

# Phenotypic Spectrum in Individuals With Pathogenic *GABRG2* Loss- and Gain-of-Function Variants

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## Abstract

### Background and Objectives

Variants in the *GABRG2* gene encoding the  $\gamma 2$  subunit of the  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor are associated with a spectrum of epilepsy phenotypes. These range from simple febrile seizures to more severe conditions, including developmental and epileptic encephalopathies (DEEs). Despite previous analyses suggesting that pathogenic variants may lead to loss-of-function (LoF) receptors, a correlation between functional analysis and clinical phenotypic diversity remains elusive. We, therefore, aimed to determine why variants in the *GABRG2* gene can lead to highly diverse phenotypes.

### Methods

We assembled a cohort of unreported probands carrying presumed pathogenic *GABRG2* variants. Electroclinical information was systematically collected, and electrophysiologic measurements were conducted for missense variants to explore potential alterations in receptor function.

### Results

We examined 44 individuals with 35 *GABRG2* variants (18 null and 17 missense). Functional assessments of the missense variants revealed that 9 caused LoF and 3 caused gain-of-function (GoF). The remaining 5 did not alter receptor function and are likely not pathogenic. Based on functional analysis and electroclinical data, 37 affected individuals were categorized into 3 groups: null LoF, missense LoF, and GoF variants. Among 19 individuals with null variants, epilepsy was diagnosed in 13, with a median onset of 14 months. The remaining 6 of 19 only had febrile seizures. Developmental delay/intellectual disability (DD/ID) was observed in 1 of 19 and psychiatric features in 4 of 18. By contrast, all 12 individuals with missense LoF variants suffered from epilepsy with a median onset of 15 months. Most common epilepsy diagnoses were febrile seizures plus in 4 of 12 and DEE in 4 of 12. DD/ID affected 9 of 12, and psychiatric features were diagnosed in 8 of 12. Statistical comparisons revealed that null variants were associated with a milder phenotype than missense LoF variants. Finally, 5 of 6 individuals with GoF variants had DEE characterized by early infancy onset at 2 months and severe/profound DD/ID. The sixth individual exhibited mild DD/ID and hypotonia without seizures.

### Discussion

Our findings indicate that the severity of disease associated with pathogenic *GABRG2* variants depends on the functional consequences of the variants. Null variants are associated with a mild phenotype and missense LoF variants with an intermediate phenotype while GoF variants can lead to severe phenotypes.

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## Glossary

**ACMG** = American College of Medical Genetics and Genomics; **ANOVA** = analysis of variance; **ASD** = autism spectrum disorder; **DD/ID** = developmental delay/intellectual disability; **DEE** = developmental and epileptic encephalopathy; **FS** = febrile seizures; **GABA** =  $\gamma$ -aminobutyric acid; **GABA<sub>A</sub>R** =  $\gamma$ -aminobutyric acid type A receptor; **GEFS+** = genetic epilepsy with febrile seizures plus; **GGE** = genetic generalized epilepsy; **GoF** = gain-of-function; **IQR** = interquartile range; **LoF** = loss-of-function; **MAE** = myoclonic atonic epilepsy; **ND** = not determined; **OR** = odds ratio; **ROC** = receiver operating characteristic.

## Introduction

The *GABRG2* gene is enriched for rare missense and protein-truncating variants that lead to epilepsy.<sup>1</sup> Initially, disease-causing variants were identified in an Australian family with genetic epilepsy with febrile seizures plus (GEFS+)<sup>2</sup> and in a French family with variable phenotypes consistent with GEFS+.<sup>3</sup> Since then, a broad range of epilepsy syndromes, from simple febrile seizures (FS)<sup>4,5</sup> and genetic generalized epilepsies (GGEs)<sup>6-10</sup> to developmental and epileptic encephalopathies (DEEs), have been reported.<sup>11-16</sup> Phenotypes include various seizure types,<sup>6,7,17,18</sup> and comorbidities include hypotonia, developmental delay (DD) and/or intellectual disability (ID), and behavioral disorders.<sup>13,16,17</sup> *GABRG2* variants may also raise risk of self-limited focal epilepsies of childhood.<sup>19,20</sup>

