

Musto Elisa (Orcid ID: 0000-0002-3298-2390)
Cantalupo Gaetano (Orcid ID: 0000-0003-1343-8434)
Chebib Mary (Orcid ID: 0000-0001-6204-3178)
Møller Rikke Steensbjerre (Orcid ID: 0000-0002-9664-1448)
Gardella Elena (Orcid ID: 0000-0002-7138-6022)

GABRA1-related disorders: from genetic to functional pathways

Elisa Musto^{1,2,3*}, Vivian W. Y. Liao^{4*}, Katrine M. Johannesen^{1,5}, Christina D. Fenger^{1,6}, Damien Lederer⁷, Kavitha Kothur⁸, Katrina Fisk⁹, Bruce Bennetts^{9,10}, Pascal Vrielynck¹¹, Delphine Delaby¹¹, Berten Ceulemans¹², Sarah Weckhuysen^{13,14,15}, Peter Sparber¹⁶, Arjan Bouman¹⁷, Simone Ardern-Holmes^{8,18}, Christopher Troedson¹⁸, Domenica I. Battaglia², Himanshu Goel¹⁹, Timothy Feyma²⁰, Somayeh Bakhtiari²¹, Linda Tjoa²², Martin Boxill²³, Nina Demina¹⁶, Olga Shchagina¹⁶, Elena Dadali¹⁶, Michael Kruer²¹, Gaetano Cantalupo^{24,25,26}, Ilaria Contaldo², Tilman Polster²⁷, Bertrand Isidor²⁸, Stefania M. Bova²⁹, Walid Fazeli³⁰, Leen Wouters³¹, Maria J. Miranda³², Francesca Darra^{24,25,26}, Elisa Pede², Diana Le Duc³³, Rami Abou Jamra³³, Sébastien Küry³⁴, Jacopo Proietti^{24,35}, Niamh McSweeney³⁶, Elly Brokamp³⁷, Peter Ian Andrews³⁸, Marie Gouray Garcia³⁹, Mary Chebib⁴⁰, Rikke S. Møller^{1,26,41}, Philip K. Ahring⁴⁰✉, Elena Gardella^{1,26,41}✉

* These authors contributed equally

- 1) Department of Epilepsy Genetics and Personalized Medicine, Danish Epilepsy Centre, Dianalund, Denmark.
- 2) Pediatric Neurology, Department of Woman and Child Health and Public Health, Child Health Area, Catholic University UCSC, Rome, Italy.
- 3) Epilepsy and Movement disorder Neurology, Ospedale Pediatrico Bambino Gesù IRCCS, Rome, Italy.
- 4) Brain and Mind Centre, Sydney Pharmacy School, Faculty of Medicine and Health, The University of Sydney, Sydney, New South Wales, Australia.
- 5) Department of Genetics, University Hospital of Copenhagen, Rigshospitalet, Copenhagen, Denmark.
- 6) Amplexa Genetics, Odense, Denmark.
- 7) Centre for Human Genetics, IPG, Gosselies, Belgium.
- 8) Kids Neuroscience Centre, The Children's Hospital at Westmead, The University of Sydney, NSW, Australia.
- 9) Sydney Genome Diagnostics, Western Sydney Genetics Program, The Children's Hospital at Westmead, Sydney, Australia.
- 10) Specialty of Genomic Medicine, Children's Hospital at Westmead Clinical School, Faculty of Medicine and Health, University of Sydney, Sydney NSW, Australia
- 11) Reference Center for Refractory Epilepsy, Catholic University of Louvain, William Lennox Neurological Hospital, Ottignies, Belgium.
- 12) Department of Pediatric Neurology, Antwerp University Hospital, University of Antwerp, Belgium.
- 13) Applied & Translational Neurogenomics Group, VIB-Department of Molecular Genetics, University of Antwerp, Antwerp, Belgium.
- 14) Department of Neurology, Antwerp University Hospital, Antwerp, Belgium.

- 15) Translational Neurosciences, Faculty of Medicine and Health Science, University of Antwerp, Antwerp, Belgium.
- 16) Research Centre for Medical Genetics Moskvorechie 1, Moscow, Russia.
- 17) Department of Clinical Genetics, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands.
- 18) T.Y. Nelson Department of Neurology and Neurosurgery, The Children's Hospital at Westmead, Westmead, New South Wales 2145, Australia.
- 19) Hunter Genetics, Newcastle, New South Wales, Australia.
- 20) Gillette Children's Specialty Healthcare, University Avenue St. Paul, MN, USA.
- 21) Pediatric Movement Disorders Program, Division of Pediatric Neurology, Barrow Neurological Institute, Phoenix Children's Hospital, Phoenix, Arizona, USA; Departments of Child Health, Neurology, Cellular & Molecular Medicine and Program in Genetics, University of Arizona College of Medicine, Phoenix, Arizona, USA.
- 22) Townsville University Hospital, Queensland, Australia.
- 23) Department of Pediatrics, Viborg Regional Hospital, Viborg, Denmark.
- 24) Child Neuropsychiatry Unit, Department of Engineering for Innovation Medicine, University of Verona.
- 25) Center for Research on Epilepsies in Pediatric age (CREP), Azienda Ospedaliero-Universitaria Integrata, Verona, Italy.
- 26) Full Member of ERN, Epicare.
- 27) Department of Epileptology (Krankenhaus Mara), Bielefeld University Medical School, Germany.
- 28) CHU Nantes, Service de Génétique Médicale, Nantes, France.
- 29) Pediatric Neurology Unit, V. Buzzi Children's Hospital, Milan, Italy.
- 30) Department of Neuropediatrics, Children's Hospital, University of Bonn, Bonn, Germany.
- 31) Department of Paediatrics, Ziekenhuis Oost-Limburg, Genk, Belgium.
- 32) Department of Pediatrics, Pediatric Neurology Herlev Hospital, Copenhagen University Hospital Herlev Denmark.
- 33) Department of Human Genetics, University of Leipzig Faculty of Medicine, Leipzig, Sachsen, Germany.
- 34) CHU Nantes, Service de Génétique Médicale, 44093 Nantes, France; l'Institut du Thorax, INSERM, CNRS, Université de Nantes, Nantes, France.
- 35) Irish Centre for Fetal and Neonatal Translational Research (INFANT), Cork, Ireland; Child Neuropsychiatry.
- 36) Department of Paediatrics, Cork University Hospital, Cork, Ireland.
- 37) Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN, USA.
- 38) Department of Neurology, Sydney Children's Hospital, Randwick, Australia.
- 39) Centre Hospitalier de Cholet 1 rue Marengo 49325 Cholet, Cedex, France.

40) Brain and Mind Centre, Sydney Pharmacy School, Faculty of Medicine and Health, The University of Sydney, Sydney, New South Wales, Australia.

41) Department of Regional Health Research, Faculty of Health Sciences, University of Southern Denmark.

Correspondence to:

✉ elga@filadelfia.dk; philip.ahring@sydney.edu.au

Elena Gardella, MD, PhD

IRS, University of Southern Denmark

Department of Clinical Neurophysiology, Danish Epilepsy Centre

Visbys Allé 5

4293 Dianalund, Denmark

Ph: 0045 58 27 11 92/93

Fax: 0045 58 27 11 88

Email: elga@filadelfia.dk

And

Philip K. Ahring

Brain and Mind Centre,

Sydney Pharmacy School,

Faculty of Medicine and Health,

The University of Sydney,

Sydney, New South Wales, Australia.

Email: philip.ahring@sydney.edu.au

Running head: *GABRA1* genotype-phenotype correlations

Summary for Social Media If Published

Variants in *GABRA1* have been associated with a broad epilepsy spectrum, however our understanding of what determines the phenotype severity and best treatment options remains inadequate. In this collaborative work, we aimed to analyse the electro-clinical features of 27 unpublished individuals harbouring 20 different *GABRA1* variants and to explore functional effects of 19 variants.

Our genotype-phenotype correlations permit us to delineate specific sub-phenotypes for LoF and GoF variants, with different epileptic and neurodevelopmental features. Going forward, this study will pave the way for a better understanding of the patho-mechanism of *GABRA1*-related disorder and for a precision medicine approach.

Draft Tweet

Investigation of 27 novel patients expand the field of *GABRA1*-related neurological disease and highlights the importance of functional analysis and genotype-driven deep phenotyping

ABSTRACT

Objective:

Variants in *GABRA1* have been associated with a broad epilepsy spectrum, ranging from genetic generalized epilepsies to developmental and epileptic encephalopathies. However, our understanding of what determines the phenotype severity and best treatment options remains inadequate. We therefore aimed to analyse the electro-clinical features and the functional effects of *GABRA1*-variants to establish genotype-phenotype correlations.

Methods:

Genetic and electro-clinical data of 27 individuals (22 unrelated and 2 families) harbouring 20 different *GABRA1* variants were collected and accompanied with functional analysis of 19 variants.

Results:

Individuals in this cohort could be assigned into different clinical subgroups based on the functional effect of their variant and its structural position within the *GABRA1* subunit. A homogenous phenotype with mild cognitive impairment and infantile-onset epilepsy (focal seizures, fever sensitivity and EEG posterior epileptiform discharges) was described for variants in the extra-cellular domain and the small transmembrane loops. These variants displayed loss-of-function (LoF) effects and the patients generally had a favourable outcome. A more severe phenotype was associated with variants in the pore-forming transmembrane helices. These variants displayed either gain-of-function (GoF) or LoF effects. GoF-variants were associated with severe early-onset neurodevelopmental disorders, including early infantile developmental and epileptic encephalopathy.

Interpretation:

Our data expand the genetic and phenotypic spectrum of *GABRA1*-epilepsies and permit to delineate specific sub-phenotypes for LoF and GoF variants, though the heterogeneity of phenotypes and variants. Generally, variants in the transmembrane helices cause more severe phenotypes, in particular GoF variants. These findings establish the basis for a better understanding of the patho-mechanism and precision medicine approach in *GABRA1*-related disorders. Further studies in larger populations are needed to provide a conclusive genotype-phenotype correlation.

Keywords: *GABRA1*, neonatal encephalopathy, infantile epilepsy, febrile seizures, juvenile myoclonic epilepsy

1. Introduction

The *GABRA1* gene is located on chromosome 5 and encodes the $\alpha 1$ subunit of the gamma-aminobutyric acid type A receptor (GABA_AR). GABA_ARs are pentameric chloride channels assembled from 1–5 of 19 subunits. The most common GABA_AR expressed in the mammalian brain is composed of two $\alpha 1$, two $\beta 2/\beta 3$ and one $\gamma 2$ subunits (Figure 1A & 1B). Each subunit shares common structure, consisting of (i) an extracellular N-terminus domain, (ii) a transmembrane domain (TM) made up of four transmembrane helices (TM1–4) and two loops TM1-TM2 and TM2-TM3, and (iii) an intracellular domain (intracellular loop TM3-TM4)¹.

GABA_AR mediate crucial inhibitory synaptic transmission in the central nervous system (CNS)². Pathogenic variants in *GABRA1* were first identified in unrelated individuals and families with genetic generalized epilepsies, including juvenile myoclonic epilepsy (JME)^{3 4} and childhood absence epilepsy⁵. More recently, *GABRA1* variants have been reported in severe developmental and epileptic encephalopathies (DEE)⁶, including Dravet-like phenotypes^{7 8 9}, Ohtahara Syndrome, epilepsy of infancy with migrating focal seizures (EIMFS) and infantile spasms syndrome (ISS)^{10 11 12 13}. Overall, this represents a very diverse phenotypic spectrum.

While variants in *GABR* genes can be observed along the entire length of the subunit, they are typically enriched in paralog-conserved sites with clustering around key functional structures such as the extracellular GABA binding site, transmembrane helices supporting or lining the pore, and loop regions that couple ligand binding to channel gating. Functional analysis of variants, including *GABRA1* variants, traditionally reported only loss-of-function (LoF) effects. Variants were found to reduce or abolish GABAergic function by either lowering the channel gating efficiency or receptor surface expression, thereby limiting inhibitory GABAergic transmission resulting in epileptic phenotypes¹⁴. Phenotypic severity has then been speculated to be a result of the degree of LoF traits¹⁵. However, the assumption of the same functional effect of all variants (LoF) in a gene such as *GABRA1* does not correlate well with the unusual diverse range of severities in clinical phenotypes.

We recently reported that the phenotypic diversity associated with epilepsy-causing *GABRB3*, *GABRA4* and *GABRD* variants is not only explained by LoF but can also be explained by the presence of variants displaying a GoF effect^{16 17 18 19}. These GoF GABA_AR variants were associated with treatment resistant epilepsy and typically more severe phenotypes. Almost all *GABRA1* variants tested to date have been reported as LoF¹⁴, but recently one p.(Thr292Ser) was reported as GoF²⁰ and another one p.(Ala332Val) was inferred to have GoF traits²¹. Given that GABA_AR $\alpha 1$ subunits structurally resemble $\beta 3$ subunits and assemble into the same receptor complexes, we speculated that historic data might not fully reflect the consequences of *GABRA1* variants. Therefore, in this study we present a cohort of 27 novel individuals carrying 20 (11 novel) presumed pathogenic *GABRA1* variants and aimed to establish possible explanations for the heterogeneity of the

phenotypic spectrum using deep phenotyping and EEG characterisation coupled with functional analysis of each variant.

