Gain-of-function variants in *GABRD* reveal a novel pathway for neurodevelopmental disorders and epilepsy

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Abstract

A potential link between *GABRD* encoding the δ subunit of extrasynaptic $GABA_A$ receptors and neurodevelopmental disorders has largely been disregarded due to conflicting conclusions from early studies. However, we identified seven heterozygous missense *GABRD* variants in 10 patients with neurodevelopmental disorders and generalized epilepsy. One variant occurred in two sibs of healthy parents with presumed somatic mosaicism, another segregated with the disease in three affected family members, and the remaining five occurred *de novo* in sporadic patients.

Electrophysiological measurements were used to determine the functional consequence of the seven missense δ subunit variants in receptor combinations of α1β3δ and α4β2δ $GABA_A$ receptors. This was accompanied by analysis of electro-clinical phenotypes of the affected individuals.

We determined that five of the seven variants caused altered function of the resulting α1β3δ and α4β2δ $GABA_A$ receptors. Surprisingly, four of the five variants led to gain-of-function effects whereas one led to a loss-of-function effect. The stark differences between the gain-of-function and loss-of function effects were mirrored by the clinical phenotypes. Six patients with gain-of-function variants shared common phenotypes: neurodevelopmental disorders with generalized epilepsy, behavioral issues, and various degrees of intellectual disability. Six patients with gain-of-function variants shared common phenotypes: neurodevelopmental disorders with behavioral issues, various degrees of intellectual disability, generalized epilepsy with atypical absences and generalized myoclonic and/or bilateral tonic-clonic seizures. The EEG showed qualitative analogies among the different gain-of-function variant carriers

consisting of focal slowing in the occipital regions often preceding irregular generalized epileptiform discharges, with frontal predominance. In contrast, the one patient carrying a loss-of-function variant had normal intelligence, no seizure history but has a diagnosis of autism spectrum disorder and suffering from elevated internalizing psychiatric symptoms.

We hypothesize that increase in tonic GABA-evoked current levels mediated by δ-containing extrasynaptic $GABA_A$ receptors lead to abnormal neurotransmission, which represent a novel mechanism for severe neurodevelopmental disorders. In support of this, the electro-clinical findings for the gain-of-function *GABRD* variants resemble the phenotypic spectrum reported in patients with missense *SLC6A1* (GABA uptake transporter) variants. This also indicates that the phenomenon of extrasynaptic receptor over-activity is observed in a broader range of patients with neurodevelopmental disorders, since *SLC6A1* loss-of-function variants also lead to overactive extrasynaptic δ-containing $GABA_A$ receptors. These findings have implications when selecting potential treatment options, since a substantial portion of available anti-seizure medication act by enhancing GABAergic function either directly or indirectly, which could exacerbate symptoms in patients with gain-of-function *GABRD* variants.

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Abbreviations: ADHD = attention deficit hyperactivity disorder; ASD = autism spectrum disorder; DEE = developmental and epileptic encephalopathy; GAT1 = GABA uptake

transporter encoded by the *SLC6A1* gene; GGE = genetic generalized epilepsy; ID = intellectual disability; $P_{O,max}$ = estimated maximum open probability.

Introduction

Ion channels play crucial roles in normal mammalian brain development and function. They are prerequisites for neuronal excitability and regulate the intricate balance between excitatory and inhibitory neurotransmission. Malfunction of ion channels (channelopathies) are therefore often associated with neurodevelopmental disorders and epilepsies, ranging from treatable focal or genetic generalized epilepsies (GGE) to severe developmental and epileptic encephalopathies (DEE).[1]

γ–Aminobutyric acid type A receptors ($GABA_A$Rs) are the primary inhibitory ion channel of the brain. Pathogenic variants of $GABA_A$R subunits (*GABRA1-A5*, *GABRB1-B3* and *GABRG2*)[1-6] have been found in many GGE and DEE patients. $GABA_A$Rs are pentameric proteins comprised of combinations of 1-5 of the 19 identified subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3), with the most common subtypes containing two α subunits, two β subunits, and one γ2 or δ subunit.[7] Whether the receptor complex contains a γ2 or δ subunit has substantial influence on receptor function and $GABA_A$Rs are broadly divided into synaptic γ2-containing receptors, responsible for fast phasic inhibition, and extrasynaptic δ-containing receptors, responsible for persistent tonic inhibition.

Many studies have indicated that disease-causing variants in synaptic γ2-containing $GABA_A$Rs lead to loss-of-function molecular phenotypes (*i.e.* decreased receptor activity) through either impaired protein synthesis, impaired protein translocation to the cell surface, or impairments in receptor activation.[7] Loss-of-function of δ-containing receptors due to variants in the coding *GABRD* gene have also previously been suggested to be implicated in generalized epilepsy.[8] Further analysis, however, could not confirm the importance of these variants in humans.[9] A recent analysis of targeted gene sequencing efforts in ~7000 patients with neurodevelopmental disorders and epilepsy therefore concluded that *GABRD* variants are unlikely to be associated with epilepsy.[10]

Contrary to previous studies, we report 10 individuals harboring *de novo* or inherited variants in *GABRD* with clinical manifestations ranging from autism spectrum disorder (ASD) to DEE.

Phenotypic spectrum characterization was complemented with detailed analysis of the functional characteristics of each variant. Based on the collective data, we propose that gain-of-function (*i.e.* increased receptor activity) *GABRD* variants represent a novel mechanism that cause severe neurodevelopmental disorder and lead to generalized epilepsy. Since many anti-seizure medications act by enhancing GABAergic tone, this can exacerbate symptoms in patients with gain-of-function variants, hence determining whether *GABR* variants result in either gain- or loss-of-function is an essential process to ensure the correct medication is prescribed.

Materials and methods

Patients

We screened the *GABRD* gene in a cohort of 933 individuals with various childhood-onset epilepsies sequentially referred for diagnostic gene panel testing. Genomic DNA from blood was extracted with standard methods, and next-generation sequencing libraries were prepared and sequenced as previously described.[11] Furthermore, we collected additional patients with epilepsy or neurodevelopmental disorders through international collaborations and GeneMatcher.[12] Epilepsy, developmental and medical histories were collected, with review of medical files, EEG and MRI reports, and administration of a validated epilepsy questionnaire where possible. Where available, video-EEG recordings were reviewed by a neurologist with EEG expertise. Seizure types were classified using International League Against Epilepsy terminology based on the description provided by the treating physician.[13] The study was approved by the local ethics committees of the collaborating centers. Informed consent was obtained from all patients and/or parents/legal guardians.

