be a useful target for GABAergic drugs to confer the most benefit for their specific mutation.

Experimental Procedures

**Molecular Biology.**

Human cDNA for monomeric α1, β3 and γ2 GABA_A subunits were kind gifts from Saniona A/S, Copenhagen, Denmark. The γ2, β3, and α1 subunits were initially subcloned into five separate in-house vectors. Linker sequences were then added through standard PCR reactions where the antisense oligonucleotides caused deletion of the stop codon and in-frame fusion to the AGS linker sequence, and the sense β3 or α1 oligonucleotides caused omission of the respective β3 or α1 signal peptide and in-frame fusion to the AGS linker sequence. The remaining sequence of the sense and antisense oligonucleotides were designed to match the respective wild-type sequences and included unique restriction sites within each linker region and at the beginning and end of each gene sequence. Standard PCR reactions with the γ2, β3 or α1 sequences as template were performed using Q5 polymerase (Genesearch, Gold Coast Australia) and PCR products were cloned into in-house vectors using restriction enzyme digestion and ligation. Correct introduction of linker sequences and fidelity of all coding sequences were verified by double stranded sequencing. The γ2-β3-α1-β3-α1 concatenated construct was then created by a restriction enzyme digest of the five vectors and ligation of the five subunits with linker sequences and subcloned into an in-house vector. The resulting construct contained the subunits with linker sequences in the order of γ2-(AGS)5-β3-(AGS)5-LGS(AGS)5-α1-(AGT(AGS)5-β3-(AGS)5-LATG(AGS)5)-α1. The vector was transformed into E. coli 10-beta bacteria for plasmid amplification and purifications were performed with standard kits (QIAGEN, Chadstone, Australia). Mutations were made using the QuikChange II Site Directed Mutagenesis Kit (Agilent Technologies, Mulgrave, Australia) in

**Experimental Procedures**
vectors containing single subunits and flanking linker sequences and then confirmed through double stranded DNA sequencing. A restriction digest was then performed on subunit DNA containing the mutation and the concatenated construct to remove the appropriate wild-type subunit, and then ligated to introduce the mutant subunit. DNA gel electrophoresis was performed to ensure incorporation of the five subunits. cRNA was produced from linearised cDNA using the mMessage mMachine T7 Transcription kit (Thermo Fisher, Scoresby, Australia) according to manufacturers description and stored at -80 °C until use.

**Xenopus surgery and Oocyte Preparation**

All procedures using *Xenopus laevis* frogs were approved by the animal ethics committee of The University of Sydney (AEC No. 2013/5269) and are in accordance to the National Health and Medical Research Council (NHMRC) of Australia code for the care and use of animals. In brief, a section of ovarian lobe from *Xenopus laevis* was surgically removed while the frog was under anaesthesia induced by tricaine, cut into smaller portions and digested with 35 mg of collagenase-A diluted in 15 ml OR2 (in mM: NaCl, 82.5; HEPES, 5; MgCl₂, 2; and KCl, 2; pH 7.4) at 18 °C for ~1 hour until the oocytes were fully detached from the follicles and the ovary tissue. Oocytes were then injected with a total of 2 ng of cRNA per cell that encoded concatenated wild-type or mutant receptors and were incubated for 2–4 days on an oscillator at 18 °C in ND96 solution (in mM: NaCl, 96; KCl, 2; MgCl₂, 1; CaCl₂, 1.8; HEPES, 5; pH 7.4) supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 µg/mL gentamycin.

**Two-Electrode Voltage-Clamp Recording**

Cell currents were recorded using the two-electrode voltage-clamp method as previously described (47). Briefly, oocytes were continuously superfused at room temperature with ND96 at approximately 5 mL/min. Cells were impaled with microelectrodes fashioned from capillary glass (Harvard Apparatus, Holliston, USA) that were prepared with a micropipette puller (Narishige, Tokyo, Japan) and filled with 3 M KCl (0.3-2.0 MΩ), and then voltage clamped at -60 mV. A semi-automated three-channel oocyte recording system was used where the application of solution was controlled through programming of a Powerlab 8/36 data acquisition system (ADI Instruments, Sydney Australia) that switched solutions through a VC-8 eight-channel perfusion system (Warner Instrument Corp., Hamden, USA) and then applied solution to three recording chambers. Currents were recording 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 clobazam concentration-response curves, a 10 µM concentration was applied as a reference and the responses were normalized to the mean current of the second two GABA concentrations. For all other experiments, a 3 mM concentration of GABA was applied as a reference three times during the experiment and for concentration-response curves peak currents were normalised to the mean current of the second two GABA applications. When estimating the maximal Po, after three consecutive applications of the reference 3 mM GABA solution, the solution containing 10 mM GABA, 1 µM diazepam and 3 µM etomidate was applied and peak currents were normalized to the mean current of the second two GABA applications. A washout period of 10-12 minutes was performed between GABA applications to prevent effects from desensitization. All experiments were performed over a minimum of two different batches of oocytes and a minimum of 10 individual experiments were performed. The order that GABA applications were applied are in the identical sequence as to those shown in the representative data figures. Data was acquired at 1 kHz and, for the purposes of displaying representative traces, the data was converted to 10 Hz offline through Microsoft excel.