2. Materials and Methods

Ethics

All institutions involved in human participant research received local IRB approval (main IRB: The ethics committee of Region Zealand, Denmark). Written informed consent, including authorization for reproduction of video images, was obtained for all patients (or legal guardians) and family members where necessary. Patient data were collected according to local ethics committee guidelines.

Data collection and analysis

Through an international collaboration including different clinical epilepsy centres in Europe, Australia and in the United States, we collected data of individuals with presumed pathogenic *GABRA1* variants. The American College of Medical Genetics and Genomics/Association of Molecular Pathology guidelines were used to assess variant pathogenicity and the *GABRA1* transcript NM_000806.5 to code variant nomenclature. Clinical information was collected by face-to-face interviews with patients and their families and from clinical charts, and data have been collected in a structured phenotyping table. The epilepsy syndromes were classified according to the guidelines of ILAE classification (2017)²². All the EEG and cerebral MRI reports were collected. Two epileptologists with EEG expertise (E.M., E.G.) reviewed the raw EEG data of 15 patients (including long-term monitoring video-EEGs) for background activity, interictal epileptiform abnormalities, ictal EEG discharges and clinical manifestations.

Functional studies

$\alpha 1\beta 3\gamma 2$ GABA_A receptors have a pentameric stoichiometry consisting of two $\alpha 1$, two $\beta 3$ and one $\gamma 2$ subunits. All patients in this cohort are heterozygous for their variant. Assuming a Mendelian distribution for the missense variants, this results in 50% of the expressed receptors having a single variant $\alpha 1$ subunit, 25% of expressed receptors having two variant $\alpha 1$ subunits, while the remaining 25% of expressed receptors are wild-type. Hence, the most important receptors to investigate are the ones that are heterozygous for the variant, as these constitute the bulk of expressed receptors. Pentameric concatenated constructs represent a highly efficient method to obtain robust expression of such receptors in *Xenopus laevis* oocytes^{23 24}. Thus, we generated 19 pentameric concatenated constructs containing a variant and a wild-type $\alpha 1$ subunit (Figure

2A). For functional analysis, the cRNAs of concatenated $\alpha 1\beta 3\gamma 2$ variant receptors and the wild-type (control) were injected into oocytes (25 ng/oocyte) and subjected to electrophysiological investigation using the two-electrode voltage clamp technique as previously described²³. Functional electrophysiological experiments were conducted using a custom-built two-electrode voltage-clamp apparatus. Inter-day variation between oocyte batches were controlled by performing wild-type experiments in parallel to variants on each experimental day. GABA concentration–response relationships ($n > 10$) were obtained by applying increasing concentrations of GABA. Maximum current amplitudes (I_{max} , $n > 20$) were determined by applications of 10 mM GABA solution and normalized against wild-type value of the day. All experiments were conducted in at least two batches of oocytes.

To obtain the EC_{50} values from the GABA concentration–response relationships, the Hill equation was fitted to the GABA-evoked current amplitudes for individual oocytes where EC_{50} is the concentration eliciting half-maximum response, and nH is the Hill slope:

$$I = I_{max} \left(\frac{[GABA]^{nH}}{[GABA]^{nH} + EC_{50}^{nH}} \right)$$

Responses were then normalized to the fitted maximum response of individual curves. Full concentration-response curve of individual oocytes was recorded as a single determination (n). Average pEC_{50} values (where $p = -\text{LOG}$) for the wild-type control ($pEC_{50}(\text{wt})$) was calculated and the ΔpEC_{50} values for each variant experimental determination on the same day was derived by the equation:

$$\Delta pEC_{50} = pEC_{50} - pEC_{50}(\text{wt})$$

Typically, in this assay a ΔpEC_{50} value of ≥ 0.2 indicates a GoF and ≤ -0.2 a LoF variant. The higher the number towards either the positive or negative spectrum the more altered receptor sensitivity to GABA.

3. Results

Patients and genetics

We collected data from 27 individuals (22 unrelated, and 2 families) harbouring 20 presumed pathogenic *GABRA1* variants of which 11 are novel. All variants were heterozygous missense, except for 1 frameshift p.(Gly222Aspfs*4). The variants occurred *de novo* in 15/27 (55%) subjects and were inherited from a symptomatic parent in 6/27 (22%), with high penetrance. Segregation data were not available for 6 subjects.

17/20 variants were predicted as damaging by at least two different prediction tools (SIFT, PolyPhen, MutationTaster). The missense variants were distributed among the various domains of the $\alpha 1$ subunit protein: 9 in the extracellular domain, 9 in the transmembrane domain and 1 in the intracellular domain (that

is not resolved in the Cryo-EM structures of the GABA_AR) (Figure 1C & 1D). The frameshift variant (Gly222Aspfs*4) was located in the extracellular domain.

Functional analysis of GABRA1 variants in $\alpha 1\beta 3\gamma 2$ receptors

As described in the methods, functional analysis was performed on receptors that are heterozygous for the GABRA1 variants (Figure 2A). Wild-type receptors and receptors containing missense variants all responded to GABA applications in a concentration-dependent manner (Figure 2B & 2C). However, in most cases the sensitivity to GABA was different from that of the wild-type receptor (Figure 2D). Four variants (p.(Tyr252His), p.(Ser299Asp), p.(Ala332Thr) and p.(Tyr438Cys)) had significantly increased sensitivity to GABA, which constitute a GoF effect (Figure 2D, Table 1). In contrast 12 variants displayed decreased sensitivity to GABA, which constitute a LoF effect. The remaining three variants (p.(Glu63Lys), p.(Ser96Cys) and p.(Glu403Gln)) did not display any significant difference relative to the wild-type receptor. Next, maximally GABA-evoked current amplitudes were assessed to investigate whether the variants might affect surface levels or gating efficiency of expressed receptors. Of the 19 tested variants, only the p.(Val270Ala) variant displayed significantly lower maximal current amplitude (Figure 2B, Table 1). As this variant also displayed LoF for the GABA sensitivity, this variant essentially displays LoF traits for both investigated parameters.

Reclassification of variants using the ACMG guidelines

The p.(Gly222Aspfs*4) variant will likely lead to nonsense mediated decay of the mRNA and limited or no protein synthesis. In case the protein is expressed, the synthesis would terminate early leading to a partial subunit that is missing the entire transmembrane domain and therefore is non-functional. This variant can be classified as likely pathogenic according to the American College of Medical Genetics (ACMG) guidelines²⁵ based on a null variant in a gene where LoF is a known mechanism of disease (PVS1). Of the remaining 19 tested variants, 12 variants were confirmed to occur *de novo* (PS1) and show deleterious effects in a well-established assay (PS3), which leads to a classification as pathogenic (Table 1). Two variants [p.(Asn275Lys) and p.(Pro305Leu)] were inherited from an affected parent and showed deleterious effects in the functional assay leading to a likely pathogenic classification. For two variants [p.(Arg147Gln) and p.(Val162Met)], the segregation status was not confirmed but deleterious effects were noted in the functional assay leading to a likely pathogenic classification.

Finally, the 3 variants [p.(Glu63Lys), p.(Ser96Cys) and p.(Glu403Gln)] that were predicted to not be damaging using *in silico* tools, did not display any deleterious effects in the functional assay. These variants have been reported in 3 unrelated subjects with different phenotypes: (1) one, with intellectual disability (ID) and

generalised epilepsy, inherited the variant p.(Glu63Lys) from the affected father; (2) one, with generalised epilepsy and severe ID, inherited the variant p.(Ser96Cys) from the asymptomatic mother; (3) the last one, with childhood absence epilepsy, harbours a *de novo* p.(Glu403Gln) variant. Although it is not possible to exclude a potential pathogenic cause that were missed with our experimental system, it is likely that these represent benign polymorphisms not responsible for the clinical phenotype of the individuals. This is supported by the observation that two of these three amino acid positions have entries in gnomAD database [p.(Glu63Ala), p.(Glu403Gln) and p.(Glu403Lys)]. The 3 patients have therefore been excluded from our genotype-phenotype analysis.

GABRA1 cohort

The remaining 24 individuals (15 females / 9 males), included for the genotype-phenotype analysis encompass 21 probands and 3 affected family members. Their age at inclusion ranged from 12 months to 34 years (median age 8.5 years). We investigated the clinical phenotype with a focus on epilepsy, EEG features and the cognitive and neurological outcomes (Table 2). Comprehensive electro-clinical and genetic details of the whole cohort are summarized in the supplementary table.

Epilepsy was diagnosed in 23/24 (96%) subjects, with a median age at onset of 9.5 months (range: 1.5 month - 15 years). Seizure types included focal and generalized tonic-clonic seizures, typical and atypical absences, myoclonic and atonic seizures, and epileptic spasms. Most individuals (21/24, 88%) had various degrees of ID or developmental delay. Other neurodevelopmental disorders were observed: autism spectrum disorder (ASD) (5/24, 21%) and behavioural disturbances or attention deficit/hyperactivity (ADHD) (7/24, 29%). Movement disorders, including ataxia, poor coordination, tremor, dystonia, and cerebral palsy were also reported (7/24, 29%).

Genotype-phenotype correlations

Functionally, the 7 missense variants located in extracellular domain had a LoF effect. In the transmembrane domain, we observed 4 missense variants with GoF effect and 5 variants with LoF effect of which two were inherited. Apart from the inherited variants, the variants in the transmembrane domain generally caused more severe and varied phenotypes compared to the variants in the extracellular domain. Based on the position and on the functional effect of the missense variants, we distinguished different subgroups with different phenotypic features (Table 2, Figure 3).

Extracellular domain missense variants (LoF)

11 individuals, aged between 1 to 19 years, harboured 7 missense variants in the extracellular domain. The modality of inheritance was *de novo* in 8. The segregation analysis was not available for 3 individuals. All subjects showed a homogenous phenotype.

Mild-to-moderate ID was described in 9/11 (82%) and severe ID in 1. Early motor milestones were slightly delayed in 7/11 (64%), however all subjects reached normal motor autonomy, and achieved independent walking within the age of 24 months. Early language development delay was reported for 9/11 (82%) subjects; 10 reached afterward normal verbal skills, while one (9 years old) was a nonverbal child with ASD. Pyramidal or extrapyramidal signs were not observed; broad-based ataxic gait, poor coordination and mild hypotonia were reported in 8/11 (73%) during infancy / early childhood, with progressive improvement.

Comorbid neuropsychological and neuropsychiatric conditions included ASD in 3/11 (27%), behavioural dysregulation in 2/11 (18%) and hyperactivity in 1/11 (9%). All the patients were at least partially autonomous in everyday life, apart from a child with ASD who showed poor independence in daily activities, personal and social autonomy.

Epilepsy was reported in all, typically with onset in the first year of life (9/11, 82%) and with focal hemiclonic seizures (6/11, 55%) elicited by high fever in 4 of them. Less frequently, brief febrile generalized tonic-clonic (GTC) (3/11, 27%), or myoclonic seizures (1/11, 9%) were observed at onset. During late-childhood, focal or focal-to-bilateral tonic clonic seizures were observed in 7/11 (64%), while GTC and myoclonic seizures, including eyelid myoclonia, in 5/11 (46%). Fever sensitivity was reported in 9/10 (90%) individuals (data not available for 1). After a "stormy phase", 6/11 (55%) subjects became seizure-free on monotherapy at an age ranging from 9 months to 6 years, while 4/11 (36%) achieved partial seizure control on 1-2 anti-seizure medications (ASM), with a maximum seizure frequency of 1 seizure/month. Only 1 subject had severe epilepsy with the need for polytherapy. LEV was the most effective drug (8/8 subjects who tried the drug), although 2 experienced negative behavioral side effects. Benzodiazepines were not tested in this group.

Interictal EEG was normal at epilepsy onset in 4/7 (57%) and showed focal (parieto-temporal or occipital) interictal epileptiform discharges (IED) with activation during sleep in 3/7 (43%) (data not available for 4 individuals). At follow up, intermittent slow background with diffuse superimposed fast rhythms or excess of beta activities in the central regions were recurrent findings (3/9, 33%; data not available for 2 patients). No IED were observed in 6/9 (66%), while 2/9 (22%) subjects had posterior spikes with tendency to diffuse spreading and sleep activation, and 1/9 (11%) had diffuse spike-and-slow waves (Figure 4A).