Genotypic analysis

The genetic findings were obtained through either targeted epilepsy panels (n = 4), whole exome sequencing (n = 3) or Sanger sequencing (n = 3; family 1: patient 6 and 7, family 2: patient 10). Variant classification was performed using the most recent American College of Medical Genetics and Genomics criteria (ACMG). All variants are reported based on transcript

NM_000815.4. The presumed pathogenicity of the variants was based on their *de novo* status or their segregation in related patients; their absence from appropriate population controls or their detection at extremely low frequencies; their location in critical and conserved domains of the *GABRD* protein; the level of *in silico* predictive evidence (SIFT, PolyPhen, MutationTaster) to support a deleterious effect; as well as the similarity in the phenotypes.

Functional studies

Electrophysiological studies were conducted using *Xenopus laevis* oocytes expressing α1β3δ or α4β2δ receptors. For α1β3δ receptors, concatenated pentameric α1β3δ constructs encoding wild type and variant *GABRD* subunits were designed using our recently described concatenation methodology and complementary RNAs (cRNA) were produced from linearized cDNA.[14] For α4β2δ receptors, cRNA mixtures of free subunits were made using a biased ratio of α4:β2:δ, 3:1:3 to limit expression of polluting binary α4β2 receptors. The cRNA mixtures encoding α1β3δ or α4β2δ $GABA_A$ receptors were then injected into *Xenopus* oocytes (25 ng/oocyte). Injected oocytes were incubated for 2-4 days in modified Barth's solution. This was followed by two-electrode voltage clamp electrophysiology using techniques previously described.[15-18] Briefly, oocytes were placed in a recording chamber, voltage clamped at a holding potential of -60 mV and continuously perfused with a saline solution (ND96). Increasing concentrations of GABA solutions were applied and the electrophysiological responses were recorded to generate the GABA concentration-response relationship of each of the variant and wild type receptors. The estimated maximum open probability ($P_{O,max}$) of each receptor was obtained as described previously.[14] In brief, estimated $P_{O,max}$ values were obtained by co-applying a cocktail of a maximally efficacious concentration of GABA (316 μM) with the allosteric modulators allopregnanolone (3.16 μM), etomidate (31.6 μM) and delta-selective compound 2 (DS2, 10 μM). Further methodological details are described in Supplementary 1.

Data and statistical analysis for *Xenopus laevis* oocytes experiments

GABA concentration-response relationship datasets were compiled from a minimum of six (n = 6) individual experiments conducted on a minimum of two batches of oocytes. Baseline-subtracted peak current amplitudes (I) of all responses were measured and the Hill equation was fitted to the data. Following this, the data were normalized to the maximal fitted response

($I_{max\_fit\_GABA}$) for each individual oocyte ($I/I_{max\_fit\_GABA}$). Final GABA concentration-response relationship datasets were obtained by combining the data for all the individual experiments and the Hill equation was fitted to the final datasets by nonlinear regression using GraphPad Prism 8. Unless a more complex model with four variables was statistically preferrable ($P < 0.05$, F-test in GraphPad Prism 8), a simple monophasic model with three variables was used (*i.e.*, fixed Hill slope of 1) with efficacy at infinitely low compound concentrations was set to 0. All statistical analysis was performed using GraphPad Prism 8. Statistical tests used for comparing GABA pEC_{50} ($-LogEC_{50}$) values: parametric ANOVA, *post hoc* Dunnett's test. Statistical test used for comparing $GABA_{max}$-evoked current amplitudes and estimated $P_O(GABA_{max})$ values: non-parametric ANOVA, Kruskal-Wallis, *post hoc* Dunn's test.

Data availability

De-identified data will be made available to those eligible. This includes the *GABRD* database and data used for all analysis in the manuscript and its supplementary material. Data will be stored for a minimum of seven years.

Results

Patients

Our initial cohort consisted of 933 individuals referred for genetic testing at the Danish Epilepsy Centre, Filadelfia. Within this cohort, we identified presumed pathogenic *GABRD* variants in three individuals from two unrelated families. Alerted by this association, we identified another seven individuals with epilepsy or neurodevelopmental disorders through international collaborations and GeneMatcher. In total we report a cohort of 10 individuals (three male, seven female), from eight unrelated families.

Genetics

All 10 individuals harbored presumed pathogenic *GABRD* missense variants and all variants either occurred *de novo* or segregated with a homogeneous phenotype in one family. One variant occurred presumably *de novo* in two sibs (p.V442I), thus one of the parents must be mosaic for the variant. Another variant was inherited in a family with affected mother and her affected twin boys (p.T291I). The remaining variants (p.M87L, p.P122A, p.P257L, p.L260V, and p.I284T) occurred *de novo* in sporadic patients. All seven variants were predicted as damaging by at least two different prediction tools (SIFT, PolyPhen, MutationTaster), and have CADD scores above 20. Six out of seven variants where absent in gnomAD and our internal dataset, whereas one variant (p.M87L) were seen once in gnomAD.

The position of the different variants spans a large part of the δ subunit protein sequence from the N-terminal end (p.M87L) to the final transmembrane domain M4 (p.V442I) (Fig. 1A). Notably, four of the variants (p.P257L, p.L260V, p.I284T and p.T291I) reside in the M1 and M2 transmembrane domains that are key to forming a functional ion channel. Five of the seven variants cause changes in amino acid residues that are fully (p.P122A, p.P257L and p.T291I) or highly (p.L260V and p.I284T) conserved among human $GABA_AR$ subunits (Fig. 1B). For the remaining two variants (p.M87L and p.V442I), the amino acid residue in the wild type δ subunit differs from the consensus of the other human $GABA_AR$ subunits.

In vitro expression of δ-containing receptors

Electrophysiological experiments were performed to decipher the effects of the seven δ subunit variants on $GABA_AR$ function. δ subunits are associated with different $GABA_AR$ α subunits depending on the specific brain region. For example, the δ subunit has been shown to assemble with the α1 subunit in hippocampal interneurons whereas it assembles with the α4 subunit in the thalamus.[19, 20] To ensure that the analysis include the major δ-containing receptor classes in brain, initial analysis was performed at α1β3δ receptors and key variants were further investigated at α4β2δ receptors.

It is widely accepted that δ-containing receptors are extremely difficult to express and study reliably in heterologous expression systems.[21] The main reason for this relates to the fact that these receptors give rise to very low current amplitudes when activated by GABA, and as a consequence of this, even low percentages of polluting receptor populations, such as binary α1β3 or α4β2 receptors, can significantly influence electrophysiological measurements. To circumvent this problem, optimized expression procedures were used for the two receptor subtypes. For α1β3δ receptors, we used our recently described concatenated construct design.[14] This entails linking the cDNA for each of the five subunits that make up the pentameric receptor such that the expressed receptors have a predetermined stoichiometry in the canonical arrangement (Fig. 2A). For α4β2δ receptors, we used the method of biased ratios of free subunits.[22] Hence, *Xenopus laevis* oocytes were injected with cRNA for concatenated α1β3δ receptors or α4β2δ receptors in a 3-1-3 ratio and subjected to two-electrode voltage clamp electrophysiology (note that an extended description of the methods and electrophysiological results are presented in Supplementary 1).