**Data Analysis and Statistics**

Concentration–response curves were fitted using GraphPad Prism 7 to a monophasic Hill equation of the form:

\[ I = I_{max} \frac{[A]^{n_H}}{EC_{50}^{n_H}} \]

Where \( I_{max} \) is the maximum current, \( EC_{50} \) is the concentration that produces the half-maximum response, \([A]\) is the concentration of ligand 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 concentration-response curves. The EC\textsubscript{50} shown is from the fitting of Hill equations to all data, while the logEC\textsubscript{50}, \(I_{\text{max}}\) and \(n_H\) values are the mean and standard error derived from fitting curves to individual experiments.

For clobazam concentration response curves, the percent modulation of clobazam was derived by the equation:

\[
\text{Percent Modulation} = 100 \times \frac{I_{\text{Clobazam}} - I_{10 \mu M \text{GABA}}}{I_{10 \mu M \text{GABA}}}
\]

This data was then fitted to the Hill equation, as above, to determine the parameters of the concentration-response curves. The Estimated \(P_{Omax}\) for individual experiments was derived by the equation:

\[
\text{Est } P_{Omax} = \text{Correction Factor} \times \frac{I_{3 \text{mM GABA}}}{I_{10 \text{mM GABA} \text{ 1} \mu \text{M Diazepam 3} \mu \text{M Etomidate}}}
\]

The Correction Factor was determined for each mutation to correct for the fact that 3 mM GABA did not always elicit the maximum response to GABA. This was derived from rearranging the Hill equation:

\[
\text{Correction Factor} = 1 + \frac{0.003^{n_H}}{\text{EC}_{50}^{n_H}}
\]

Where the EC\textsubscript{50} was in M.

When comparing the wild-type and mutation concentration-response curves, all data was transformed to the Est \(P_{Omax}\).

For statistical analysis, Est \(P_{Omax}\) 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.
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References