A syndromic classification of genetic epilepsy with febrile seizures plus (GEFs+) was made for most patients, because of onset with febrile seizures in the first year of life and afebrile polymorphic seizures appearing later in life, with some atypical features (e.g. mild ID). For some patients, Dravet syndrome (DS) was suspected, as previous normal infants presented with prolonged, febrile, hemiclonic seizures followed by

developmental slowing. However, prolonged seizures and status epilepticus were not common in this cohort, gait abnormalities and motor impairment were non-progressive, and an encephalopathic course was not observed. To the opposite, complete seizure control on monotherapy was achieved. EEG are normal or with an occipital focus with activation during sleep (similar to focal idiopathic epilepsy). For these reasons, the developmental course does not underpin the clinical diagnosis of DS.

Transmembrane domain missense variants (LoF)

Three individuals harbouring LoF variants located in the TM1-TM3 helices encompassed a range of neurodevelopmental disorders associated with early-onset epilepsy with prominent photosensitivity.

Pt.15 [p.(Val270Ala), TM1] had severe hypotonia and global developmental delay as an infant; she never sat nor walked independently. She developed focal seizures at 1.5 months of age, and at 14 years multi-focal myoclonus that has increased over time. After multiple ASM trials, seizures were ultimately controlled with primidone, started for kinetic tremor. At latest control (16 years old), she had severe ID, behavioural dysregulation and generalized hypotonic-dystonic cerebral palsy (GMFCS level 5). Comorbidities included dysphagia and poor weight gain, treated by gastrostomy tube placement. EEG showed slow background, abundant beta activity and photosensitivity; at latest follow-up continuous delta slowing, and superimposed beta activity was described.

Pt.19 [p.(Thr295Ile), TM2] presented with developmental delay from early infancy and focal hemiclonic seizures from the age of 7 months, followed by focal autonomic seizures. After a “stormy phase”, he achieved seizure control on LEV from the age of 16 months. Epilepsy relapsed at 6 years with recurrent unprovoked absences with eyelid myoclonia. EEG at onset was normal, then showed occipital and diffuse spike-and-waves and photosensitivity.

Pt.22 [p.(Ile317Leu), TM3] is a 17 months old boy, with hypotonia, developmental and motor delay from early infancy, and GTC from the age of 7 months. Longer follow up is needed to determine epilepsy and cognitive outcome. Long-term monitoring EEG showed slow background and excess of beta activity in the central regions (Figure 4B).

Individuals harbouring inherited LoF-variants located in TM1-TM2 and TM2-TM3 loops presented with milder phenotypes similar to the ones carrying variants in the extracellular domain. Photosensitivity seems to be a common feature during late childhood/adolescence.

Pt. 16-18 [p.(Asn275Lys), TM1-TM2 loop] were members of a GEFs+ family, sharing a quite similar phenotype. All 3 had mild ID, normal behaviour and normal neurological examination. All 3 started having febrile seizures within 18 months of life, followed by afebrile GTC (proband and mother) and typical absences (sister) during

infancy. The interictal EEG initially showed focal IED in the posterior regions, then diffuse spike and slow waves at follow up (Figure 4C). All 3 achieved seizure-freedom on LEV, however seizures with photosensitivity relapsed in the mother from the age of 12 until 30 years (good control on carbamazepine).

Pt.21 [p.(Pro305Leu), TM2-TM3 loop, inherited from a symptomatic father], is a 10-year-old girl with mild ID, learning disabilities, normal behaviour and normal neurological examination. She presented with childhood onset epilepsy; Panayiotopoulos syndrome was suspected, due to the age of epilepsy onset (4 years), the seizure semiology (prolonged focal autonomic seizures), and the posterior spike-and-waves with photosensitivity.

Transmembrane domain missense variants (GoF)

The 4 individuals included in this group encompasses a range of severe clinical phenotypes, ranging from infantile spasms syndrome (ISS) to isolated neurodevelopmental disorders without epilepsy.

Pt.14 [p.(Tyr252His), TM1] presented with infantile spasms, developmental regression and hypsarrhythmia at the age of 5 months. Infantile spasms were responsive to ACTH and prednisolone and the patient achieved seizure freedom, although the persistence of almost continuous EEG abnormalities, treated with VPA, LEV and sulthiame with no benefit. His EEG showed multifocal IED (4 year old), fronto-temporal IED (7 year old) and was normal by the age of 9 years. His motor development was delayed, but no seizures were reported at follow up. At the age of 18 years, he was ambulant, but still presented with impairment of speech competencies (severe language disorder), cognitive abilities (moderate ID) and behaviour regulation.

Pt.20 [p.(Ser299Asn), TM2] presented with severe ID, movement disorders and autism, but never had seizures nor IED on the EEG. However, since she is only 3 years and 6 months old, a longer follow up is needed to define her phenotype.

Pt.23 [p.(Ala332Thr), TM3] presented with global developmental delay from early infancy, evolving to moderate ID associated with mild dystonic posturing, pyramidal signs, and poor language. GTC occurred at the age of 10 years and were not completely controlled by VPA.

Pt.24 [p.Tyr438Cys, TM4] had developmental delay evolving to mild ID, ASD and ADHD with anxiety disorder. Myoclonic seizures had onset at the age of 27 months, and subsequently focal motor seizures and atypical absences appeared (5 years old). At latest control (8 years old), he still had seizures despite multiple ASM (VPA, clobazam, rufinamide, lamotrigine). The EEG showed posterior or diffuse slowing and generalized 2-3 Hz spike-and-slow waves (Figure 4D).

Frameshift variant

The only protein truncating variant [p.(Gly222Aspfs*4)] was observed in a proband and her mother (pt.10 and pt.11), both with pharmacoresponsive epilepsy, normal neurological examination and normal cognition. The syndromic classification is difficult because epilepsy showed both features of genetic generalized epilepsy as well as of focal epilepsy. The mother had two GTC during adolescence, whilst her daughter had adolescence-onset focal seizures (autonomic manifestations, automatisms, staring) and GTC, well controlled with carbamazepine. The EEG of the daughter showed sporadic IED in the frontal region bilaterally, and myoclonic seizures with generalized spike-and-slow waves.

4. Discussion

In this study, we defined the electro-clinical features of 24 novel patients harbouring 17 different disease-causing *GABRA1* variants including 11 novel variants. We distinguished different electro-clinical phenotypes and described the complex genotype-phenotype correlations. Our data confirm the broad clinical spectrum of *GABRA1*, encompassing both epilepsy and movement disorders²⁶, including pyramidal and extra-pyramidal motor impairment. Interestingly, the different *GABRA1* phenotypic expression depends on the type (missense vs nonsense), the position (extracellular vs transmembrane domains) and ultimately on the functional effect (LoF vs GoF) of the variants (Figure 3). The combined impact of these three aspects, appear to define the overall clinical features including the severity of epilepsy, cognitive and motor development.

Functional analysis

The 19 *GABRA1* missense variants were functionally evaluated as $\alpha 1\beta 3\gamma 2$ GABA_A receptors in heterozygous assemblies containing one variant and one wild-type *GABRA1* subunit. 12 variants displayed LoF traits, and 4 variants displayed GoF traits which enables classifications of likely pathogenic or pathogenic according to the ACMG guidelines. The remaining 3 missense variants did not cause any significant functional change and likely represent benign variants. Based on multiple entries in the gnomAD database, one of these three variants, p.(Glu403Gln), might have been excluded from the study onset, however, inclusion of expected benign variants is critical when performing functional analysis. This validates that the assay can be used to distinguish between the three categories of GoF, LoF and benign variants²⁷. Historically, epilepsy-associated *GABR* variants were all assumed to be LoF¹⁵, but the data presented here add the *GABRA1* subunit to a growing list of *GABR* subunits for which both LoF and GoF variants have been confirmed. This list currently includes *GABRA4*¹⁸, *GABRB3*^{16 17} and *GABRD*¹⁹. In the current study, LoF variants were found in both the extracellular and transmembrane domains while GoF variants were only found in the transmembrane helices.

While this suggests that extracellular domain *GABRA1* variants are more likely to display LoF traits, GoF variants could still emerge once more variants are functionally characterised.

Frameshift variant

Protein truncating *GABRA1* variants are not commonly described, with only three previously reported cases: 1 deletion-insertion in a family with genetic generalized epilepsy⁴, 1 patient with childhood absence epilepsy⁵, and 1 case in comorbidity with Williams-Beuren Syndrome²⁸. Apart from the comorbidity, protein truncating variants seem to be related to normal cognition and mild, late-onset epilepsy with a good outcome. This is also what we observed for the single frameshift variant [p.(Gly222Aspfs*4)] in this study (Figure 3). From a protein function perspective, protein truncating variants can cause nonsense-mediated mRNA degradation²⁹ and/or protein truncation, hence they are expected to lead to no protein or non-functional proteins, which ultimately are not incorporated into a pentameric receptor complex. These variants are thus *de facto* LoF and resemble haploinsufficiency. It is quite likely that upregulation of expression of the subunit using the healthy gene copy leads to some degree of compensation, however, it is still noteworthy that haploinsufficiency with up to 50% of normal receptor expression leads to epilepsy with a relatively mild and treatable phenotype.

Missense extracellular domain variants

Missense variants located in the extracellular domain were all found to have LoF effect and presented with a homogenous phenotype, with seizure onset in the first year of life in the majority of cases (82%), typically with focal hemiclonic seizures elicited by high fever (Figure 3). Fever sensitivity is a hallmark for this group throughout their epilepsy course, and it is particularly remarkable for some specific variants [p.(Arg112Gln), p.(Arg214His)]. These features are similar to what has been previously described for *GABRA1* patients with syndromic classification of Dravet syndrome, Dravet syndrome-like, early-onset epileptic encephalopathy and GEFs^{6 7 8 9 12 13}. However, the cognitive and epilepsy outcomes for this group were much better than what is expected for Dravet syndrome or for most early-onset DEE. During infancy, they typically developed mild-to-moderate ID and focal autonomic seizures and eventually focal-to-bilateral tonic-clonic or myoclonic seizures, achieving seizure freedom on ASM monotherapy. Prolonged seizures and status epilepticus were exceptional and an encephalopathic course was never observed. The EEG pattern of this group consists of focal posterior slowing and IED, and diffuse spike-and-waves. This is also clearly distinguishable from the EEG of Dravet syndrome³⁰. While formally not part of the extracellular domain, LoF variants located in the transmembrane loops TM1-TM2 or TM2-TM3 overall resembled the extracellular domain group.

Missense transmembrane helices variants

Missense variants located in the transmembrane helices were associated with more severe phenotypes. These observations align well with previously reported cases, where *GABRA1* variants with the most severe phenotypes (*e.g.*, early-onset ISS, Ohtahara syndrome¹⁰ or EIMFS¹¹) were found in the TM1 and TM2 transmembrane helices. Variants in the TM1 and TM2 thus appear to share a clinical phenotype of EIDEE with early-onset epileptic spasms, evolving into severe refractory epilepsy, severe ID, and severe neurological impairment, and inauspicious outcome (Figure 3).

Interestingly, variants in helices had either a LoF or a GoF effect. We identified four GoF variants, which represent the first description of *GABRA1*-GoF DEE [p.(Tyr252His), p.(Ala332Thr) and p.(Tyr438Cys)], whereby the phenotype mainly consist of early-onset neurodevelopmental disorder and early life epilepsy (*e.g.* ISS). The individual with the remaining GoF variant p.(Ser299Asn) also has developmental delay and severe ID, however, so far without epilepsy (current age: 42 months). This is similar to a recently reported single *GABRA1* GoF variant (p.(Thr292Ser)) in a 2-year-old subject with severe neurodevelopmental delay and no seizures²⁰. LoF variants located in the transmembrane helical regions also have severe phenotypes that are not clearly distinguishable from *GABRA1*-GoF DEE, except for photosensitivity which seems to be a prominent feature of DEE *GABRA1*-LoF variants. Photosensitivity is not very frequent in DEEs³¹ or subjects with variants in other GABR subunits^{17 19}. If confirmed in a larger population, this feature could contribute to addressing the diagnosis of *GABRA1*-LoF DEE. The LoF variant p.(Val270Ala) located in TM1 is particularly interesting because it causes a large loss in GABA sensitivity (~10-fold) coupled with a significant loss in GABA-elicited current amplitude (~60% loss) (Table 1). While the detriment of “double losses” in protein function correlates with the severe phenotype in our patient, it is noteworthy that this variant was recently described in another subject with early-onset treatable epilepsy and moderate developmental delay¹², suggesting heterogeneity in phenotypic outcome for this variant.