Functional analysis of variants at α1β3δ receptors

The wild type α1β3δ receptor and receptors with variant δ subunits all responded to applications of GABA albeit with substantial differences in observed current amplitudes (Fig. 2B, top row). The average $GABA_{max}$-evoked current amplitude for the wild type α1β3δ receptor was 62 nA (Fig. 2C, Table 1). Similar values of 38-50 nA were observed with the δ^{M87L} and δ^{V442I} variants and a marginally higher value of 180 nA was seen with the δ^{P257L} variant. In contrast, a significantly lower value of 12 nA was observed with the δ^{P122A} variant, and significantly higher values ranging 560-1100 nA were observed with δ^{L260V}, δ^{I284T} and δ^{T291I} variants. δ-containing $GABA_ARs$ receptors typically display no or limited desensitization upon prolonged exposure to GABA. Hence, it is rarely meaningful to analyze desensitization

characterizations for these receptors, but substantial changes can be visually observed from the current decay profiles. The wild type receptor along with six of the seven variant-containing receptors exhibited no noticeable current decay in their response during $GABA_{max}$ applications, only the receptor containing the δ^{P122A} variant displayed current decay suggesting that this receptor is prone to desensitization upon prolonged exposure to GABA (Fig. 2B, bottom row). The fitted GABA concentration-response relationship for the wild type α1β3δ receptor revealed an EC_{50} value of 27 μM (Fig. 2D, Table 1). Similar results were observed for six of seven variant receptors with EC_{50} values in the 27-33 μM range, while the fitted value for receptors with the δ^{P257L} variant was 12 μM, which represents a significant increase in GABA sensitivity.

To decipher how the variants cause changes to GABA-evoked current amplitudes, the ability of GABA to open (gate) the various receptors was determined. δ-containing receptors are unique among $GABA_A$Rs for having a low open-probability even in the presence of saturating concentrations of GABA. This fundamental intrinsic property ensures that current flow is measured throughout long-lasting fluctuations in ambient GABA concentrations. Hence, the estimated receptor open probability upon $GABA_{max}$ applications, $P_O(GABA_{max})$, was obtained as previously described (further details in Supplementary 1).[14] The estimated $P_O(GABA_{max})$ for the wild type receptor was 0.37% and data for δ^{M87L} and δ^{V442I} variants revealed similar values of 0.33-0.34% (Fig. 2E, Table 1). The δ^{P122A} variant revealed a significantly lower value of 0.21%, while the δ^{P257L}, δ^{L260V}, δ^{I284T} and δ^{T291I} variants all revealed significantly higher values ranging 2.8-6.0%. Thus, five of the seven variants led to significant changes in estimated $P_O(GABA_{max})$ with decreased GABA-evoked gating observed for the δ^{P122A} variant and increased GABA-evoked gating observed for variants in the transmembrane M1 and M2 domains (δ^{P257L}, δ^{L260V}, δ^{I284T} and δ^{T291I}). This demonstrate that the observed changes in $GABA_{max}$-activated current amplitudes are the result of alterations in receptor gating efficiency.

Based on the functional assessments, it is possible to conclude how the individual variants affect resulting α1β3δ receptors. The δ^{P122A} variant resulted in a 5-fold decrease in the average maximal current amplitude and combined with its increased propensity to desensitize, this represents a loss-of-function trait. The four variants in transmembrane domains M1 and M2 (δ^{P257L}, δ^{L260V}, δ^{I284T} and δ^{T291I}) all resulted in a 3-18-fold increase in current amplitudes, which represent a gain-of-function trait. While the current amplitude increase of the δ^{P257L} variant was

only 3-fold, this receptor also displayed increased sensitivity to GABA reinforcing the gain-of-function traits. Finally, no functional changes were noted for the δ^{M87L} and δ^{V442I} variants.

Functional analysis of variants at α4β2δ receptors

The five δ subunit variants that displayed significant changes to α1β3δ receptor function was next evaluated in combination with α4 and β2 subunits. Maximal average $GABA_{max}$-evoked current amplitude for the wild type α4β2δ receptor was 130 nA and a similar value of 91 nA was observed for the δ^{P257L} variant (Fig. 3A, Table 1). A significantly lower value of 57 nA was observed for the δ^{P122A} variant and significantly higher values ranging 600-700 nA was observed for the δ^{L260V}, δ^{I284T} and δ^{T291I} variants. The fitted GABA concentration-response relationship for the wild type α4β2δ receptor revealed an EC_{50} value of 1.6 μM (Fig. 3B, Table 1). A larger value of 2.5 μM was observed for the δ^{P122A} variant signifying decreased GABA sensitivity, while significantly smaller values ranging 0.33-0.57 μM were observed for the δ^{P257L}, δ^{L260V}, δ^{I284T} and δ^{T291I} variants signifying increased GABA sensitivity.

The estimated $P_O(GABA_{max})$ for the wild type receptor was 7.0% (Fig. 3C, Table 1). A lower value of 3.8% was observed with the δ^{P122A} variant while higher values ranging 31-58% were observed for the δ^{P257L}, δ^{L260V}, δ^{I284T} and δ^{T291I} variants (further details in Supplementary 1). Overall, the five variants led to significant changes in estimated $P_O(GABA_{max})$ with a pattern mirroring that observed with α1β3δ receptors. Decreased GABA-evoked gating was observed for the δ^{P122A} variant while increased GABA-evoked gating was observed for variants in the transmembrane M1 and M2 domains (δ^{P257L}, δ^{L260V}, δ^{I284T} and δ^{T291I}).

In summary, the δ^{P122A} variant displayed a decrease in current amplitudes and sensitivity to GABA which represents loss-of-function traits. In contrast, the transmembrane domain M1 and M2 variants (δ^{P257L}, δ^{L260V}, δ^{I284T} and δ^{T291I}) caused increase in the average maximal current amplitude and/or increased sensitivity to GABA which represent gain-of-function traits. Hence, conclusions from experiments with α4β2δ receptors match those with α1β3δ receptors.

Does the wild type δ subunit reflect population variants?