1. Howard, M. A., and Baraban, S. C. (2017) Catastrophic Epilepsies of Childhood. Annu Rev Neurosci 40, 149-166
2. Cossette, P., Liu, L., Brisebois, K., Dong, H., Lortie, A., Vanasse, M., Saint-Hilaire, J. M., Carmant, L., Verner, A., Lu, W. Y., Wang, Y. T., and Rouleau, G. A. (2002) Mutation of GABRA1 in an autosomal dominant form of juvenile myoclonic epilepsy. Nat Genet 31, 184-189
3. Gontika, M. P., Konialis, C., Pangalos, C., and Papavasiliou. A. (2017) Novel SCN1A and GABRA1 Gene Mutations With Diverse Phenotypic Features and the Question on the Existence of a Broader Spectrum of Dravet Syndrome. Child Neurol Open 4, 2329048X17706794
4. Hernandez, C. C., Klassen, T. L., Jackson, L. G., Gurba, K., Hu, N., Noebels, J. L., and Macdonald, R. L. (2016) Deleterious Rare Variants Reveal Risk for Loss of GABAA Receptor Function in Patients with Genetic Epilepsy and in the General Population. PLoS One 11, e0162883
5. Johannesen, K., Marini, C., Pfieffer, S., Moller, R. S., Dorn, T., Ntirud, C. E., Gardella, E., Weber, Y., Sondergard, M., Hjalgrim, H., Nikanorova, M., Becker, F., Larsen, L. H., Dahl, H. A., Maier, O., Mei, D., Biskup, S., Klein, K. M., Reif, P. S., Rosenow, F., Elias, A. F., Hudson, C., Helbig, K. L., Schubert-Bast, S., Scordo, M. R., Craiu, D., Djemie, T., Hoffman-Zacharska, D., Caglayan, H., Helbig, I., Serratos, J., Striano, P., De Jonghe, P., Weckhuysen, S., Suls, A., Muru, K., Talvik, I., Talvik, T., Muhle, H., Borggraefe, I., Rost, I., Guerrini, R., Lerche, H., Lemke, J. R., Rubboli, G., and Maljevic, S. (2016) Phenotypic spectrum of GABRA1: From generalized epilepsies to severe epileptic encephalopathies. Neurology 87, 1140-1151
6. Kodera, H., Ohba, C., Kato, M., Maeda, T., Araki, K., Tajima, D., Matsuo, M., Hino-Fukuyo, N., Kohashi, K., Ishiyama, A., Takeshiba, S., Motoi, H., Kikuchi, A., Tsurusaki, Y., Nakashima, M., Miyake, N., Sasaki, M., Kure, S., Sasaki, M., Kure, S., Hagiyoua, K., Kato, M., Maeda, T., Araki, K., Tajima, D., Matsuo, M., and Hino-Fukuyo, N. (2016) De novo GABRA1 mutations in Ohtahara and West syndromes. Epilepsia 57, 566-573
7. Lachance-Touchette, P., Brown, P., Meloche, C., Kinirons, P., Lapointe, L., Lacasse, H., Lortie, A., Carmant, L., Bedford, F., Bowie, D., and Cossette, P. (2011) Novel alpha1 and gamma2 GABAA receptor subunit mutations in families with idiopathic generalized epilepsy. Eur J Neurosci 34, 237-249
8. Moller, R. S., Larsen, L. H., Johannesen, K. M., Talvik, I., Talvik, T., Vaeruer, U., Miranda, M. J., Farooq, M., Nielsen, J. E., Svendsen, L. L., Kjellgaard, D. B., Linnet, K. M., Hao, Q., Uldall, P., Frangoul, M., Tommerup, N., Baig, S. M., Abdulla, U., Born, A. P., Gellert, P., Nikanorova, M., Olofsson, K., Jepsen, B., Marjanovic, D., Al-Zehhawi, L. I., Penalva, S. J., Krag-Olsen, B., Brusgaard, K., Hjalgrim, H., Rubboli, G., Pal, D. K., and Dahl, H. A. (2016) Gene Panel Testing in Epileptic Encephalopathies and Familial Epilepsies. Mol Syndromol 7, 210-219
9. Stossor, M. B., Lindy, A. S., Butler, E., Retterer, K., Piccirillo-Stossor, C. M., Richard, G., and McKnight, D. A. (2018) High frequency of mosaic pathogenic variants in genes causing epilepsy-related neurodevelopmental disorders. Genet Med 20, 403-410
10. Epi, K. C., Epilepsy Phenome/Genome, P., Allen, A. S., Berkovic, S. F., Cossette, P., Delanty, N., Dlugos, D., Eichler, E. E., Epstein, M. P., Glauser, T., Goldstein, D. B., Han, Y., Heinezen, E. L., Hitomi, Y., Howell, K. B., Johnson, M. R., Kuzniecky, R., Lowenstein, D. H., Liu, Y. F., Madou, M. R., Marson, A. G., Mefford, H. C., Esmaeeli Nieh, S., O'Brien, T. J., Ottman, R., Petrovski, S., Poduri, A., Ruzzo, E. K., Scheffer, I. E., Sherr, E. H., Yuskaitis, C. J., Abou-Khalil, B., Alldredge, B. K., Bautista, J. F., Berkovic, S. F., Boro, A., Cascino, G. D., Consalvo, D., Crumrine, P., Devinsky, O., Dlugos, D., Epstein, M. P., Fiol, M., Fountain, N. B., French, J., Friedman, D., Geller, E. B., Glauser, T., Glynn, S., Haut, S. R., Hayward, J., Helmers, S. L., Joshi, S., Kanner, A., Kirsch, H. E., Knowlton, R. C., Kossoff, E. H., Kuperman, R., Kuzniecky, R., Lowenstein, D. H., Mcguire, S. M., Motika, P. V., Novotny, E. J., Ottman, R., Paolicchi, J. M., Parent, J. M., Park, K., Poduri, A., Scheffer, I. E., Shellhaas, R. A., Sherr, E. H., Shih, J. J., Singh, R., Sirven, J., Smith, M. C., Sullivan, J., Lin Thio, L., Venkat, A., Vining, E. P., Von Allmen, G. K., Weisenberg, J. L., Widdess-Walsh, P., and Winawer, M. R. (2013) De novo mutations in epileptic encephalopathies. Nature 501, 217-221