GoF and LoF variants in GABR subunits

Similar to our genotype-phenotype and functional correlation studies in *GABRB3* and *GABRD*^{32 19 17}, GoF and LoF variants in *GABRA1* overall display distinctive phenotypic features that can be distinguished and separated into clinical categories. In all three genes, LoF variants including protein truncating variants are generally mild, with good prognosis and can be inherited. Severe phenotypes such as DEE and Dravet-like phenotype are typically only described for LoF variants in transmembrane helices. In contrast, GoF variants are *de novo* and are typically associated with the more severe, drug resistant epilepsy with poorer clinical outcome. Currently, our understanding of how GoF GABA_AR variants cause severe neurodevelopmental

disorders is limited. That said, both GoF and LoF variants have recently been described in several epilepsy-causing genes including *SCN1A*, *SCN8A* and *CACNA1A* indicating that this is not a unique phenomenon^{33 34 35}. A commonality for all these genes is that patient phenotypes and treatment options vary significantly between GoF and LoF variants. Hence, future studies are urgently needed to unravel how the different variant types affect neurodevelopment and normal brain circuitry.

Unlike variants in *GABRB3* and *GABRD*, the individuals with GoF *GABRA1* variants in this cohort achieve seizure control with or even without ASM. This observation could simply be due to the small sample size, or it could be related to the differences in the role the different subunits play in receptor function, and regions of the brain where the resulting receptors are found. A larger sample size and more *GABRA1* variants will need to be functionally characterised to establish more solid genotype-phenotype correlations and improve our understanding of the role this subunit plays in the pathogenesis of epilepsy.

Finally, given that the degree of detrimental change in receptor function can be successfully quantified and encapsulated using our functional parameters (e.g., ΔpEC_{50}) it is tempting to speculate that disease severity is linked to the magnitude of functional change. This notion is supported by the observation that highly altered receptor function in either direction of gain or loss caused a severe phenotype, exemplified by p.(Val270Ala) and p.(Ser299Asn). However, across the cohort there is no strong linkage between the magnitude of functional change and disease severity. While this is likely related to the size of the cohort, it is also important to note that different patients with the same *GABR* variant can vary substantially in their clinical presentation.

Treatment options

Our current knowledge of identifying the best treatment option for patients with either LoF or GoF variants is poor. However we can speculate from our *GABRB3* study, that seizure freedom is more common in subjects with LoF variants, particularly for variants located in the extracellular domain. Interestingly the most effective treatment for LoF variants in *GABRA1* and *GABRB3* vary with LEV identified for *GABRA1* individuals versus VPA in *GABRB3*³². Conversely, ASMs are ineffective in patients with GoF *GABRA1* variants with severe epilepsy, and this was also observed for individuals harbouring pathogenic *GABRB3* variants located in TMD domain³². Benzodiazepines, that specifically enhance the effects of endogenous and exogenous GABA mediated by GABA_A receptors, have not been tried on any of the subjects in our cohort.

5. Conclusion

It is well known that LoF variants in the *GABRA1* gene can cause epilepsy, however, here we demonstrate that variants with a GoF effect can also cause neurodevelopmental disorders, including epilepsy. This observation aligns with recent observations for *GABRB3* and *GABRD*. *GABRA1* LoF and GoF variants lead to different epilepsy phenotypes of different severity, also depending on their protein position. In general, variants in the transmembrane helices present with severe phenotypes, especially GoF variants that are associated with the most severe neurodevelopmental disorders, autistic features and early-onset epilepsy. LoF variants in the extracellular domain as well as variants in the transmembrane loop regions give rise to a quite homogenous and relatively benign phenotype (Figure 3). These findings pave the way for testing the possibility of a precision medicine approach to the treatment of *GABRA1*-epilepsies. Larger cohort studies are needed to confirm and better quantify these results.

Acknowledgements

We thank the patients and their families for participating in this study, the Australian National Health & Medical Research Council grants APP1185122 and APP1081733 (to M.C. and P.K.A.) and The Novo Nordisk Foundation (NNF19OC0058749 to R.S.M.)

Author Contributions

R.S.M., P.K.A., M.C. and E.G. contributed to the conception and design of the study. E.M., K.M.J., C.D.F., D.L., K.K., K.F., B.B., P.V., D.D., B.C., S.W., P.S., A.B., S.A.H., C.T., D.I.B., H.G., T.F., S.B., L.T., M.B., N.D., O.S., E.D., M.K., G.C., I.C., T.P., B.I., S.M.B., W.F., L.W., M.J.M., F.D., E.P., D.L.D., R.A.J., S.K., J.P., N.M., E.B., V.W.Y.L., P.K.A., M.C., E.G. and R.S.M. contributed to the acquisition and analysis of data. E.M., V.W.Y.L., E.G., M.C., R.S.M. and P.K.A. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

Data availability

De-identified data, including the *GABRA1* database and data used for functional studies, will be made available to those eligible and be stored for 10 years.

References

1. Chua HC, Chebib M. GABAA Receptors and the Diversity in their Structure and Pharmacology. *Adv Pharmacol* 2017;79:1–34.
2. Farrant M, Nusser Z. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nat Rev Neurosci* 2005;6(3):215–229.
3. Cossette P, Lortie A, Vanasse M, et al. Autosomal dominant juvenile myoclonic epilepsy and GABRA1. *Adv Neurol* 2005;95:255–263.
4. Lachance-Touchette P, Brown P, Meloche C, et al. Novel $\alpha 1$ and $\gamma 2$ GABAA receptor subunit mutations in families with idiopathic generalized epilepsy. *Eur J Neurosci* 2011;34(2):237–249.
5. Maljevic S, Krampfl K, Cobilanschi J, et al. A mutation in the GABA(A) receptor $\alpha 1$ -subunit is associated with absence epilepsy. *Ann Neurol* 2006;59(6):983–987.
6. Johannesen K, Marini C, Pfeiffer S, et al. Phenotypic spectrum of GABRA1: From generalized epilepsies to severe epileptic encephalopathies. *Neurology* 2016;87(11):1140–1151.
7. Carvill GL, Weckhuysen S, McMahon JM, et al. GABRA1 and STXBP1: novel genetic causes of Dravet syndrome. *Neurology* 2014;82(14):1245–1253.
8. Gontika MP, Konialis C, Pangalos C, Papavasiliou A. 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* 2017;4:2329048X17706794.
9. Hernandez CC, Tian X, Hu N, et al. Dravet syndrome-associated mutations in GABRA1, GABRB2 and GABRG2 define the genetic landscape of defects of GABAA receptors. *Brain Commun* 2021;3(2):fca033.
10. Kadera H, Ohba C, Kato M, et al. De novo GABRA1 mutations in Ohtahara and West syndromes. *Epilepsia* 2016;57(4):566–573.
11. Burgess R, Wang S, McTague A, et al. The Genetic Landscape of Epilepsy of Infancy with Migrating Focal Seizures. *Ann Neurol* 2019;86(6):821–831.
12. Zhang L, Liu X. Clinical phenotype and genotype of children with GABAA receptor $\alpha 1$ subunit gene-related epilepsy. *Front Neurol* 2022;13:941054.
13. Maillard P-Y, Baer S, Schaefer É, et al. Molecular and clinical descriptions of patients with GABAA receptor gene variants (GABRA1, GABRB2, GABRB3, GABRG2): A cohort study, review of literature, and genotype-phenotype correlation. *Epilepsia* 2022;
14. Hernandez CC, Macdonald RL. A structural look at GABAA receptor mutations linked to epilepsy syndromes. *Brain Research* 2019;1714:234–247.
15. Qu S, Zhou C, Howe R, et al. The K328M substitution in the human GABAA receptor $\gamma 2$ subunit causes GEFS+ and premature sudden death in knock-in mice. *Neurobiol Dis* 2021;152:105296.
16. Absalom NL, Liao VWY, Kothur K, et al. Gain-of-function GABRB3 variants identified in vigabatrin-hypersensitive epileptic encephalopathies [Internet]. *Brain Communications* 2020;2(fca162)[cited 2021 Jan 4] Available from: <https://doi.org/10.1093/braincomms/fcaa162>

17. Absalom NL, Liao VWY, Johannesen KMH, et al. Gain-of-function and loss-of-function GABRB3 variants lead to distinct clinical phenotypes in patients with developmental and epileptic encephalopathies. *Nat Commun* 2022;13(1):1822.
18. Ahring PK, Liao VWY, Lin S, et al. The de novo GABRA4 p.Thr300Ile variant found in a patient with early-onset intractable epilepsy and neurodevelopmental abnormalities displays gain-of-function traits. *Epilepsia* 2022;63(9):2439–2441.
19. Ahring PK, Liao VWY, Gardella E, et al. Gain-of-function variants in GABRD reveal a novel pathway for neurodevelopmental disorders and epilepsy. *Brain* 2022;145(4):1299–1309.
20. Chen W, Ge Y, Lu J, et al. Distinct Functional Alterations and Therapeutic Options of Two Pathological De Novo Variants of the T292 Residue of GABRA1 Identified in Children with Epileptic Encephalopathy and Neurodevelopmental Disorders. *Int J Mol Sci* 2022;23(5):2723.
21. Steudle F, Rehman S, Bampali K, et al. A novel de novo variant of GABRA1 causes increased sensitivity for GABA in vitro. *Sci Rep* 2020;10(1):2379.
22. Scheffer IE, Berkovic S, Capovilla G, et al. ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. *Epilepsia* 2017;58(4):512–521.
23. Liao VWY, Chua HC, Kowal NM, et al. Concatenated γ -aminobutyric acid type A receptors revisited: Finding order in chaos. *J Gen Physiol* 2019;151(6):798–819.
24. Liao VWY, Chebib M, Ahring PK. Efficient expression of concatenated $\alpha 1\beta 2\delta$ and $\alpha 1\beta 3\delta$ GABAA receptors, their pharmacology and stoichiometry. *Br J Pharmacol* 2021;178(7):1556–1573.
25. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015;17(5):405–424.
26. Papandreou A, Danti FR, Spaul R, et al. The expanding spectrum of movement disorders in genetic epilepsies. *Dev Med Child Neurol* 2020;62(2):178–191.
27. Brnich SE, Abou Tayoun AN, Couch FJ, et al. Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework. *Genome Med* 2019;12(1):3.
28. Popp B, Trollmann R, Büttner C, et al. Do the exome: A case of Williams-Beuren syndrome with severe epilepsy due to a truncating de novo variant in GABRA1. *Eur J Med Genet* 2016;59(10):549–553.
29. Baker KE, Parker R. Nonsense-mediated mRNA decay: terminating erroneous gene expression. *Current Opinion in Cell Biology* 2004;16(3):293–299.
30. Bureau M, Dalla Bernardina B. Electroencephalographic characteristics of Dravet syndrome. *Epilepsia* 2011;52 Suppl 2:13–23.
31. Karkare KD, Menon RN, Radhakrishnan A, et al. Electroclinical characteristics and syndromic associations of “eye-condition” related visual sensitive epilepsies—A cross-sectional study. *Seizure - European Journal of Epilepsy* 2018;58:62–71.
32. Johannesen KM, Iqbal S, Guazzi M, et al. Structural mapping of GABRB3 variants reveals genotype-phenotype correlations [Internet]. 2021.[cited 2021 Dec 7] Available from: <https://www.medrxiv.org/content/10.1101/2021.06.04.21256727v1>

33. Jiang X, Raju PK, D'Avanzo N, et al. Both gain-of-function and loss-of-function de novo CACNA1A mutations cause severe developmental epileptic encephalopathies in the spectrum of Lennox-Gastaut syndrome. *Epilepsia* 2019;60(9):1881–1894.
34. Berecki G, Bryson A, Terhag J, et al. *SCN1A* gain of function in early infantile encephalopathy. *Ann Neurol* 2019;85(4):514–525.
35. Pan Y, Cummins TR. Distinct functional alterations in SCN8A epilepsy mutant channels. *J Physiol* 2020;598(2):381–401.
36. Masiulis S, Desai R, Uchański T, et al. GABAA receptor signalling mechanisms revealed by structural pharmacology. *Nature* 2019;565(7740):454–459.
37. Pettersen EF, Goddard TD, Huang CC, et al. UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Sci* 2021;30(1):70–82.

Legends

Table 1 Fitted and recorded data for $\alpha 1\beta 3\gamma 2$ GABA_A receptors containing *GABRA1* variants

Xenopus laevis oocytes were injected with cRNA for concatenated constructs containing the indicated variants and subjected to two-electrode voltage-clamp electrophysiology as described in the methods. A Hill equation was fitted to GABA concentration–response datasets by non-linear regression. The difference in fitted GABA sensitivities (EC_{50} values) for variant and wild-type receptors is presented as $\Delta pEC_{50} \pm SD$ where $p = -\log$ for the indicated number (n) of individual oocytes. Analysis for statistical significance was obtained using One-way ANOVA with *post hoc* Dunnett’s test relative to the wild-type receptor. The average maximal current obtained with GABA_{max} (10000 μ M) applications is presented as GABA_{max} $\pm SD$ in nA for the indicated number (n) of individual oocytes. Analysis for statistical significance was obtained using Mann-Whitney U-test comparing variant to wild-type receptor data from the same experimental days. For both statistical analysis, significance is claimed when $P < 0.0001$.