Two variants (p.M87L and p.V442I) did not reveal any significant functional changes. Interestingly, one of these two variants (p.M87L) is also seen in the gnomAD database. More

than 200 supposedly benign *GABRD* variants are found in the gnomAD database of which the more common ones can be seen as population variants. To investigate whether some of the common *GABRD* variants differ functionally from the canonical wild type δ subunit, two additional variants (p.R220H and p.V259I) were tested. Overall, the functional differences between receptors with the wild type δ subunit and the three tested gnomAD variants were marginal to non-existent (Supplementary 1). This show that the wild type δ subunit reflect the function of common population variants and underscore that the substantial changes observed with the p.P122A, p.P257L, p.L260V, p.I284T and p.T291I variants above represent true divergence from *GABRD* function observed in the normal population.

Phenotypic characterization

In contrast to the loss-of-function (p.P122A) or gain-of-function (p.P257L, p.L260V, and p.I284T and p.T291I) effect of five of the variants, the p.M87L and p.V442I variants did not show any detectable functional changes. The three individuals, carrying these two variants, are therefore not included in our phenotypic analysis. The excluded individuals included two siblings with difficult to treat multifocal epilepsy, intellectual disability, autism spectrum disorder, attention deficit hyperactivity disorder (ADHD), and congenital immunodeficiency, and a sporadic patient with intractable generalized epilepsy, fine motor abnormalities, and slow processing speed. The phenotypes of the three individuals are described in more details in Supplementary 2.

The remaining seven patients had a median age of 10 years, ranging from 3 to 37 years. Their clinical features are summarized in Table 2. All six patients with a gain-of-function variant suffered from generalized epilepsy and various degrees of learning difficulties or intellectual disability, whereas the patient with a loss-of-function variant had ASD, normal intelligence and no seizure history. Due to this clear phenotypic difference, we describe the electroclinical feature of the patients with gain-of-function *GABRD* variants separately from the individual with a loss-of-function variant.

Patients with gain-of-function *GABRD* variants

Median age at epilepsy onset was 10.5 months, ranging from 4 months to 4 years. The most common seizure types included atypical absences, generalized myoclonic seizures, tonic seizures and generalized tonic-clonic seizures. Seizures occurred daily in 4/6 patients. Fever sensitivity was reported in one patient. Epilepsy was medically refractory in 5/6 patients. Syndrome classification was atypical generalized epilepsy in 4, and DEE in 2. The interictal EEGs showed similar qualitative features among different patients, including background slowing in 5/6 patients, high voltage slow waves in the occipital regions in 4/6 patients and epileptiform discharges in 5/6 patients, mainly consisting of irregular generalized 3 Hz spike and slow waves with frontal predominance in 4/5 patients (Fig. 4). Patient 3 who suffered from an intractable DEE with myoclonic and generalized tonic clonic seizures, passed away at the age of three years.

Motor delay was identified in 4/6 patients, with regression or stagnation at seizure onset in at least two patients. Median age of walking was 18 months (range months 12 – 32 months). One patient could not walk at 3 years of age. Learning difficulties or intellectual disability (ID) was present in all. One had learning difficulties requiring special education, two had mild ID, one had mild to moderate ID, whereas two had severe to profound ID. The two most severely affected patients were non-verbal, whereas the remaining four were verbally fluent. The behavioral and psychiatric profile included four with ADHD, and one with autistic features including ritualistic behavior and hair pulling. Brain MRI was unremarkable in two of three patients for whom data were available; mild non-specific abnormalities in the frontal regions were reported in one patient.

Patient with loss-of-function *GABRD* variant

We only found one individual with a loss-of-function variant in *GABRD*. This individual was a 10 year, 5-month-old female. She meets the criteria for ASD using gold standard diagnostic tools (ADOS + ADI). Her full-scale IQ was in an average range (FSIQ = 87), with consistent nonverbal (NVIQ = 89) and verbal (VIQ = 86) scores. However, her adaptive skills were in the low range (adaptive behavior composite = 66). She had elevated internalizing psychiatric symptoms. She was verbally fluent and had no seizure or regression history.

Discussion

Here we demonstrate that heterozygous missense variants in the *GABRD* gene encoding the δ subunit of extrasynaptic $GABA_AR$s cause neurodevelopmental disorders with or without generalized epilepsy. This conclusion is supported by multiple lines of evidence: variants occurred *de novo* or segregated with the phenotype in one family and were absent from control databases; variants occurred at residues that exhibit a high level of evolutionary conservation; and variants demonstrated marked $GABA_AR$ dysfunction (either loss-of-function or gain-of-function) in functional analysis.

Functional analysis

We analyzed seven different variants from a cohort of 10 patients from seven unrelated families with neurodevelopmental disorders, epilepsy and presumed pathogenic missense variants in *GABRD*. Significant and clear functional effects were observed for five of the seven variants and of these four (p.P257L, p.L260V, p.I284T and p.T291I) resulted in gain-of-function effects and one (p.P122A) in loss-of-function effect. Hence, four variants cause increased tonic GABAergic tone in the brain and only one variant decrease GABAergic tone. Both gain- and loss-of-function effects were primarily the result of alterations to the gating efficiency of GABA in resulting α1β3δ and α4β2δ receptors. Based on the magnitude of the collective changes, we assign a severity rank order of the gain-of-function variants of p.L260V > p.I284T > p.T291I > p.P257L. All the individuals, showing functional *GABRD* alterations, suffered from neurodevelopmental disorders with or without epilepsy. Two variants (p.M87L and p.V442I) did not reveal any significant functional changes. Although we cannot exclude different alterations that were missed with our experimental system, it is likely that the p.M87L and p.V442I variants represent benign polymorphisms that are not responsible for the clinical phenotype of the individuals. This is supported by the observations that the p.M87L variant is seen in the gnomAD database and that the p.V442I variant is likely inherited from a mosaic parent.

Gain-of-function *GABRD* variants

The six individuals, harboring four different gain-of-function variants (p.P257L, p.L260V, p.I284T and p.T291I) suffered from atypical-generalized epilepsy and various degrees of neurodevelopmental disorders. In classical GGE syndromes, cognitive and motor functions are usually within the normal range. However, the mutual relationships between epilepsy and neuropsychiatric comorbidities are complex and bidirectional, especially in children with a genetic etiology.[23] The EEG showed qualitative analogies among the different patients, consisting of focal slowing in the occipital regions often preceding irregular generalized epileptiform discharges, with frontal predominance. The most common seizure types included atypical absences, generalized myoclonic and generalized tonic-clonic seizures. These electroclinical findings are overlapping with the phenotypic spectrum reported for some of the variants in other $GABA_AR$ subunit genes[1-3, 24] and in particular with clinical and EEG features of patients with loss-of-function variants in *SLC6A1* which code for the GABA uptake transporter GAT1.[25]