11. Janve, V. S., Hernandez, C. C., Verdier, K. M., Hu, N., and Macdonald, R. L. (2016) Epileptic encephalopathy de novo GABRB mutations impair GABA\(_A\) receptor function. *Ann Neurol* **79**, 806-825
12. Papandreou, A., McTague, A., Trump, N., Ambegaonkar, G., Ngoh, A., Meyer, E., Scott, R. H., and Kurian, M. A. (2016) GABRB3 mutations: a new and emerging cause of early infantile epileptic encephalopathy. *Dev Med Child Neurol* **58**, 416-420
13. Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J. F., Baulac, M., Brice, A., Bruzzone, R., and LeGuern, E. (2001) First genetic evidence of GABA\(_A\) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. *Nat Genet* **28**, 46-48
14. Boillot, M., Morin-Brureau, M., Picard, F., Weckhuysen, S., Lambrecq, V., Minetti, C., Striano, P., Zara, F., Iacomino, M., Ishida, S., An-Gourfinkel, I., Daniau, M., Hardies, K., Baulac, M., Dulac, O., Leguern, E., Nabbout, R., and Baulac, S. (2015) Novel GABRG2 mutations cause familial febrile seizures. *Neurol Genet* **1**, e35
15. Shen, D., Hernandez, C. C., Shen, W., Hu, N., Poduri, A., Shiedley, B., Rotenberg, A., Datta, A. N., Leiz, S., Patzer, S., Boor, R., Ramsey, K., Goldberg, E., Helbig, I., Ortiz-Gonzalez, X. R., Lemke, J. R., Marsh, E. D., and Macdonald, R. L. (2017) De novo GABRG2 mutations associated with epileptic encephalopathies. *Brain* **140**, 49-67
16. Wallace, R. H., Marini, C., Petrou, S., Harkin, L. A., Bowser, D. N., Panchal, R. G., Williams, D. A., Sutherland, G. R., Mulley, J. C., Scheffer, I. E., and Berkovic, S. F. (2001) Mutant GABA\(_A\) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. *Nat Genet* **28**, 49-52
17. Khazipov, R. (2016) GABAergic Synchronization in Epilepsy. *Cold Spring Harb Perspect Med* **6**, a022764
18. Miller, P. S., and Aricescu, A. R. (2014) Crystal structure of a human GABAA receptor. *Nature* **512**, 270-275
19. Olsen, R. W. (2018) GABAA receptor: Positive and negative allosteric modulators. *Neuropharmacology* **136**(PtA), 10-22
20. Hevers, W., and Luddens, H. (1998) The diversity of GABAA receptors. Pharmacological and electrophysiological properties of GABAA channel subtypes. *Mol Neurobiol* **18**, 35-86
21. Farrant, M., and Nusser, Z. (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA\(_A\) receptors. *Nat Rev Neurosci* **6**, 215-229
22. Bouzat, C. (2012) New insights into the structural bases of activation of Cys-loop receptors. *J Physiol Paris* **106**, 23-33
23. Ahring, P. K., Liao, V. W. Y., and Balle, T. (2018) Concatenated nicotinic acetylcholine receptors: A gift or a curse? *J Gen Physiol* **150**, 453
24. Baumann, S. W., Baur, R., and Sigel, E. (2002) Forced subunit assembly in alpha1beta2gamma2 GABAA receptors. Insight into the absolute arrangement. *J Biol Chem* **277**, 46020-46025
25. Sigel, E., Kaur, K. H., Luscher, B. P., and Baur, R. (2009) Use of concatamers to study GABAA receptor architecture and function: application to delta-subunit-containing receptors and possible pitfalls. *Biochem Soc Trans* **37**, 1338-1342
26. Baur, R., Minier, F., and Sigel, E. (2006) A GABA\(_A\) receptor of defined subunit composition and positioning: concatenation of five subunits. *FEBS Lett* **580**, 1616-1620
27. Ramerstorfer, J., Furtmuller, R., Sarto-Jackson, I., Varagic, Z., Sieghart, W., and Ernst, M. (2011) The GABAA receptor alpha+beta- interface: a novel target for subtype selective drugs. *J Neurosci* **31**, 870-877
28. Che Has, A. T., Absalom, N., van Nieuwenhuijzen, P. S., Clarkson, A. N., Ahring, P. K., and Chebib, M. (2016) Zolpidem is a potent stoichiometry-selective modulator of alpha1beta3 GABAA receptors: evidence of a novel benzodiazepine site in the alpha1-alpha1 interface. *Sci Rep* **6**, 28674