Table 2 Characteristics of the *GABRA1* subgroups

Abbreviations: Ab: absences, aAb: atypical absences, ACTH: adrenocorticotrophic hormone, ADHD: Attention Deficit / Hyperactivity Disorder, ASD: autism spectrum disorder, Behav: behavioral, CBZ: carbamazepine, DE: developmental encephalopathy, DEE: developmental and epileptic encephalopathy, dist.: disturbance, FE: focal epilepsy, FS: febrile seizures, GEFs+: genetic epilepsy with febrile seizures plus, GoF: gain of function, GTC: generalized tonic clonic, ID: intellectual disability, IGE: Idiopathic Generalized Epilepsies, IS: infantile spasm, LEV: levetiracetam, LoF: loss of function, mod.: moderate, My: myoclonic, n.a.: not available; TC: tonic-clonic seizures, VPA: valproic acid.

Figure 1 2D and 3D-position of the variants

(A) Top-view and (B) side-view of $\alpha 1\beta 3\gamma 2$ GABA_A receptor cryo-EM structure (pdb:6hup)³⁶ visualized using ChimeraX³⁷. (C) 18 *GABRA1* variants (yellow spheres) are mapped onto the GABA_A receptor $\alpha 1$ subunit. Since the intracellular domain of the cryo-EM structure is unresolved, p.(Glu403Gln) variant cannot be mapped onto protein structure. (D) Canonical protein sequence of the GABA_A receptor $\alpha 1$ subunit with key regions highlighted (signal peptide (pink), extracellular domain (blue) transmembrane helices (green) and loops (orange), and C-terminus (pale blue)) depicting the position and 19 variants.

Figure 2 Functional analysis of $\alpha 1\beta 3\gamma 2$ GABA_A receptors containing *GABRA1* variants.

(A) cRNA for the wild-type receptor and receptors containing a single variant *GABRA1* subunit was injected into *Xenopus laevis* oocytes and subjected to two electrode voltage clamp experiments as described in the methods. **(B)** GABA concentration-response relationships (CRRs) were generated for wild-type receptors and receptors containing variant *GABRA1* subunits on each experimental day. Representative traces depict CRRs for the wild-type receptor and receptors containing the p.(Val270Ala) and p.(Ser299Asn) *GABRA1* variants. **(C)** Normalised GABA CRRs were plotted as a function of the GABA concentration and the Hill equation was fitted to each dataset by non-linear regression. Datapoints are presented as mean $\pm$ SEM for n = 12-14 independent experiments. Dotted lines indicated the concentrations that lead to half maximal activation (EC_{50}) for each receptor type. Arrows indicated whether the variant leads to an increased (GoF) or a decreased (LoF) GABA sensitivity. **(D)** The difference in GABA sensitivities between wild-type and 19 variant receptors was calculated from the logarithmic conversion of EC_{50} values (ΔpEC_{50} , where $p = -\text{Log}$). Final ΔpEC_{50} datasets for each variant contain data from n = 11-14 independent experiments performed at minimally two independent experimental days. Statistical analysis was performed as described in the Table 1 legend and **** indicates $P < 0.0001$.

Figure 3 Clinical features and genotype-phenotype correlations of *GABRA1* variants

(Left) Correlations between phenotype, genotype (variant type and position) and functional effect (GoF vs LoF) for 24 subjects, carrying 17 different *GABRA1* pathogenic variants. Abbreviations: DEE = developmental and epileptic encephalopathy, GEFs+ = genetic epilepsy with febrile seizures plus, GoF = gain of function, LoF = loss of function. **(Right)** Variant locations mapped on the *GABRA1* subunit of the cryo-EM structure (pdb:6hup)³⁶ visualized using ChimeraX. Grey, red and purple spheres indicate functionally neutral, LoF and GoF variants respectively.

Figure 4 EEG patterns in the different *GABRA1* subgroups

Extracellular domain missense variants with LoF effect p.(Gly251Asp). **(A)** Pat.12, 9 year old, with a FE/GEFs+ phenotype, with infantile onset focal epilepsy and subsequently generalised seizures, moderate ID and behavioural disturbances. The EEG background is well structured. We observed trains of spike and slow waves in the right occipito-posterior temporal region, with occasional diffuse spreading. **(B)** Transmembrane helix TM3 missense variants with LoF effect Pt.22 p.(Ile317Leu). Pat.22, 17-months-old, with developmental delay and refractory GTC seizures. The interictal EEG showed a background slowing and frequent trains of 16 Hz activity in the central regions, with maximum central in the midline. **(C)** Transmembrane loop TM1-TM2

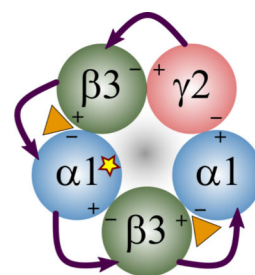
missense variants with LoF effect p.(Asn275Lys). Pat.17, 5 year old, from a family with a GEFs+ phenotype. The interictal EEG shows trains of spike and slow waves bilaterally in the parieto-occipital regions, with occasional diffuse spreading. **(D)** Transmembrane loop TM2- TM3 with LoF effect p.(Pro305Leu). Pat.21, a 10-year-old girl with mild ID and childhood onset epilepsy with prolonged focal autonomic seizures. Intermittent photic stimulation performed induces photoparoxysmal response. **(E)** Transmembrane helix TM4 missense variants with GoF effect p.(Tyr438Cys). Pat.24, 8 year old, with mild-to-moderate ID and drug resistant atypical absences, focal motor seizures and NCSE. The EEG shows generalized 2.5-3 Hz spike and slow waves. Interestingly, EEGs from subjects harbouring LoF variants in the extracellular compartment (A+C), that can be distinguished from the EEG of subjects with transmembrane variants (B+D). EEG parameters: band pass filter 0.5-70 Hz; notch off.

Supplementary Table Electro-clinical features of the *GABRA1* cohort

Abbreviations: aAb: atypical absences, Ab: absences, ADHD: attention deficit/hyperactivity disorder, ASD: autism spectrum disorder, ASM: antiseizure medications, At: atonic, BGS: back-ground slowing, BRV: brivaracetam, CLB: clobazam, CNZ: clonazepam, CT: centro-temporal, D: discharges, DD: developmental delay, DE: developmental encephalopathy, DEE: developmental and epileptic encephalopathy, DQ: development quotient, EEG: electroencephalogram, F: female, FDG PET: fluorodeoxyglucose positron emission tomography, FE: focal epilepsy, FS: febrile seizures, FT: fronto-temporal, fu: follow-up, G: generalized, GEFs+: genetic epilepsy with febrile seizures plus, GMFCS: Gross Motor Function Classification System, GoF: gain of function, GTC: generalised tonic clonic seizures, ID: intellectual disability, IED: interictal epileptiform discharges, IGE: idiopathic generalized epilepsy, IQ: intelligence quotient, IS: infantile spasms, L: left, LCS: lacosamide, LEV: levetiracetam, LMT: lamotrigine, LoF: loss-of-function, M: male, mo: months, MRI: magnetic resonance imaging, my: myoclonus, NA: not available, PB: phenobarbital, PNES: psychogenic non epileptic seizures, PO: parieto-occipital, PT: parieto-temporal, R: right, RUF: rufinamide, Sp: spike, SE: status epilepticus, STP: stiripentol, SVT: supraventricular tachycardia, SW: spike and wave, Sz: seizures, T: tonic, TC: tonic-clonic, TPM: topiramate, VPA: valproic acid, VUS: variant of uncertain significance, y: years, ZNS: zonisamide. *: already reported in literature, †: familial variant, CADD model GRCH37 v1.6

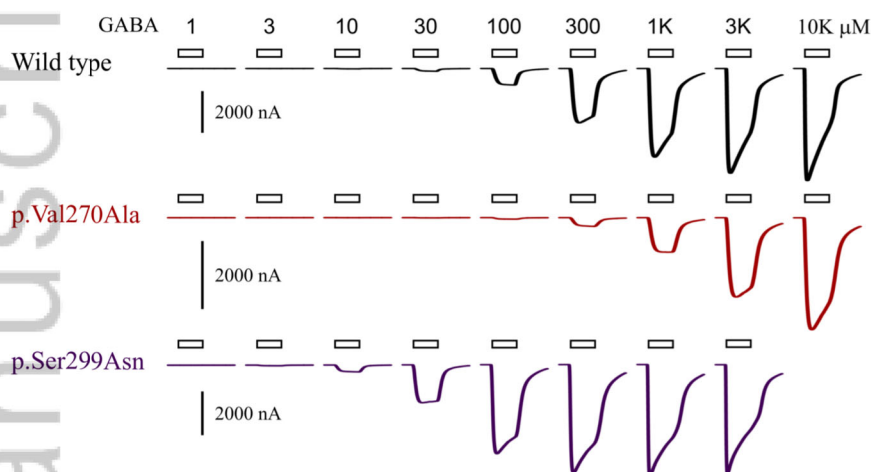
A

Concatenated cDNA construct design for expression of $\alpha 1\beta 3\gamma 2$ GABA_A receptors that are heterozygous for *GABRA1* variants

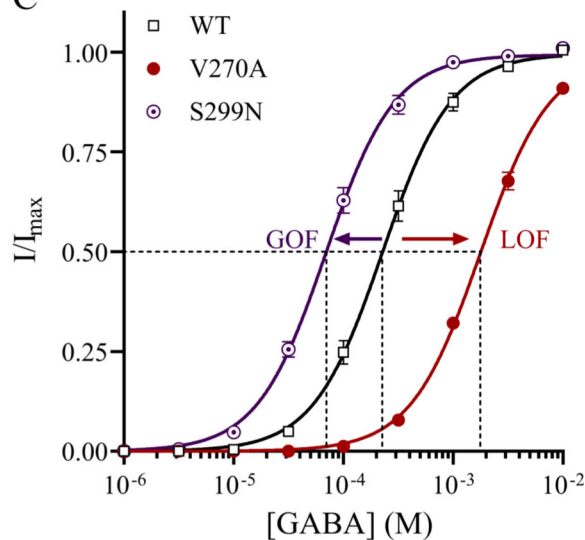


B

Representative traces for wild type and *GABRA1* variant receptors



C



D

Change in GABA sensitivity of receptors with *GABRA1* variants relative to wild type

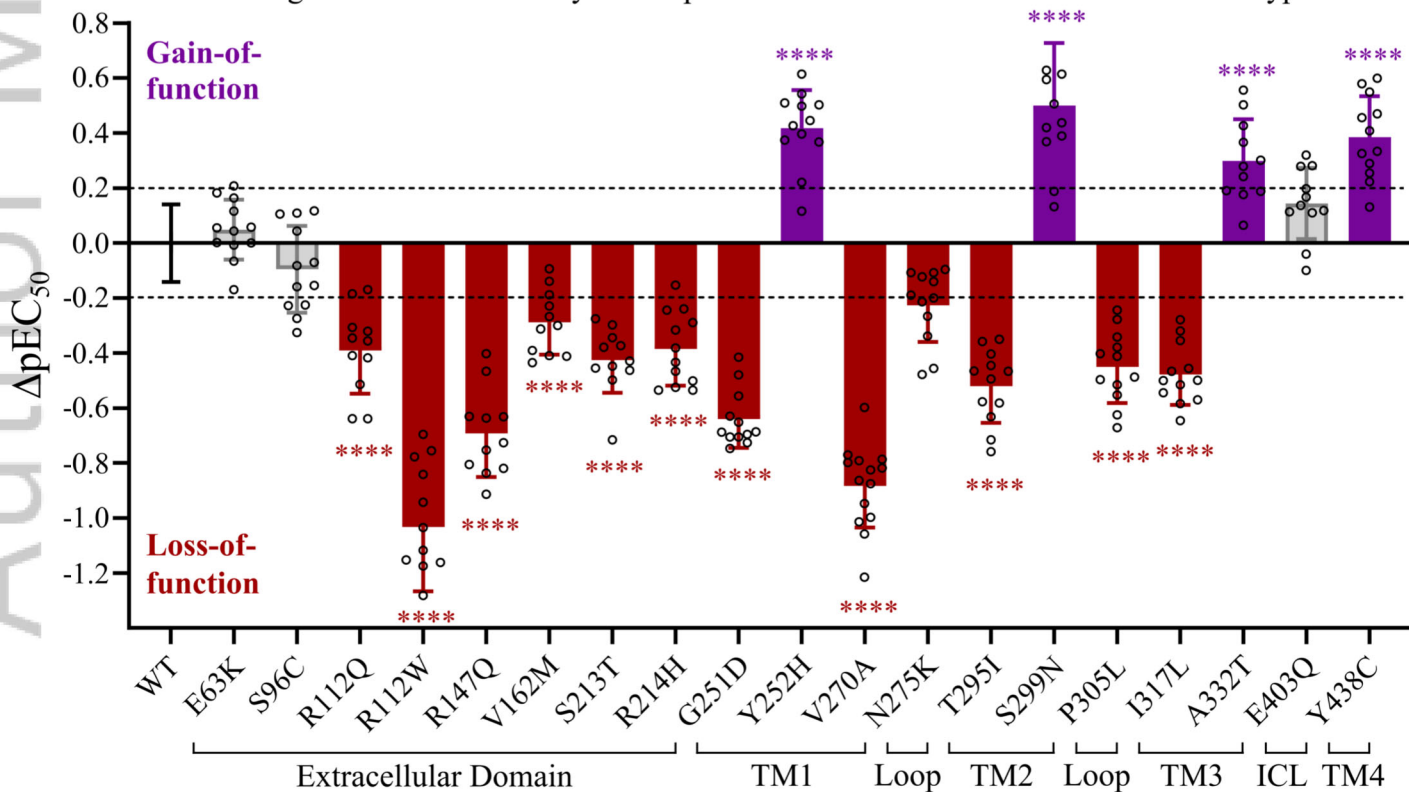
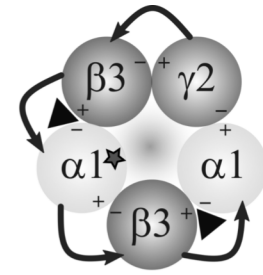