The EEG pattern suggests an extended underlying cortico-thalamic network typical for generalized epilepsies with diffuse spike and slow waves, as previously shown in both humans and in animal models.[26] Several EEG-functional MRI studies documented a similar pathophysiological brain network across different GGE syndromes, consisting of increase of the blood oxygenation level-dependent signal in thalamus and decrease in prefrontal, parietal and posterior cingulate cortex,[27] likely gated in the precuneus.[28] A transiently low posterior network synchrony may precede the generalized spike-wave onset, in agreement with the occipital EEG slowing, often preceding the generalized epileptiform discharges, both in the present patients as well as in patients with *SLC6A1* associated epilepsy.[29]

In summary, the gain-of-function *GABRD* variants resulted in epilepsy with severity ranging from treatable generalized epilepsy (p.T291I, p.P257L), to severe DEE (p.L260V, p.I284T). Although the number of patients is too small to draw firm conclusions, the data indicate a correlation between the severity of the phenotype, the severity of the EEG abnormalities, and the severity of the gain-of-function effect in electrophysiological analysis (*i.e.,* p.L260V worst and p.P257L mildest). The patient who carried the p.L260V variant passed away at the age of three years, highlighting the severity of the phenotype associated with the strong gain-of-function.

Loss-of-function *GABRD* variant

The only individual carrying a loss-of-function variant (p.P122A) had ASD, normal intelligence and no seizures. ASD is a neurodevelopmental disorder encompassing severe deficits in socialization and communication abilities, and repetitive behaviors.[30] An increasing number of studies have described several *GABR* gene deficiency associated with the occurrence of ASD, including *GABRB3, GABRG3, GABRA5,*[31, 32] and *GABRA4* both independently and through interaction with *GABRA1*.[33] Our data suggests that loss-of-function *GABRD* variants might be included as well among causative genes for ASD. The hypothesis that loss-of-function variants in *GABRD* are disease-causing is further supported by the lack of protein truncating variants in the gnomAD database (pLi=0,99), however further studies are needed to confirm this.

The concept that gain-of-function *GABRD* can cause epilepsy

δ-containing receptors are responsible for mediating long-lasting (tonic) inhibition and are abundantly expressed at extrasynaptic locations in hippocampus, amygdala, neocortex, thalamus, hypothalamus, and cerebellum. Here, they respond to ambient concentrations of GABA and spill-over from synapses and play important roles in regulating neurophysiological responses such as movement, learning and memory.[34] To serve in this role, they have a high sensitivity to GABA and a unique low open probability upon GABA binding. This ensures that receptors respond to low concentrations of GABA with a delicately measured current flow. Despite the low open probability, inhibitory neurotransmission via δ-containing receptors is still substantial and tonic inhibition is believed responsible for > 50% of the total chloride ion flux in hippocampal and thalamocortical neurons.[35] It is thus not surprising that variants that cause substantial changes in tonic current levels significantly affect neurotransmission. While the concept that epilepsy can be caused by increased tonic GABAergic tone may seem counterintuitive, recent studies have pointed to a complex role of δ-containing receptors showing that these can either reduce or increase the activity of neurons depending on the neuronal subtype and its state of excitability.[36, 37] Furthermore, three key lines of evidence from animal models and humans support the seemingly paradoxical role of these receptors.

First, four of the six individuals with gain-of-function variants in *GABRD* had absence seizures. Three individuals from one family suffered from intractable absence epilepsy, whereas the fourth individual had atypical absences as part of an intractable DEE with multiple seizure types. Increased tonic $GABA_AR$ current amplitude in thalamocortical neurons was found in an

established rat model of absence epilepsy.[38] Furthermore, pharmacological enhancement of δ-containing receptor activity in thalamocortical neurons was shown to be sufficient to elicit absence seizures in wild type animals.[26, 38] Hence, the patient data corroborate experimental evidence that enhanced *GABRD* activity in thalamus can lead to absence seizures.

Second, the electroclinical features observed in the presented patients overlap with the phenotypes described in patients with loss-of-function variants in *SLC6A1*.[25, 39] Presumably, GAT1 deficiency result in an accumulation of GABA in synapses as well as peri-synaptically, which leads to increased activation of synaptic and extrasynaptic $GABA_A$Rs.[40] In fact, due to their higher GABA sensitivity, extrasynaptic receptors are more likely to be affected by such elevated GABA levels. Patients with GAT1 deficiency are suffering from developmental delay, mild to moderate intellectual disability and epilepsy with (atypical) absences, and myoclonic atonic seizures,[25, 39] similar to what we observe in patients with gain-of-function variants in *GABRD*. Hence, it is likely that loss-of-function GAT1 variants primarily cause neurodevelopmental delay and epilepsy via enhanced tonic GABAergic activity.

Finally, in a recent study we identified gain-of-function variants in *GABRB3* in two patients with vigabatrin-hypersensitive epileptic encephalopathies.[41] This finding was surprising since all analyzed *GABRB3* variants hitherto had been characterized as causing loss-of-function. Furthermore, the drug vigabatrin acts by increasing ambient levels of GABA and it was unclear how this could affect synaptic β3-containing receptors. The *GABRD* data presented in this study might explain this conundrum. δ subunits are associated with β3 subunits in many brain regions, hence, we speculate that the observed hypersensitivity reaction to vigabatrin in the gain-of-function *GABRB3* patients was caused by further exacerbation of already elevated tonic GABAergic currents. Interestingly, one of the two *GABRB3* variants, p.T287I, is paralog to p.T291I in *GABRD,* which was found in the family with intractable absence epilepsy and mild to moderate intellectual disability. None of the three family members have tried vigabatrin, however, as for the *GABRB3* gain-of-function variants this drug should not be prescribed to patients with *GABRD* gain-of-function variants. Furthermore, we propose that drugs that increase GABAergic tone such as benzodiazepines and neurosteroids should be avoided in patients with gain-of-function $GABA_A$R variants or loss-of-function GABA transporter variants.

Conclusion

Our data underscore that changes in tonic current levels via δ-containing receptors in humans can have multifaceted impacts on neuronal development and function. Conventional thinking dictates that *GABR*-associated epilepsy in humans relates to loss-of-function perturbations in synaptic $GABA_AR$ subunits. Gain-of-function *GABRD* variants have not previously been associated with epilepsy and the observation that increased tonic currents represent a novel pathway for neurodevelopmental disorders significantly challenges our understanding of the role of the GABAergic system in epilepsy. We propose that variants in other genes such as *SLC6A1* converge on this pathway and that increased tonic currents via δ-containing receptors represent a core issue for a wide range of patients. Since gain-of-function in the GABAergic system has not been related to neurodevelopmental disorders, previous drug-discovery efforts have rarely focused on drugs that lower GABAergic activity. Current treatment options are therefore limited for these patients and new treatment strategies are greatly needed. Hence, it is of utmost importance to further investigate the role of extrasynaptic δ-containing receptors in neuronal networks and hopefully these discoveries will fuel new drug-discovery efforts.