29. Hammer, H., Ebert, B., Jensen, H. S., and Jensen, A. A. (2015) Functional characterization of the 1,5-benzodiazepine clobazam and its major active metabolite N-desmethylclobazam at human GABA\(_A\) receptors expressed in Xenopus laevis oocytes. *PLoS One* **10**, e0120239
30. Shin, D. J., Germann, A. L., Johnson, A. D., Forman, S. A., Steinbach, J. H., and Akk, G. (2018) Propofol Is an Allosteric Agonist with Multiple Binding Sites on Concatemeric Ternary GABAA Receptors. Mol Pharmacol 93, 178-189
31. Fisher, J. L., and Macdonald, R. L. (1997) Single channel properties of recombinant GABAA receptors containing gamma 2 or delta subtypes expressed with alpha 1 and beta 3 subtypes in mouse L929 cells. J Physiol 505 (Pt 2), 283-297
32. Purohit, P., Gupta, S., Jaday, S., and Auerbach, A. (2013) Functional anatomy of an allosteric protein. Nat Commun 4, 2984
33. Kash, T. L., Dizon, M. J., Trudell, J. R., and Harrison, N. L. (2004) Charged residues in the beta2 subunit involved in GABAA receptor activation. J Biol Chem 279, 4887-4893
34. Kash, T. L., Jenkins, A., Kelley, J. C., Trudell, J. R., and Harrison, N. L. (2003) Coupling of agonist binding to channel gating in the GABA(A) receptor. Nature 421, 272-275
35. Sigel, E., and Steinmann, M. E. (2012) Structure, function, and modulation of GABA(A) receptors. J Biol Chem 287, 40224-40231
36. Ahring, P. K., Bang, L. H., Jensen, M. L., Strobaek, D., Hartiadi, L. Y., Chebib, M., and Absalom, N. (2016) A pharmacological assessment of agonists and modulators at alpha4beta2gamma2 and alpha4beta2delta GABAA receptors: The challenge in comparing apples with oranges. Pharmacol Res 111, 563-576
37. Lape, R., Colquhoun, D., and Sivilotti, L. G. (2008) On the nature of partial agonism in the nicotinic receptor superfamily. Nature 454, 722-727
38. Sigel, E., Baur, R., Boulineau, N., and Minier, F. (2006) Impact of subunit positioning on GABAA receptor function. Biochem Soc Trans 34, 868-871
39. Gallagher, M. J., Song, L., Arain, F., and Macdonald, R. L. (2004) The juvenile myoclonic epilepsy GABA(A) receptor alpha1 subunit mutation A322D produces asymmetrical, subunit position-dependent reduction of heterozygous receptor currents and alpha1 subunit protein expression. J Neurosci 24, 5570-5578
40. Phulera, S., Zhu, H., Yu, J., Claxton, D. P., Yoder, N., Yoshioka, C., and Gouaux, E. (2018) Cryo-EM structure of the benzodiazepine-sensitive a1β1γ2S tri-heteromeric GABAA receptor in complex with GABA. eLife 7, 39383
41. Laverty, D., Desai, R., Uchanski, T., Masiulis, S., Stec, W. J., Malinauskas, T., Zivanov, J., Pardon, E., Steyaert, J., Miller, K. W., and Aricescu, A. R. (2019) Cryo-EM structure of the human alpha1beta3gamma2 GABAA receptor in a lipid bilayer. Nature 565, 516-520
42. Absalom, N. L., Lewis, T. M., and Schofield, P. R. (2004) Mechanisms of channel gating of the ligand-gated ion channel superfamily inferred from protein structure. Exp Physiol 89, 145-153
43. Wood, J. D. (1975) The role of gamma-aminobutyric acid in the mechanism of seizures. Prog Neurobiol 5, 77-95
44. Kang, J. Q., Shen, W., Zhou, C., Xu, D., and Macdonald, R. L. (2015) The human epilepsy mutation GABRG2(Q390X) causes chronic subunit accumulation and neurodegeneration. Nat Neurosci 18, 988-996
45. Greenfield, L. J., Jr., Zaman, S. H., Sutherland, M. L., Lummis, S. C., Niemeyer, M. I., Barnard, E. A., and Macdonald, R. L. (2002) Mutation of the GABAA receptor M1 transmembrane proline increases GABA affinity and reduces barbiturate enhancement. Neuropharmacology 42, 502-521
46. Liu, J., Tong, L., Song, S., Niu, Y., Li, J., Wu, X., Zhang, J., Zai, C. C., Luo, F., Wu, J., Li, H., Wong, A. H. C., Sun, R., Liu, F., and Li, B. (2018) Novel and de novo mutations in pediatric refractory epilepsy. Mol Brain 11, 48
47. Chua, H. C., Absalom, N. L., Hanrahan, J. R., Viswas, R., and Chebib, M. (2015) The Direct Actions of GABA, 2'-Methoxy-6-Methylflavone and General Anaesthetics at beta3gamma2L GABAA Receptors: Evidence for Receptors with Different Subunit Stoichiometries. PLoS One 10, e0141359
### Table 1. Concentration-Response curves of α1β3γ2 receptors