Figure 2 functional data ver1 (2).tif

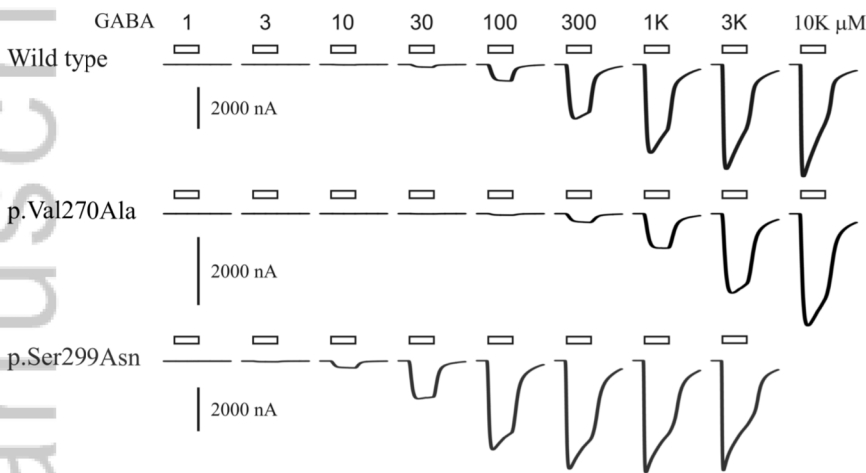
A

Concatenated cDNA construct design for expression of $\alpha 1\beta 3\gamma 2$ GABA_A receptors that are heterozygous for *GABRA1* variants

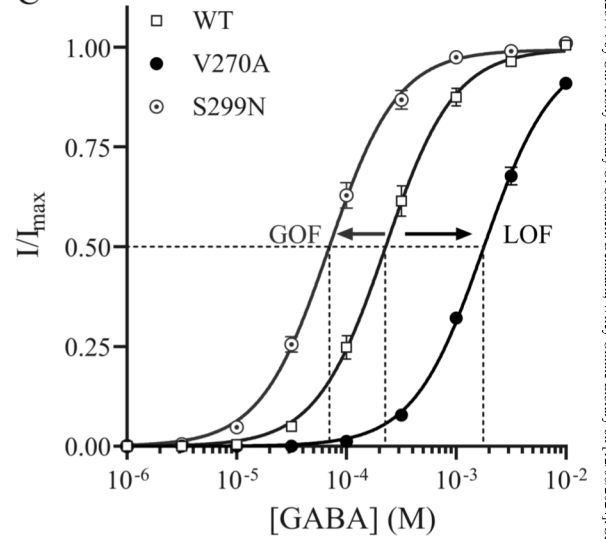


B

Representative traces for wild type and *GABRA1* variant receptors



C



D

Change in GABA sensitivity of receptors with *GABRA1* variants relative to wild type

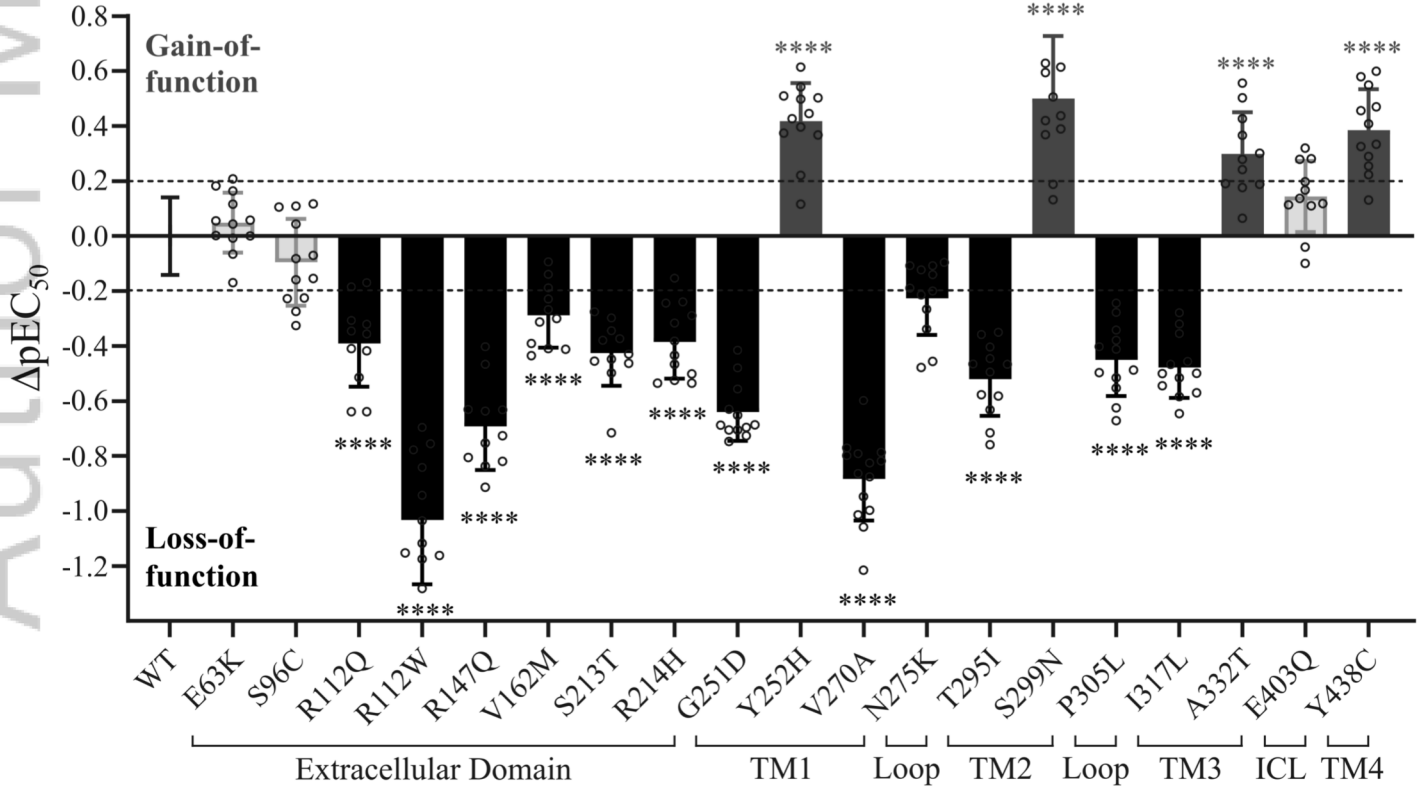


Figure 2 functional data ver1_bw (2) (1).tif

Clinical features and genotype-phenotype correlations of *GABRA1* variants

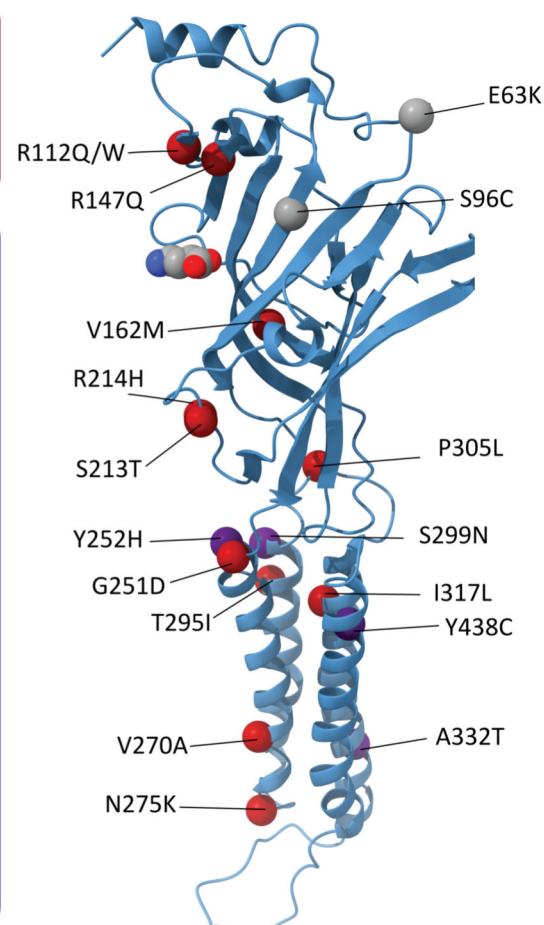
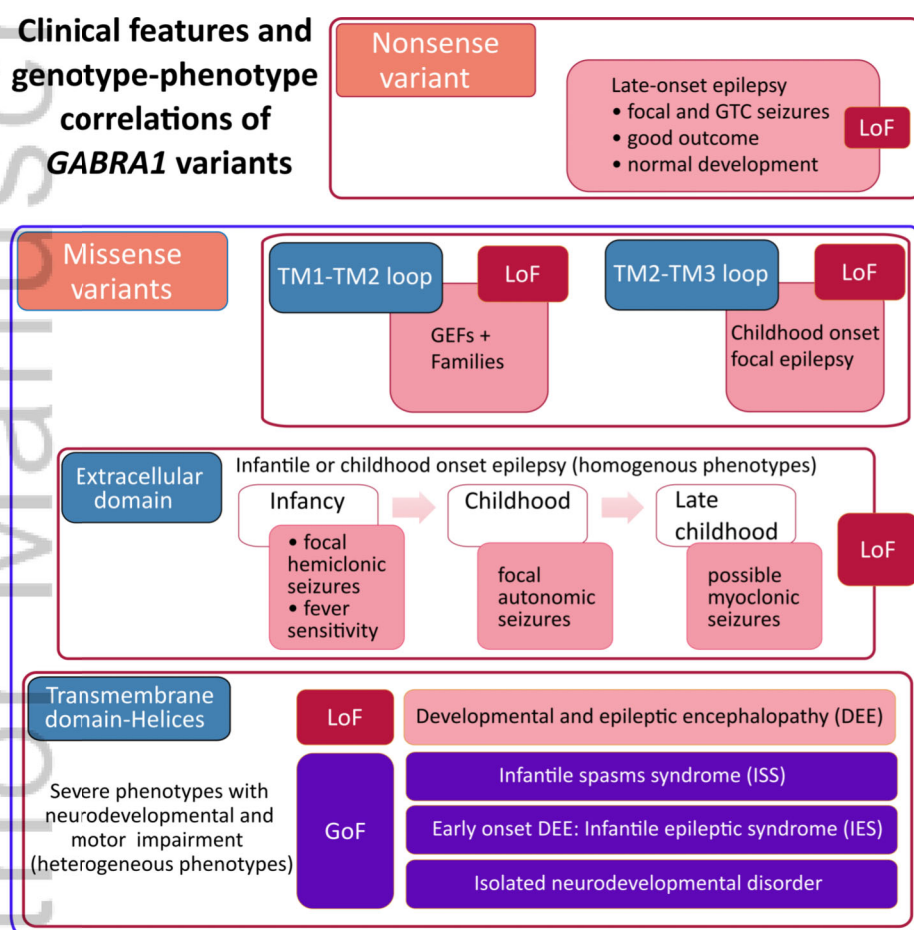