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Competing interests

TSS is an employee of GeneDx, Inc

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Supplementary material

Supplementary material is available at *Brain* online.

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Figure legends

Figure 1. Approximate location of *GABRD* variants. (**A**), Schematic representation of a single $GABA_AR$ δ subunit (encoded by *GABRD*) where the four transmembrane domains that participate in forming the receptor pore are indicated with M1-M4. The approximate location of seven missense *GABRD* variants is indicated with resulting amino acid substitutions. Results from the functional experiments (Figure 2 and 3) for each variant is indicated below. VUS = variant of unknown significance. (**B**), Alignment of the $GABA_AR$ δ subunit sequence with that of 12 commonly found human $GABA_AR$ subunits. Only sequences in the immediate vicinity of the seven variant positions are shown. Alignment was performed using MegAlign Pro 17 and is presented using the Shapely color scheme.

Figure 2. Functional analysis of seven *GABRD* variants in α1β3δ receptors. (**A**), The concatenated pentameric α1-β3-α1-δ-β3 cDNA construct is illustrated with four linkers (purple) and resulting expressed fusion protein viewed from the extracellular side. (**B**), *Xenopus laevis* oocytes were injected with cRNA for concatenated receptors and subjected to two electrode voltage-clamp electrophysiology. Representative traces show $GABA_{max}$(316 μM)-evoked currents at receptors containing the indicated δ subunit variants. Bars above the traces designate the 50-s application time. Top row depicts all traces on the same current amplitude scale (200 nA). Bottom row depicts the same traces scaled to identical size. (**C**)**,** $GABA_{max}$-evoked peak-current amplitudes for the indicated receptors are depicted as box with whiskers, where the box marks the first, second and third quartiles and whiskers extend to minimum and maximum values observed for n = 27-30 experiments. The mean value is indicated for each dataset (Table 1). Datasets were significantly different ($P < 0.0001$, ANOVA Kruskal-Wallis test) with the indicated significance levels between variant and the wild type receptor (*post hoc* Dunn's test). (**D**), Normalized GABA-evoked peak current amplitudes are depicted as mean ± SD as a function of the GABA concentration for n = 6-9 experiments for the indicated receptors. A Hill equation was fitted to the data using non-linear regression and fitted EC_{50} values are indicated in the panel. Full regression results are presented in Table 1 and Supplementary 1. (**E**), Estimated open probabilities were calculated by comparing the response of $GABA_{max}$ (316 μM) to the response of a mixture of $GABA_{max}$, allopregnanolone (3.16 μM), etomidate (31.6 μM) and DS2 (10 μM) for the indicated receptors (also see Supplementary 1). Data are depicted as box with whiskers for n = 15-19 experiments with

indication of mean values (Table 1) and were significantly different ($P < 0.0001$, ANOVA Kruskal-Wallis test, *post hoc* Dunn's test). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

Figure 3. Functional analysis of five *GABRD* variants in α4β2δ receptors. *Xenopus laevis* oocytes were injected with cRNA mixtures of free α4, β2 and δ subunits in a 3:1:3 ratio and subjected to two electrode voltage-clamp electrophysiology. (**A**), $GABA_{max}$-evoked peak-current amplitudes for the indicated receptors are depicted as box with whiskers, where the box marks the first, second and third quartiles and whiskers extend to minimum and maximum values observed for $n = 30$ experiments. The mean value is additionally indicated for each dataset (Table 1). Datasets were significantly different with the indicated significance levels between variant and the wild type receptor ($P < 0.0001$, ANOVA Kruskal-Wallis test, *post hoc* Dunn's test). (**B**), Normalized GABA-evoked peak current amplitudes are depicted as mean ± SD as a function of the GABA concentration for $n = 11$-14 experiments for the indicated receptors. A Hill equation was fitted to the data using non-linear regression and fitted EC_{50} values are indicated in the panel. Full regression results are presented in Table 1 and Supplementary 1. (**C**), Estimated open probabilities were calculated by comparing the response of $GABA_{max}$ (316 μM) to the response of a mixture of $GABA_{max}$, allopregnanolone (3.16 μM), etomidate (31.6 μM) and DS2 (10 μM) for the indicated receptors (also see Supplementary 1). Data are depicted as box with whiskers for $n = 18$-22 experiments with indication of mean values (Table 1) and were significantly different ($P < 0.0001$, ANOVA Kruskal-Wallis test, *post hoc* Dunn's test). *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

Figure 4. EEG features of patients with *GABRD* variants. In Patient 2 (p.P257L, age 6 y, 11 mo), with mild intellectual disability and treatable epilepsy, the EEG background is well structured, with superimposed irregular high voltage 4 Hz activity in the occipital-posttemporal regions (red lines), with dipole in the prefrontal derivations (arrow). This activity is recurring with high frequency, do not have the feature of Posterior Slow Waves of Youth, and is therefore clearly abnormal for age. In Patient 5 and Patient 6 (p.T291I, age 10 y, 6 mo), twin brothers with mild and mild-to-moderate intellectual disability respectively and daily absences, the EEG is characterized by discrete background slowing, abnormal high voltage 3.5-4 Hz activity in the occipital-posttemporal regions (red lines) and generalized 2.5-3 Hz spike / sharp and waves with posterior start and frontal predominance (arrow), in more (Patient 6) or less prolonged

trains. Patient 4 (p.I284T, age 6 y, 6 mo - 7 y, 6 mo), with severe developmental and epileptic encephalopathy, has a diffuse slowing and disorganization of the EEG background, as well as bursts of irregular 4 Hz activity in the occipital-posttemporal regions (red line), and very frequent irregular trains of generalized 3 Hz sharp and slow waves with frontal predominance (arrows). EEG parameters: band pass filter 1-70 Hz; 50 Hz notch on.