| Construct | EC$_{50}$ μM (log EC$_{50}$ ± SEM) | $I_{\text{mM}, \text{GABA}}$ (nA ± SEM) | $n$ | Est Po$_{\text{max}}$ (± SEM) | $n$ |
|-----------|-----------------------------------|-------------------------------|-----|------------------------------|-----|
| γ2-β3-α1-β3-α1 | 69.0 (-4.12±0.06) | 2095±126 | 13 | 0.95±0.04 | 10 |
| γ2$^{R32Q}$-β3-α1-β3-α1 | 315 (-3.42±0.04)** | 1395±214 | 10 | 0.85±0.03 | 10 |
| γ2-β3$^{D120N}$-α1-β3-α1 | 1144 (-2.77±0.05)** | 701±92*** | 10 | 1.12±0.07 | 10 |
| γ2-β3$^{T157M}$-α1-β3-α1 | 422 (-3.37±0.05)** | 1146±210† | 10 | 0.90±0.05 | 10 |
| γ2-β3$^{S254F}$-α1-β3-α1 | 181 (-3.68±0.04)** | 1230±134* | 10 | 1.00±0.04 | 10 |
| γ2-β3$^{Y302C}$-α1-β3-α1 | 164 (-3.71±0.05)** | 1826±165 | 10 | 0.40±0.06 | 10 |
| γ2-β3-α1-β3$^{D120N}$-α1 | 1473 (-2.87±0.11)** | 969±158*** | 10 | 1.00±0.05 | 10 |
| γ2-β3-α1-β3$^{T157M}$-α1 | 279 (-3.53±0.06)** | 1995±256 | 10 | 1.00±0.00 | 10 |
| γ2-β3-α1-β3$^{S254F}$-α1 | 34.2 (-4.47±0.07)** | 2480±248 | 10 | 0.95±0.03 | 10 |
| γ2-β3-α1-β3$^{Y302C}$-α1 | 471 (-3.35±0.09)** | 1336±156* | 10 | 0.74±0.05 | 10 |
| γ2-β3$^{D120N}$-α1-β3$^{D120N}$-α1 | ND† | ND† | ND† | ND† | ND† |
| γ2-β3$^{T157M}$-α1-β3$^{T157M}$-α1 | ND† | ND† | ND† | ND† | ND† |
| γ2-β3$^{S254F}$-α1-β3$^{S254F}$-α1 | 29.6 (-4.52±0.05)** | 1528±210 | 10 | 0.89±0.03 | 10 |
| γ2-β3$^{Y302C}$-α1-β3$^{Y302C}$-α1 | 6806 (-2.16±0.08)** | 77±15*** | 10 | 0.24±0.04 | 10 |