Figure 3 (2) (1).tif

Clinical features and genotype-phenotype correlations of *GABRA1* variants

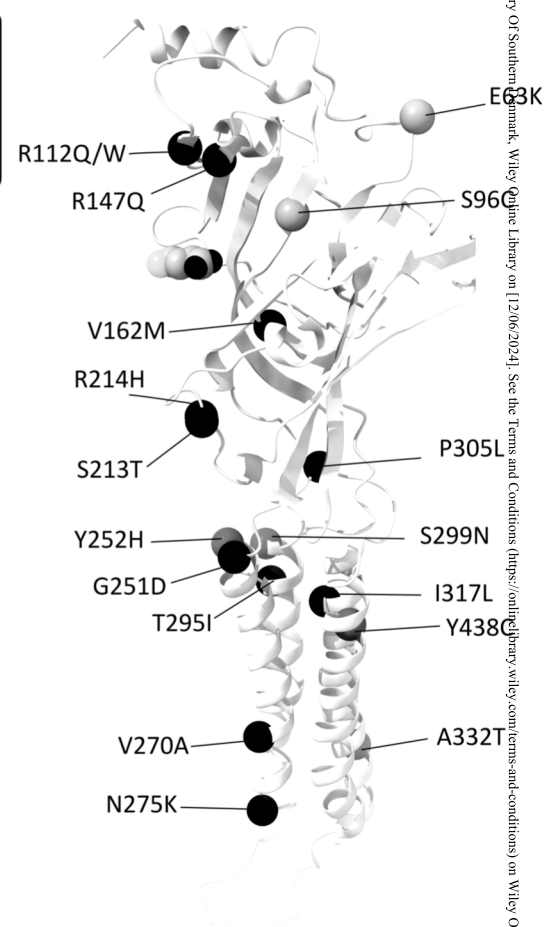
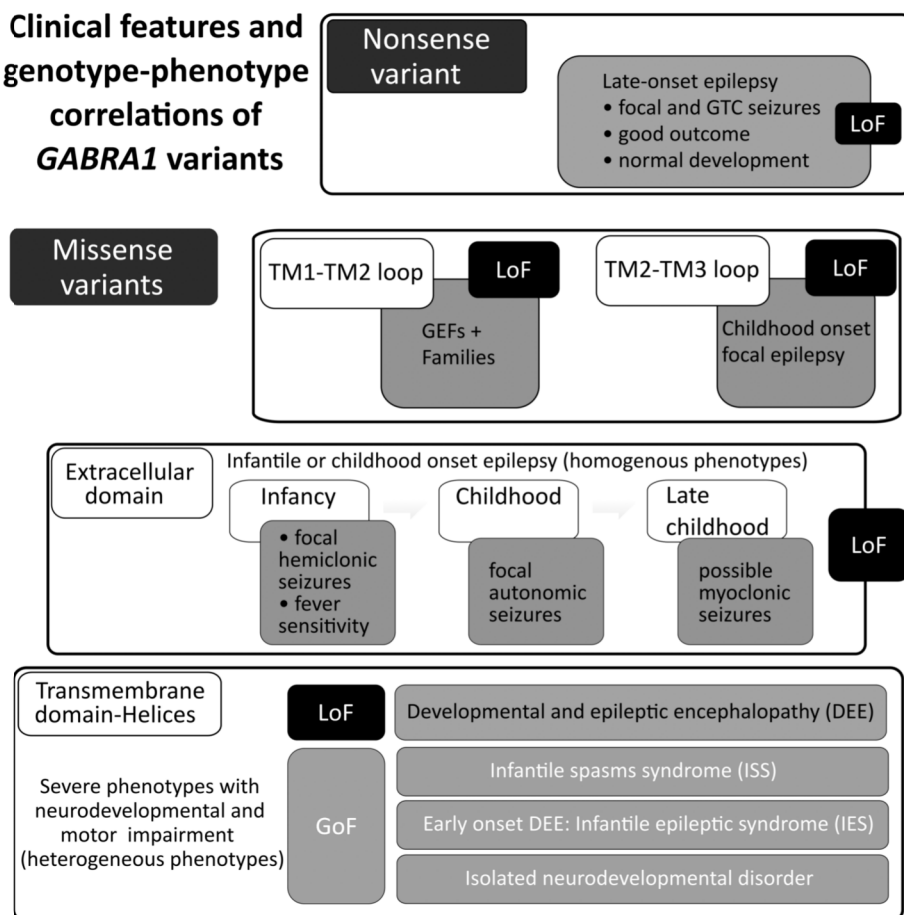


Figure 3_bw (2) (1).tif

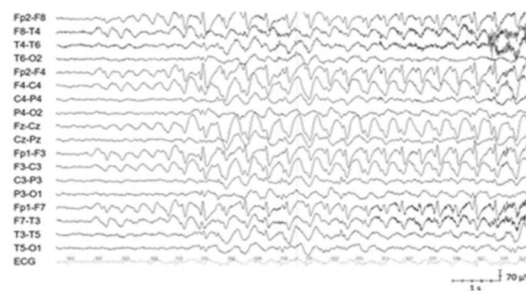
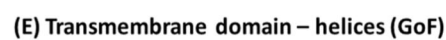
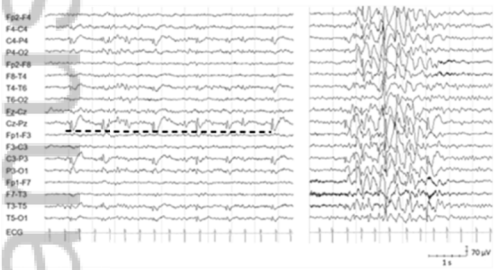
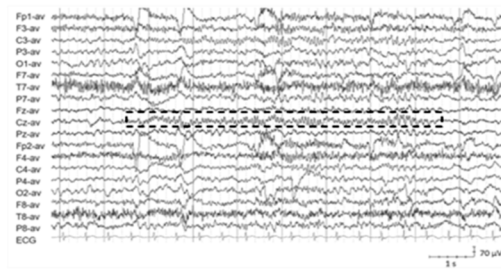


Figure 4 (2).tif

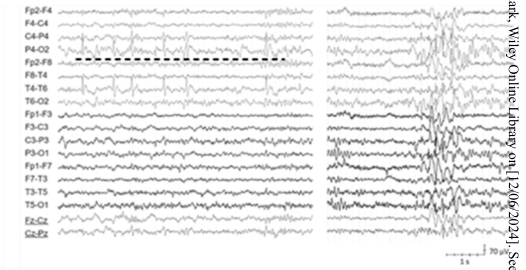
(A) Extracellular domain (LoF)



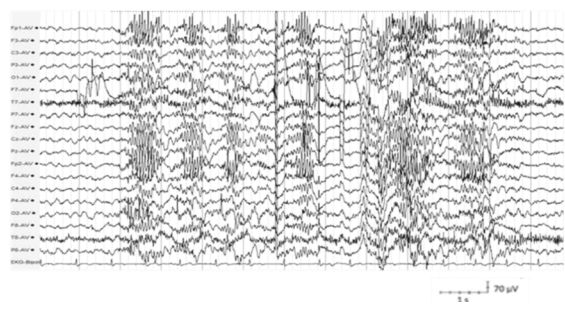
(B) Transmembrane domain – helicef (LoF)



(C) Transmembrane domain – TM1-TM2 Loop (LoF)



(D) Transmembrane domain TM2-T3 loop (LoF)



(E) Transmembrane domain – helices (GoF)

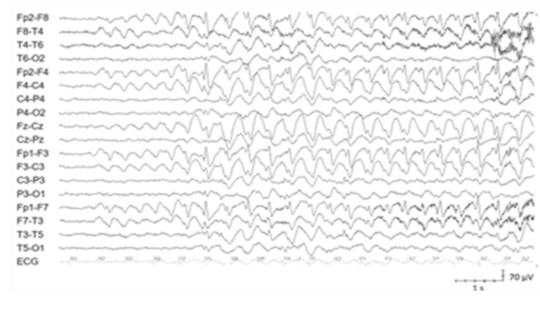
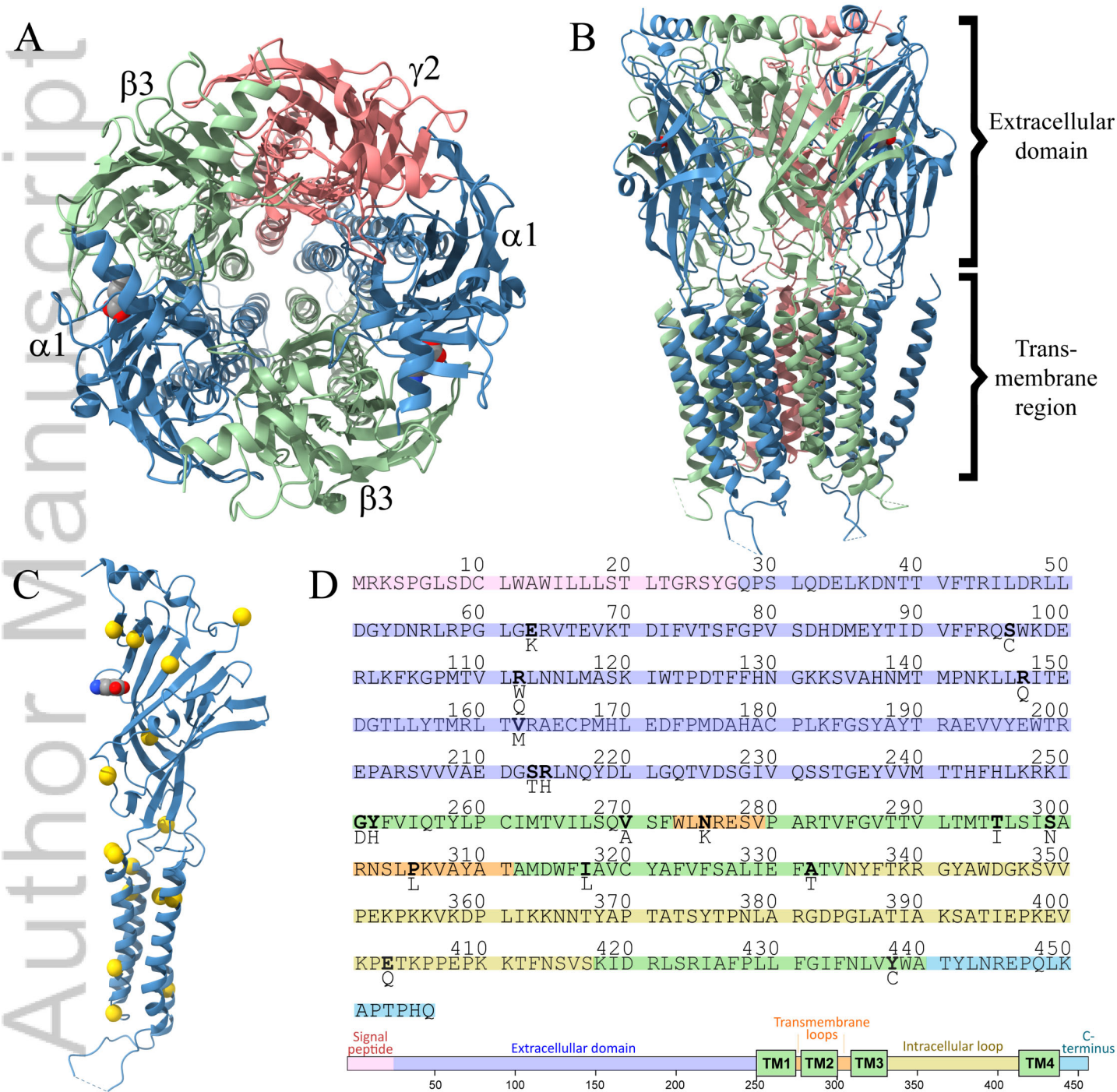