Figure 1

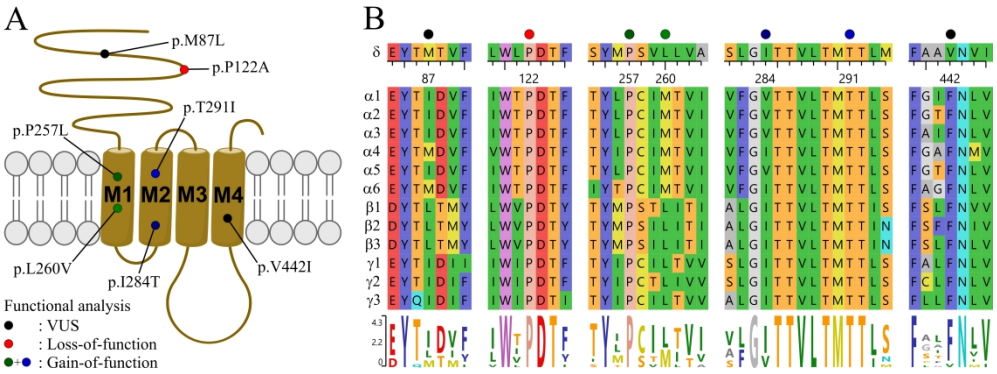

Fig. 1

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Figure 2

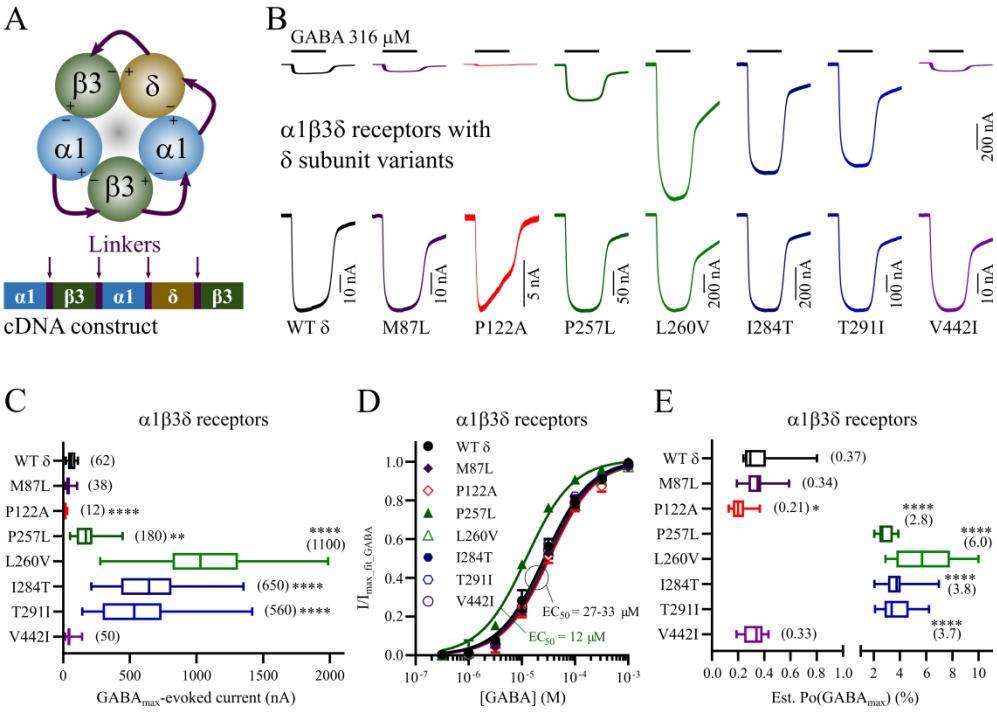

Fig. 2

527x394mm (236 x 236 DPI)

Figure 3

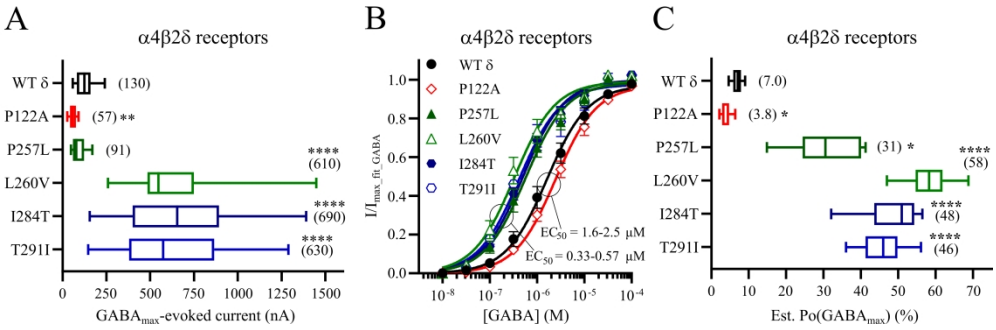

Fig. 3

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Figure 4

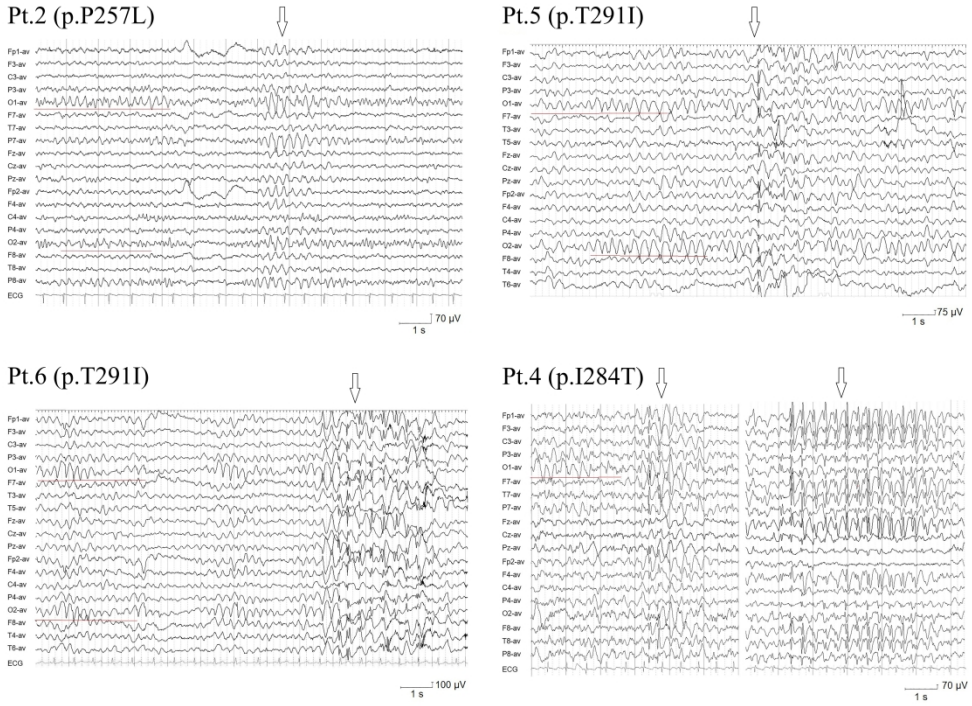

Fig. 4

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Table 1 Functional parameters of α1β3δ and α4β2δ receptors containing *GABRD* variants

