eMethods

Clinical ascertainment

Individuals with a presumed pathogenic variant in the *GABRG2* gene, detected through genetic testing for intellectual disability, febrile seizures, epilepsies, or DEEs, were recruited at the Danish Epilepsy Center and through an international network of epilepsy and genetic centers across Europe, the Middle East, America, and Australia. Recruitment was facilitated by the European Reference Network (ERN) ERN-EpiCare and the Network for Therapy in Rare Epilepsies (NETRE). Only previously unreported probands were included to ensure a diverse representation of variants and genetic backgrounds. All data were collected in a structured phenotype table hosted at the Danish Epilepsy Centre. Data are reported in line with the Strengthening Reporting of Observational Studies in Epidemiology (STROBE) statement.

Phenotypic analysis.

Probands and their families underwent detailed phenotyping including interviews, review of their medical history, neuroimaging, and EEG findings. Developmental and cognitive level was based, where possible, on evaluations or formal assessments by referring physicians or neuropsychologists and classified according to the Diagnostic and Statistical Manual of Mental Disorders 5th edition. Developmental delay (DD) was classified as mild, moderate or severe in children aged 5 years or younger. Children who were at least 6 years old were classified as having mild, moderate, severe, or profound intellectual disability (ID). Seizures, epilepsies and, where possible, epilepsy syndromes were classified according to the International League Against Epilepsy classification.[31-33] A diagnosis of DEE was defined by both (1) an epileptic encephalopathy where frequent epileptiform activity and/or seizures caused developmental slowing or regression, and (2) abnormal development due to the underlying aetiology.[31] Intellectual disability and epilepsy were diagnosed if they had developmental impairment and seizures without evidence of an epileptic encephalopathy.[31,34,35] EEG recordings in individuals with seizures, where available, were reviewed by two epileptologists (EG and GR). Effectiveness of ASMs were retrospectively assessed by the referring clinicians.

Genotype analysis

GABRG2 variants were identified by genomic analysis using targeted gene panels, whole exome sequencing or whole genome sequencing at each institution. Genomic variants and familial segregation were confirmed by direct Sanger sequencing. All variants were annotated

using the NM_198904.4 (GRCh37 / hg19) transcript of *GABRG2*, which corresponds to the γ2L splice form of the γ2 subunit. The potential impact of the *GABRG2* variants was assessed using SIFT (sorting intolerant from tolerant), PolyPhen-2 (polymorphism phenotyping-v2), CADD v1.6 (combined annotation dependent depletion), Alphamissense, and their frequency in gnomAD v v4.0.0 (genome aggregation database). Variants were classified according to the 2015 American College of Medical Genetics and Genomics (ACMG) guidelines. Concerning copy number variations, we did not include individuals with deletions/duplications involving other genes in addition to *GABRG2*.

Severity Index

In addition to comparing individual phenotypic features, we designed a simple holistic measure of disease burden. For this, a severity index was calculated as the sum of scores for all phenotypic features for each individual. The following scores were assigned to the various phenotypic features: inheritance (0 for inherited, 1 for *de novo*), epilepsy (0 for no, 1 for yes), age of seizure onset (0 for no epilepsy, 1 for onset above 6 months, 2 for onset less than 6 months), seizure frequency (0 for no seizures, 1 for monthly or rare, 2 for daily or weekly), DD/ID (0 for normal, 1 for mild to moderate, 2 for severe or profound), language delay (0 for no delay, 1 for delay, 2 for nonverbal), psychiatric issues (0 for no, 1 for yes), motor skill issues (0 for normal, 1 for delay or clumsiness, 2 for non-ambulant) and hypotonia (0 for normal, 1 for hypotonia). Further detail regarding scoring is presented in the Supplementary Table.

Molecular biology

To circumvent the common issue with non-uniform $GABA_AR$ populations that e.g., include polluting binary α1β3 receptors, concatenated pentameric γ2-β3-α1-β3-α1 receptor constructs using human subunits were used in all experiments as previously described.[24,27,28,36] These constructs lead to expression of a fusion protein containing all five subunits of a $GABA_AR$ in the correct order for assembly.[37,38] Wild-type and 17 single mutant $\gamma2^*$ subunits were purchased from GenScript (Singapore) and subcloned into the X position of a tetrameric concatenated construct (X-β3-α1-β3-α1). The incorporation of desired mutations was verified by Sanger sequencing. DNA gel electrophoresis was performed to ensure the incorporation of the five subunits. cRNA of each concatenated receptor was synthesised from linearised cDNA using the mMessage mMachine T7 transcription kit (Thermo Fisher, Scoresby, Australia). cRNA was stored at -20ºC until use.