Figure 4_BW.tif



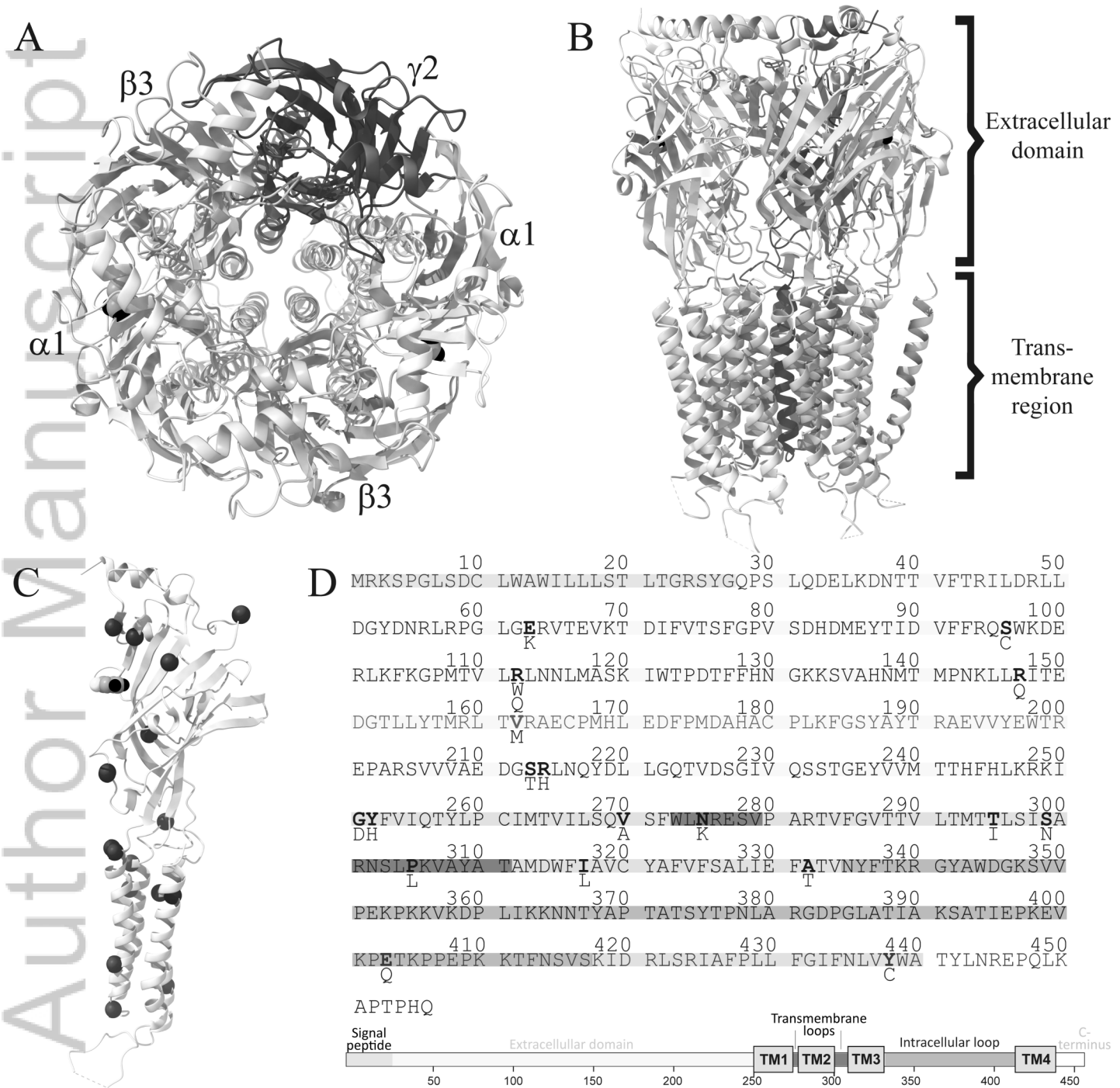


Table 1 Fitted and recorded data for $\alpha 1\beta 3\gamma 2$ receptors containing *GABRA1* variants

$\alpha 1$ variant	$\Delta pEC_{50} \pm SD$	n	<i>P</i> (ΔpEC_{50})	GABA _{max} (nA) $\pm SD$	n	<i>P</i> (GABA _{max})	Functional outcome	ACMG
WT	0.00 $\pm$ 0.14	83	NA	1.00 $\pm$ 0.38	189	NA	NA	NA
Glu63Lys	0.05 $\pm$ 0.11	12	0.995	0.94 $\pm$ 0.44	29	0.2411	No Change	Likely benign
Ser96Cys	-0.10 $\pm$ 0.16	12	0.5366	1.05 $\pm$ 0.36	24	0.7984	No Change	Likely benign
Arg112Gln	-0.39 $\pm$ 0.17	11	<0.0001	0.92 $\pm$ 0.41	33	0.2265	LOF	Pathogenic
Arg112Trp	-1.03 $\pm$ 0.23	11	<0.0001	0.88 $\pm$ 0.53	24	0.0822	LOF	Pathogenic
Arg147Gln	-0.69 $\pm$ 0.17	11	<0.0001	0.88 $\pm$ 0.37	23	0.2951	LOF	Likely pathogenic
Val162Met	-0.29 $\pm$ 0.12	11	<0.0001	0.93 $\pm$ 0.38	24	0.4803	LOF	Likely pathogenic
Ser213Thr	-0.43 $\pm$ 0.12	11	<0.0001	0.86 $\pm$ 0.40	30	0.0358	LOF	Pathogenic
Arg214His	-0.39 $\pm$ 0.14	12	<0.0001	0.86 $\pm$ 0.35	30	0.0741	LOF	Pathogenic
Gly251Asp	-0.64 $\pm$ 0.11	12	<0.0001	0.72 $\pm$ 0.29	30	0.0013	LOF	Pathogenic
Tyr252His	0.42 $\pm$ 0.14	12	<0.0001	0.89 $\pm$ 0.35	27	0.5171	GOF	Pathogenic
Val270Ala	-0.88 $\pm$ 0.15	14	<0.0001	0.42 $\pm$ 0.25	30	<0.0001	LOF/LOF	Pathogenic
Asn275Lys	-0.23 $\pm$ 0.14	12	<0.0001	1.12 $\pm$ 0.52	24	0.5229	LOF	Likely pathogenic
Thr295Ile	-0.52 $\pm$ 0.13	12	<0.0001	0.80 $\pm$ 0.43	32	0.0704	LOF	Pathogenic
Ser299Asn	0.50 $\pm$ 0.22	12	<0.0001	1.10 $\pm$ 0.59	29	0.8029	GOF	Pathogenic
Pro305Leu	-0.45 $\pm$ 0.13	12	<0.0001	0.83 $\pm$ 0.33	30	0.0821	LOF	Likely pathogenic
Ile317Leu	-0.48 $\pm$ 0.11	12	<0.0001	0.95 $\pm$ 0.39	30	0.5714	LOF	Pathogenic
Ala332Thr	0.30 $\pm$ 0.15	11	<0.0001	0.77 $\pm$ 0.37	29	0.031	GOF	Pathogenic
Glu403Gln	0.14 $\pm$ 0.14	11	0.1557	0.85 $\pm$ 0.25	24	0.6295	No Change	Likely benign
Tyr438Cys	0.39 $\pm$ 0.16	12	<0.0001	0.94 $\pm$ 0.46	26	0.7248	GOF	Pathogenic

Table 1 Fitted and recorded data for $\alpha 1\beta 3\gamma 2$ receptors containing *GABRA1* variants

$\alpha 1$ variant	$\Delta pEC_{50} \pm SD$	n	$P (\Delta pEC_{50})$	GABA _{max} (nA) $\pm SD$	n	$P (GABA_{max})$	Functional outcome	ACMG
WT	0.00 $\pm$ 0.14	83	NA	1.00 $\pm$ 0.38	189	NA	NA	NA
Glu63Lys	0.05 $\pm$ 0.11	12	0.995	0.94 $\pm$ 0.44	29	0.2411	No Change	Likely benign
Ser96Cys	-0.10 $\pm$ 0.16	12	0.5366	1.05 $\pm$ 0.36	24	0.7984	No Change	Likely benign
Arg112Gln	-0.39 $\pm$ 0.17	11	<0.0001	0.92 $\pm$ 0.41	33	0.2265	LOF	Pathogenic
Arg112Trp	-1.03 $\pm$ 0.23	11	<0.0001	0.88 $\pm$ 0.53	24	0.0822	LOF	Pathogenic
Arg147Gln	-0.69 $\pm$ 0.17	11	<0.0001	0.88 $\pm$ 0.37	23	0.2951	LOF	Likely pathogenic
Val162Met	-0.29 $\pm$ 0.12	11	<0.0001	0.93 $\pm$ 0.38	24	0.4803	LOF	Likely pathogenic
Ser213Thr	-0.43 $\pm$ 0.12	11	<0.0001	0.86 $\pm$ 0.40	30	0.0358	LOF	Pathogenic
Arg214His	-0.39 $\pm$ 0.14	12	<0.0001	0.86 $\pm$ 0.35	30	0.0741	LOF	Pathogenic
Gly251Asp	-0.64 $\pm$ 0.11	12	<0.0001	0.72 $\pm$ 0.29	30	0.0013	LOF	Pathogenic
Tyr252His	0.42 $\pm$ 0.14	12	<0.0001	0.89 $\pm$ 0.35	27	0.5171	GOF	Pathogenic
Val270Ala	-0.88 $\pm$ 0.15	14	<0.0001	0.42 $\pm$ 0.25	30	<0.0001	LOF/LOF	Pathogenic
Asn275Lys	-0.23 $\pm$ 0.14	12	<0.0001	1.12 $\pm$ 0.52	24	0.5229	LOF	Likely pathogenic
Thr295Ile	-0.52 $\pm$ 0.13	12	<0.0001	0.80 $\pm$ 0.43	32	0.0704	LOF	Pathogenic
Ser299Asn	0.50 $\pm$ 0.22	12	<0.0001	1.10 $\pm$ 0.59	29	0.8029	GOF	Pathogenic
Pro305Leu	-0.45 $\pm$ 0.13	12	<0.0001	0.83 $\pm$ 0.33	30	0.0821	LOF	Likely pathogenic
Ile317Leu	-0.48 $\pm$ 0.11	12	<0.0001	0.95 $\pm$ 0.39	30	0.5714	LOF	Pathogenic
Ala332Thr	0.30 $\pm$ 0.15	11	<0.0001	0.77 $\pm$ 0.37	29	0.031	GOF	Pathogenic
Glu403Gln	0.14 $\pm$ 0.14	11	0.1557	0.85 $\pm$ 0.25	24	0.6295	No Change	Likely benign
Tyr438Cys	0.39 $\pm$ 0.16	12	<0.0001	0.94 $\pm$ 0.46	26	0.7248	GOF	Pathogenic

Table 2 Clinical characteristics of the *GABRA1* subgroups based on location and functional effects of variants.

	Missense variants				Nonsense variant
Domain	<i>N-terminus</i>	<i>Transmembrane domain</i>			
Position	Extra cellular	Helices TM1, TM2, TM3	Helices TM1 TM2, TM3, TM4	Loops TM1-TM2 and TM2-TM3	N-terminal
Subjects / variants	11/7	3/3	4/4	4/2	2/1 (1 family)
Inheritance	8 <i>de novo</i> , 3 n.a.	2 <i>de novo</i> , 1 n.a.	4 <i>de novo</i>	3 inherited, 1 n.a.	Maternal
ID	Mild-mod 9/11 (82%) Severe 1/11 (9%) No 1/11 (9%)	Mild-mod 2/3 (67%) Severe 1/3 (33%)	Mild-mod 3/4 (75%) Severe 1/4 (25%)	Mild 4/4 (100%)	No (2/2) (100%)
Neurological examination	Normal 3/11 (27%) Poor coordination / mild ataxia 8/11 (73%)	Normal 1/3 (33%) Hypotonia 1/3 (33%) Hypotonic-dystonic cerebral palsy 1/3 (33%)	Normal 2/4 (50%) Ataxia 1/4 (25%) Pyramidal signs + dystonia 1/4 (25%)	Normal 4/4 (100%)	Normal 2/2 (100%)
Epilepsy	11/11	3/3	3/4	4/4	2/2
Age at seizure onset (median and range)	8 mo (3–48 mo)	8 mo (3–48 mo)	27 mo (5 mo-10 y)	21 mo (9 mo-4 y)	14,5 y (14-15 y)
Syndromes	GEFS+	DEE	DEE / DE	GEFS+	IGE
Seizure types at onset	Focal 6/11 (55%) FS 3/11 (27%) GTC 1/11 (9%)	Focal 2/3 (67%) GTC 1/3 (33%)	IS 1/3 (33%) My + aAb 1/3 (33%) GTC 1/3 (33%)	FS 3/4 (75%) Focal 1/4 (25%)	GTC (1) Focal to bilateral TC (1)
Seizure types at follow up	Focal 7/11 (64%) GTC + My 5/11 (46%)	My 2/3 (66%) Ab 1/3 (33%) GTC 1/3 (33%)	aAb 1/3 (33%) Focal 1/3 (33%) GTC 1/3 (33%)	GTC 2/4 (50%) Ab 1/4 (25%) My 1/4 (25%)	GTC 1/2 (50%) Focal to bilateral TC 1/2 (50%)
Seizure outcome	Sz free 6/11 (55%) Treatable 4/11 (36%) Refractory 1/11 (9%)	Treatable 1/3 (33%) Refractory 2/3 (67%)	Sz free 1/3 (33%) Treatable 2/3 (67%)	Sz free 3/4 (75%) Treatable 1/4 (25%)	Sz free 2/2 (100%)
Fever sensitivity	9/10 (90%)	1/3 (33%)	0/2	3/4 (75%)	0/2
Photosensitivity	0/11	2/3 (67%)	0/2	2/4 (50%)	0/2
Most effective ASM	LEV (5/6), VPA (3/4)	LEV (2/3), VPA (1/1)	ACTH (1/1), VPA (2/2)	LEV (4/4)	CBZ (1/1)
Additional features	Behav dist 2/11 (18%) ADHD 1/11 (0,9%) ASD 2/11 (18%)	Behav dist 1/3 (33%) ADHD 1/3 (33%)	Behav dist 1/3 (33%) ASD 2/3 (67%)	ASD 1/4 (25%)	-
Recurrent variants	p.Arg112Gln p.Gly251Asp				