Construct	GABA EC_{50} (pEC_{50}) (n) μM (M ± SEM)	$GABA_{max}$ (n) nA ± SD	Est. $P_O(GABA_{max})$ (n) % ± SD
α1-β3-α1-δ-β3	**27 (4.58 ± 0.02) (9)**	**62 ± 29 (29)**	**0.37 ± 0.18 (18)**
α1-β3-α1-δ(M87L)-β3	31 (4.51 ± 0.02) (8)	38 ± 20 (28)	0.34 ± 0.10 (16)
α1-β3-α1-δ(P122A)-β3	33 (4.48 ± 0.02) (6)	12 ± 6 (30) ****	0.21 ± 0.06 (18) *
α1-β3-α1-δ(P257L)-β3	12 (4.91 ± 0.02) (9)	180 ± 90 (27) **	2.8 ± 0.6 (19) ****
α1-β3-α1-δ(L260V)-β3	29 (4.54 ± 0.01) (9)	1100 ± 400 (28) ****	6.0 ± 2.4 (18) ****
α1-β3-α1-δ(I284T)-β3	29 (4.55 ± 0.01) (8)	650 ± 270 (30) ****	3.8 ± 1.1 (17) ****
α1-β3-α1-δ(T291I)-β3	27 (4.57 ± 0.02) (9)	560 ± 310 (29) ****	3.7 ± 1.2 (16) ****
α1-β3-α1-δ(V442I)-β3	30 (4.53 ± 0.02) (9)	50 ± 30 (29)	0.34 ± 0.10 (15)
α4β2δ	**1.6 (5.79 ± 0.02) (12)**	**130 ± 50 (30)**	**7.0 ± 1.3 (22)**
α4β2δ(P122A)	2.5 (5.61 ± 0.01) (14)	57 ± 20 (30) **	3.8 ± 1.2 (22) *
α4β2δ(P257L)	0.57 (6.24 ± 0.02) (13)	91 ± 37 (30)	31 ± 9 (18) *
α4β2δ(L260V)	0.33 (6.48 ± 0.02) (12)	610 ± 250 (30) ****	58 ± 5 (18) ****
α4β2δ(I284T)	0.50 (6.30 ± 0.01) (13)	690 ± 350 (30) ****	48 ± 7 (18) ****
α4β2δ(T291I)	0.42 (6.38 ± 0.03) (11)	630 ± 280 (30) ****	46 ± 5 (18) ****

Xenopus laevis oocytes were injected with the indicated cRNA 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. Fitted GABA sensitivities are presented as EC_{50} in μM and pEC_{50} ± SEM where p = −Log for the indicated number (n) of individual oocytes. Full regression results are presented in Supplementary 1. The average maximal current obtained with $GABA_{max}$ (316 or 1000 μM) applications is presented as $GABA_{max}$ ± SD in nA for the indicated number (n) of individual oocytes. Est. $P_O(GABA_{max})$ denotes the estimated maximum open probability upon $GABA_{max}$ applications and is presented in % ± SD for the indicated number (n) of individual oocytes (also see Supplementary 1). Analysis of statistical significance was obtained with an ANOVA Kruskal-Wallis test and *post hoc* Dunn's test relative to the respective wild type receptor combinations. *P < 0.05, **P < 0.01, ****P < 0.0001.

Table 2 Main phenotypic characteristics of patients carrying disease-causing variants in *GABRD*

	Patient 1	Patient 2	Patient 3	Patient 4	Family 1 Patient 5 (Twin of pt. 6)	Family 1 Patient 6 (Twin of pt. 5)	Family 1 Patient 7 (Mother of twins)
GABRD variant	p.P122A *de novo*	p.P257L *de novo*	p.L260V *de novo*	p.I284T *de novo*	p.T291I maternal	p.T291I maternal	p.T291I *de novo*
CADD score	25.4	28.5	24.7	23.4	23.8	23.8	23.8
Functional analysis	LOF	GOF	GOF	GOF	GOF	GOF	GOF
Sex, age	Female, 10y	Female, 9y	Female, 3y deceased	Female, 16y	Male, 10y	Male, 10y	Female, 37y
Developmental delay	No	Mild	Severe	Severe	Mild	Moderate	Mild
Intellectual disability	No (FSIQ = 87)	Mild (FSIQ=70)	Moderate-severe	Profound	Mild	Mild-moderate (FSIQ=50)	Learning difficulties
Language abilities	Verbally fluent	Verbal dyspraxia	Non-verbal	Non-verbal	Verbally fluent	Verbally fluent	Verbally fluent
Behavior	ASD, adaptive skills are in the low range	Attention deficit, anxious	Limited interaction, irritability	Autistic traits, hair pulling, tantrums, hyperactivity	ADHD	ADHD	NA
Neurological exam	NA	Normal	NA	Ataxic gait, truncal ataxia, intention tremor	NA	NA	NA
Epilepsy	No	Yes	Yes	Yes	Yes	Yes	Yes
Age of onset, seizure types	NR	9mo, FS	4mo, GTCS	9mo, atypical absences	12mo, absences	12mo, absences	4y, absences
Seizure types	NR	FS, myoclonic, GTC	Myoclonic, GTC	Tonic, atonic, myoclonic, atypical absences, GTC	Atypical absences	Atypical absences	Absences
Triggering factors	NR	None	NA	Constipation	NA	NA	NA
Seizure outcome	NR	Seizure free	Intractable	Intractable	1-2 absences /day	Daily absences	Daily absences
EEG at onset; follow-up	NA	Normal; high voltage slow waves occipito-post-temporal	Multifocal epileptiform discharges mainly right temporal	Background slowing, high voltage SW-activity occipito-parietal; Continuous generalized epileptiform discharges mainly frontal	Background slowing, rare generalized epileptiform discharges	Background slowing, generalized epileptiform discharges mainly frontal	NA
Current treatment	NR	LEV	CLZ	TPM, NTZ	VPA, OXC	VPA, OXC	VPA
Brain MRI	NA	Slight enlargement of the subarachnoidal-spaces frontally in both hemispheres	Normal	Normal	NA	NA	NA
Additional features	Elevated internalizing psychiatric symptoms	Clumsy. Episodes with diffuse muscle/ joint pain and increased muscle tension, myopia (+8, +7.5), slight hypermetropia. Episodes of double vision with blackouts. Decreased muscular strength in hands	Feeding difficulties, on NG feeds	Severe constipation	Pes planovalgus, hyperopia, strabismus, hypertelorism	Pes planovalgus, hypertelorism	-

Abbreviations: ADHD: attention deficit hyperactivity disorder; ASD: autism spectrum disorder; CADD: Combined Annotation-Dependent Depletion; CLZ: clonazepam; GOF: gain-of-function; GTC: generalized tonic-clonic seizure; LEV: levetiracetam; LOF: loss-of-function; mo: months; MRI: magnetic resonance imaging; NA: not available; NTZ: nitrazepam; OXC: oxcarbazepine; TPM: topiramate; VPA: valproic acid; y: years.