Xenopus laevis oocyte preparation

Oocytes were obtained from an ovarian lobe of *Xenopus laevis* from the University of Wollongong under animal ethics protocol AE2003. The ovarian lobe was treated with 2 mg/mL of collagenase A dissolved in modified Barth's solution (in mM, 82.5 NaCl, 2 KCl, 1 MgCl, 5 HEPES hemisodium pH 7.4) until oocytes were detached. Oocytes were then stored in ND96 (in mM, 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES hemisodium pH 7.4, 1.8 $CaCl_2$) supplemented with gentamicin and ampicillin. For each oocyte, 25 ng of cRNA encoding for mutant or wild-type concatenated α1β3γ2 receptors were microinjected into oocytes and the oocytes were stored in ND96 solution supplemented with gentamicin and ampicillin at 18ºC for 2 days before recording.

Two-electrode voltage clamp electrophysiology

Electrophysiological recordings were carried out using a custom-built two-electrode voltage clamp apparatus. Briefly, cells were impaled with 3M-KCl-filled glass borosilicate microelectrodes with a resistance of 0.2-1.4 MΩ, then voltage clamped at -50 mV. Cells were continuously perfused with ND96 during the experiment. Currents were recorded using GeneClamp 500B (Axon instrument, Foster City, USA), digitised using the Digidata 1440A (Axon instrument) and stored on a PC using Clampex (Axon instrument). All experiments were conducted at room temperature with at least two batches of oocytes. To control for variations between oocyte batches and experimental days, wild-type and mutant receptors were assessed on each experimental day (fully parallel experiments).

Functional data analysis

To determine the functional effects of the $\gamma2^*$ subunit mutations on receptor function, EC_{50} values derived from GABA concentration-response relationships (n = 12-29), and maximum current amplitudes (n = 27-65) were determined as previously described.[26,28-30] Briefly, increasing concentrations of GABA were applied to oocytes to generate a concentration–response curve. The EC_{50} value for GABA concentration-response relationships was determined by nonlinear regression of the Hill equation to the maximal GABA-elicited amplitudes for each oocyte:

$$I = Abs.I_{max}\frac{([A]^{n_H})}{([A]^{n_H}+[EC_{50}]^{n_H})}$$

Where Abs. I_{max} is the absolute maximum current, [A] is the agonist concentration, n_H is the Hill slope, and EC_{50} is the concentration that elicits a half-maximal response. Data were fitted

for each completed concentration-response curve to determine the EC_{50} value at each single oocyte. The $LogEC_{50}$ value was then calculated from the EC_{50} value.

To determine the relative change in the $LogEC_{50}$ value of each mutant from the wild-type receptor, the $LogEC_{50}$ of the mutant was compared to the mean $LogEC_{50}$ of wild-type values determined on the same experimental day. The $\Delta LogEC_{50}$ for each mutant on the same day is given by the equation:

$$\Delta LogEC_{50} = LogEC_{50}(wt) - LogEC_{50}$$

Where the $LogEC_{50}(wt)$ is the $LogEC_{50}$ of wild-type and $LogEC_{50}$ of mutant receptors.

The maximum current (I_{max}) was determined by the peak current amplitude elicited by 10 mM GABA for wild-type and each mutant. The maximum current for each single experiment was given by the equation:

$$I_{max} = (Abs.I_{max})/(Abs.I_{max}(wt))$$

Where $Abs.I_{max}$ is the maximum current for mutant and $Abs.I_{max}(wt)$ is the maximum current elicited at 10 mM for wild-type.

Statistics

Curve fitting of GABA concentration-response relationships was performed by nonlinear regression using GraphPad Prism 10 (Dotmatics, Boston, MA) and were compared with a one-way ANOVA and multiple corrections with Dunnett's post-hoc test. Maximum GABA-evoked current amplitudes and ages of onset were compared by Mann-Whitney test. A conservative threshold for significance in $\Delta LogEC_{50}$ values was set at $P < 0.0001$ and a $\Delta LogEC_{50}$ shift exceeding ± 0.2. A similarly conservative threshold at I_{max} was set at $P < 0.0001$ and a I_{max} reduction exceeding 2 fold (less than 50% remaining current amplitude). A Fisher's Exact test was used to compare clinical features where appropriate, with a threshold of $P < 0.05$. The age of onset for epilepsy was compared with a Mann-Whitney test and a Mantel-Cox test to account for patients without epilepsy. The severity index was compared with a Mantel-Cox test and a receiver operating characteristic analysis with a Wilson-Brown test for significance.

Standard Protocol Approvals, Registrations, and Patient Consents

The study was conducted according to the ethical principles for medical research outlined in the Declaration of Helsinki. The study was approved by the local ethics committee in the Zealand region of Denmark (number SJ-91), and by the Institutional Review Board at the

Danish Epilepsy Centre, Filadelfia (EMN-2024-01998). Informed consent of individuals or their responsible relatives and approval by local ethical committees were obtained. Clinicians and caregivers filled anonymised data in an online questionnaire and standardised phenotyping sheets. The database was stored at the Danish Epilepsy Centre.

Data availability

All phenotypic data for the individuals in this study are available in the main text or the Supplementary material. Raw data for the functional analyses are available upon request from the corresponding authors. Data will be stored for a minimum of 7 years.