*p<0.05, **p<0.01, ***p<0.001, One-way ANOVA with Tukey’s post-hoc test
†Currents too low to determine concentration-response curve
Figure 1. (A) Pentameric structure of the GABA and diazepam-bound α1β3γ2 receptor (pdb 6hup) from above showing the orientation of the subunits. The subunits are coloured with respect to their order in the pentamer, 1st γ2 (green), 2nd β3 (maroon), 3rd α1 (blue), 4th β3 (red) and 5th α1 (dark blue). (B) Side-view showing the 1st γ2-subunit adjacent to the 2nd β3 subunit. The γ2R323 residue on the M2-M3 loop is depicted with the sidechain in black in a transparent sphere. (C) Side-view showing the 4th β3-subunit adjacent to the 5th α1 subunit, with the GABA-binding site highlighted. The sidechains of the β3D120N residue at the GABA-binding site, the β3T157M residue within an internal β-sheet, the β3Y302C residue in the M2-M3 loop and the β3S254F residue in the M1 region are shown in black.
Figure 2. (A) Schematic of coding region of concatenated receptor containing the DNA construct. Linker lengths are 15 amino acids ((AGS)_3), 27 amino acids ((AGS)_3LGS(AGS)_3), 18 amino acids (AGT(AGS)_3) and 27 amino acids ((AGS)_4AGT(AGS)_4). (B) Schematic of the expected arrangement of the concatenated receptor where the subunits arrange in a counter-clockwise orientation. GABA and clobazam binding sites are shown. (C) Representative data (above) from a single two-electrode voltage clamp experiment where different concentrations of GABA (open bars) were applied to construct a concentration-response curve to GABA (below). Filled bars represent reference 3 mM GABA applications, open bars represent GABA applications at concentrations shown. Peak currents were measured and the mean ± s.e.m plotted (open circles) and fitted to the Hill equation (below). (D) Representative data (above) from a single two-electrode voltage clamp experiment constructing a modulation curve to clobazam (below). Three pulses of reference 10 µM GABA (closed bars) were applied prior to co-application of 10 µM GABA and clobazam at concentrations shown (closed bars). Percent modulation of the control GABA response was calculated and the mean ± s.e.m plotted and fitted to the Hill equation (below). The fitted EC_{50} of clobazam was 86 nM (log EC_{50} = -4.03 ± 0.06, mean ± s.e.m, n=10) and the fitted E_{max} was 306 % (320 ± 32, mean ± s.e.m, n=10).
Figure 3. (A) Schematic of concatenated receptor indicating location of mutations when they are introduced into the γ2 or distinct β3 subunits. ● indicates location of mutations on the 1st γ2 subunit and ●, ■ and ● indicate location of mutations on the 2nd, 4th or both 2nd and 4th β3 subunits, respectively. (B) Representative traces of wild-type and γ2 R323Q, β3 D120N, β3 T157M, β3 S254F and β3 Y302C mutant receptors with mutation(s) in the labelled locations after application of reference 3 mM GABA (filled bars). Scale bars represent 500 nA and 100 s. (C) Absolute current elicited by 3 mM GABA after injection of 2 ng RNA. Individual data points are depicted as either open circles or squares with wild-type as black bars and grey circles and identical colour and pattern scheme to Panel A. Bars and error bars represent mean ± s.d of 10-13 individual cells. *p < 0.05, **p < 0.01, ***p < 0.001 compared to wild-type, one-way ANOVA with Tukey’s post-hoc test.
Figure 4. (A) Schematic of concatenated receptor indicating location of $\gamma_{2}^{R323Q}$ mutation (●) within the concatenated construct (left). Representative data (right) from a single two-electrode voltage clamp experiment where different concentrations of GABA (open bars) were applied to construct a concentration-response curve to GABA at $\gamma_{2}^{R323Q}$-$\beta_{3}$-$\alpha_{1}$-$\beta_{3}$-$\alpha_{1}$ receptors. Filled dark red bars and traces represent reference 3 mM GABA applications, open red bars and traces represent GABA applications at concentrations shown. Concentration-response curve to GABA (below) of wild-type $\gamma_{2}$-$\beta_{3}$-$\alpha_{1}$-$\beta_{3}$-$\alpha_{1}$ (○) and $\gamma_{2}^{R323Q}$-$\beta_{3}$-$\alpha_{1}$-$\beta_{3}$-$\alpha_{1}$ (○) receptors normalized to the Est $\mathrm{Po}_{\text{max}}$ and fitted to the Hill equation. Dots represent mean ± s.e.m of 10-13 individual experiments. The EC$_{50}$ of the mutant receptor derived from the curvefit is shown, *p < 0.05, **p < 0.01, ***p < 0.001 compared to wild-type, one-way ANOVA with Tukey’s post-hoc test. (B) Representative traces of wild-type (black) and $\gamma_{2}^{R323Q}$ (red) mutant receptors after application of reference 3 mM GABA (filled bars) and 10 mM GABA, 1 $\mu$M diazepam and 3 $\mu$M etomidate (open bars), respectively. Scale bars represent 500 nA and 100 s and the Est. GABA $\mathrm{Po}_{\text{max}}$ of wild-type (○) and $\gamma_{2}^{R323Q}$ (○) mutant receptors (below) was determined by dividing the current elicited by 3 mM GABA by the current elicited by 10 mM GABA, 1 $\mu$M Diazepam and 3 $\mu$M etomidate and corrected for the reference 3 mM GABA-current. Lines and bars represent mean ± s.d of 10 individual cells.
Figure 5. (A) Schematic of concatenated receptor indicating location of $\beta_3^{D120N}$ and $\beta_3^{T157M}$ mutations introduced within the second (●), fourth (■) or the second and fourth (○) subunits within the resulting pentameric receptor. (B) Concentration-response curve to GABA of wild-type $\gamma_2$-$\beta_3$-$\alpha_1$-$\beta_3$-$\alpha_1$ (○), $\gamma_2$-$\beta_3^{D120N}$-$\alpha_1$-$\beta_3$-$\alpha_1$ (●), $\gamma_2$-$\beta_3^{D120N}$-$\alpha_1$-$\beta_3^{D120N}$-$\alpha_1$ (□) and $\gamma_2$-$\beta_3^{D120N}$-$\alpha_1$-$\beta_3^{D120N}$-$\alpha_1$ (●) receptors and (C) Concentration-response curve to GABA of wild-type $\gamma_2$-$\beta_3$-$\alpha_1$-$\beta_3$-$\alpha_1$ (○), $\gamma_2$-$\beta_3^{T157M}$-$\alpha_1$-$\beta_3$-$\alpha_1$ (○), $\gamma_2$-$\beta_3$-$\alpha_1$-$\beta_3^{T157M}$-$\alpha_1$ (□) and $\gamma_2$-$\beta_3^{T157M}$-$\alpha_1$-$\beta_3^{T157M}$-$\alpha_1$ (●) receptors normalized to the Est $P_{o}$max and fitted to the Hill equation. The EC$_{50}$ of the mutant receptor derived from the curvefit is shown, *p < 0.05, **p < 0.01, ***p < 0.001 compared to wild-type, one-way ANOVA with Tukey’s post-hoc test. (D) Representative traces of $\beta_3^{D120N}$ and $\beta_3^{T157M}$ receptors after application of 3 mM GABA (filled bars) and 10 mM GABA, 1 µM diazepam and 3 µM etomidate (open bars), respectively. Red traces indicate mutation at second subunit location and purple at fourth. Scale bars represent 500 nA and 100 s. (E) Est. GABA $P_{o}$max of wild-type, $\beta_3^{D120N}$ and $\beta_3^{T157M}$ receptors was determined by dividing the current elicited by 3 mM GABA by the current elicited by 10 mM GABA, 1 µM Diazepam and 3 µM etomidate and corrected for the reference 3 mM GABA. ○ indicates wild-type, ○ indicates receptors with a mutation in the second position and □ in the fourth. Lines and bars represent mean ± s.d of 10 individual cells.
Figure 6. (A) Concentration-response curve to GABA of wild-type γ2-β3-α1-β3-α1 (○), γ2-β3<sup>Y302C</sup>-α1-β3-α1 (∙), γ2-β3-α1-β3<sup>Y302C</sup>-α1 (□) and γ2-β3<sup>Y302C</sup>-α1-β3<sup>Y302C</sup>-α1 (●) receptors normalized to the Est Po<sub>max</sub> and fitted to the Hill equation. Dots represent mean ± s.e.m of 10-13 individual experiments. The EC<sub>50</sub> of the mutant receptor derived from the curvefit is shown, *p < 0.05, **p < 0.01, ***p < 0.001 compared to wild-type, one-way ANOVA with Tukey’s post-hoc test. (B) Representative traces of β3<sup>Y302C</sup> mutant receptors after application of reference 3 mM GABA (filled bars) and 10 mM GABA, 1 µM diazepam and 3 µM etomidate (open bars), respectively. Red traces indicate mutation at second subunit location, purple at fourth and blue indicates mutation at both second and fourth locations. Scale bars represent 500 nA and 100 s. (C) Estimated GABA Po<sub>max</sub> of wild-type γ2-β3-α1-β3-α1 (○), γ2-β3<sup>Y302C</sup>-α1-β3-α1 (○), γ2-β3-α1-β3<sup>Y302C</sup>-α1 (□) and γ2-β3<sup>Y302C</sup>-α1-β3<sup>Y302C</sup>-α1 (●) mutant receptors. Est Po<sub>max</sub> was determined by dividing the current elicited by 3 mM GABA by the current elicited by 10 mM GABA, 1 µM Diazepam and 3 µM etomidate and corrected where 3 mM GABA was not at the maximum of the concentration response curves. Lines and bars represent mean ± s.d of 10 individual cells, *p < 0.05, **p < 0.01, ***p < 0.001 compared to wild-type, one-way ANOVA with Tukey’s post-hoc test. (D) Concentration-response curve to GABA of wild-type γ2-β3-α1-β3-α1 (○), γ2-β3<sup>S254F</sup>-α1-β3-α1 (○), γ2-β3-α1-β3<sup>S254F</sup>-α1 (□) and γ2-β3<sup>S254F</sup>-α1-β3<sup>S254F</sup>-α1 (●) receptors normalized to the Est Po<sub>max</sub> and fitted to the Hill equation. Dots represent mean ± s.e.m of 10-13 individual experiments. The EC<sub>50</sub> of the mutant receptor derived from the curvefit is shown, *p < 0.05, **p < 0.01, ***p < 0.001 compared to wild-type, one-way ANOVA with Tukey’s post-hoc test. (E) Representative traces of β3<sup>S254F</sup> mutant receptors after application of reference 3 mM GABA (filled bars) and 10 mM GABA, 1 µM diazepam and 3 µM etomidate (open bars), respectively. Red traces indicate mutation at second subunit location, purple at fourth and blue indicates mutation at both second and fourth locations. Scale bars represent 500 nA and 100 s. (F) Estimated GABA Po<sub>max</sub> of wild-type γ2-β3-α1-β3-α1 (○), γ2-β3<sup>S254F</sup>-α1-β3-α1 (○), γ2-β3-α1-β3<sup>S254F</sup>-α1 (□) and γ2-β3<sup>S254F</sup>-α1-β3<sup>S254F</sup>-α1 (●) mutant receptors. Est Po<sub>max</sub> was determined by dividing the current elicited by 3 mM GABA by the current elicited by 10 mM GABA, 1 µM Diazepam and 3 µM etomidate and corrected where 3 mM GABA was not at the maximum of the concentration response curves. Lines and bars represent mean ± s.d of 10 individual cells, *p < 0.05, **p < 0.01, ***p < 0.001 compared to wild-type, one-way ANOVA with Tukey’s post-hoc test.
Figure 7. (A)-(C) Enlarged view of the α1β3γ2 Cryo-EM structure (pdb 6hup) (41) showing the β3 mutant residues (A) β3D120, (B) β3T157 and (C) β3Y302 in the two different subunit locations of the 2nd β3 subunit (left) and 4th β3 subunit (right). The subunits are coloured with the 1st γ2 subunit in green, the 2nd β3 subunit in maroon, the 3rd α1 subunit in light blue, the 4th β3 subunit in red and the 5th α1 subunit in dark blue and GABA molecule in blue, red and green. Residues from adjacent α1 or γ2 subunits are indicated. At (A) β1D120, (B) β1T157 and (C) β1Y302 residues, the interacting partners are either identical residues on the adjacent subunit or within the β3 subunit itself.
Figure 8. (A)-(C) Enlarged view of the (A) GABA and diazepam-bound α1β3γ2 Cryo-EM structure (pdb 6hup), (B) apo α1β3γ2 (pdb 6i53) and (C) α1β1γ2 (pdb 6dw0) (40,41) showing the β3S254 or β1S254 mutant residues in the two different subunit locations of the 2nd β3 subunit (left) and 4th β3 subunit (right). The subunits are coloured with the 1st γ2 subunit in green, the 2nd β3 subunit in maroon, the 3rd α1 subunit in light blue, the 4th β3 subunit in red and the 5th α1 subunit in dark blue. Residues from adjacent α1 or γ2 subunits are indicated. Although the interacting partners of the S254 residue are within the β subunit, the increased volume of the phenylalanine residue that substitutes for the serine at position 254 will cause the helix to occupy the space closer to the M3 helix of the adjacent subunit. When in the 2nd position in the apo or α1β1γ2 structures, the M3 helix of the γ2 helix is kinked rather than parallel to the M1 helix of the β subunit. The residues of the M1 and M3 that face each other are the β1M253, β1L256 and β1I259 residues for both subunits, the γ2V341, γ2F344, γ2F345 and γ2S348 residues of the 1st subunit (left), and the α1Y321, α1F323 and α1Y325 residues of the 3rd subunit (right). At the locations of all these mutations, the sequence between β1 and β3 is identical.
Functional genomics of epilepsy-associated mutations in the GABA<sub>A</sub> receptor subunits reveal that one mutation impairs function and two are catastrophic
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