*GABRG2* encodes the  $\gamma 2$  subunit of the  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub>R) that mediates fast inhibitory synaptic neurotransmission.<sup>21,22</sup> Activation of GABA<sub>A</sub>Rs typically leads to chloride influx and hyperpolarization of the postsynaptic cell, inhibiting action potential generation. Nineteen GABA<sub>A</sub>R subunits exist ( $\alpha 1-6$ ,  $\beta 1-3$ ,  $\gamma 1-3$ ,  $\delta$ ,  $\epsilon$ ,  $\theta$ ,  $\pi$ , and  $\rho 1-3$ ) with the most prevalent receptor isoform comprising 2  $\alpha 1$ , 2  $\beta 2$  or  $\beta 3$ , and 1  $\gamma 2$  subunit. Loss-of-function (LoF) effects have been proposed as the primary disease mechanism in *GABRG2*-related disorders.<sup>15</sup> These effects can result from impaired receptor assembly, trafficking, or channel activation,<sup>5,9,23</sup> leading to either reduced surface expression or dysfunctional GABA receptors and thus diminished GABA-evoked responses. This decreases inhibitory inputs in neurons, increasing neuronal excitability. However, although LoF explains how *GABRG2* variants may cause epilepsy, it does not fully account for the significant severity differences among variants.

The lack of convincing genotype-phenotype associations hinders our understanding of *GABRG2*-related epilepsies and their underlying pathophysiologic mechanisms. The broad range of neurologic phenotypic expressions and variable responses to antiseizure medications (ASMs) challenge the assumption that *GABRG2* variants only cause LoF effects. Recent reports show that epilepsy-associated variants in other GABA<sub>A</sub>R subunit genes including *GABRA1*,<sup>24,25</sup> *GABRA4*,<sup>26</sup> *GABRB2*,<sup>27</sup> *GABRB3*,<sup>28,29</sup> and *GABRD*<sup>30</sup> can cause both gain-of-function (GoF) and LoF effects. Although GoF variants have not been reported for *GABRG2*, their existence is thus plausible. Furthermore, certain protein-truncating variants

(null variants) are speculated to cause more severe phenotypes than missense variants because of dominant negative effects.<sup>12</sup> However, direct comparisons between cohorts of individuals with null and missense variants are currently lacking.

In this study, we present a comprehensive analysis of the phenotypic and genotypic spectrum of *GABRG2* in 44 previously unreported cases, including individuals with null and missense variants. Functional analysis identified missense variants with either GoF or LoF effects. Although there is some overlap in phenotypic spectra, our genotype-phenotype analysis suggests that the diversity in phenotypes correlates with the specific type and functional impact of the variants.

## Methods

Full methodological descriptions are available in the eMethods.

### Clinical Ascertainment

Individuals with a presumed pathogenic variant in the *GABRG2* gene, detected through genetic testing for ID, febrile seizures, epilepsies, or DEEs, were recruited at the Danish Epilepsy Centre and through an international network of epilepsy and genetic centers. Only previously unreported probands were included to ensure a diverse representation of variants and genetic backgrounds. Probands and their families underwent detailed phenotyping including interviews, review of their medical history, neuroimaging, and EEG findings as previously described.<sup>31-35</sup> Data are reported in line with the Strengthening Reporting of Observational Studies in Epidemiology statement.

### Severity Index

The severity index was calculated as the sum of scores for all phenotypic features for each individual. The following scores were assigned to the various phenotypic features: inheritance (0 for inherited, 1 for de novo), epilepsy (0 for no, 1 for yes), age at seizure onset (0 for no epilepsy, 1 for onset above 6 months, 2 for onset less than 6 months), seizure frequency (0 for no seizures, 1 for monthly or rare, 2 for daily or weekly), DD/ID (0 for normal, 1 for mild to moderate, 2 for severe or profound), language delay (0 for no delay, 1 for delay, 2 for nonverbal), psychiatric issues (0 for no, 1 for yes), motor skill issues (0 for normal, 1 for delay or clumsiness, 2 for non-ambulant), and hypotonia (0 for normal, 1 for hypotonia).

## Functional Assessment

cDNA constructs with variant  $\gamma 2$  subunits were created using classical molecular biology techniques as previously reported.<sup>36-38</sup> Oocytes were obtained from ovarian lobes of *Xenopus laevis* frogs from the University of Wollongong under animal ethics protocol AE2003. Treatment and storage of and recording from oocytes using two-electrode voltage clamp electrophysiology followed previously reported methods.<sup>24,27,28</sup> Functional analysis focused on  $EC_{50}$  and  $I_{max}$  of GABA.<sup>26,28-30</sup>

## Statistics

Curve fitting of GABA concentration-response relationships was performed by nonlinear regression using GraphPad Prism 10 (Dotmatrix, Boston, MA) and was compared with a one-way analysis of variance (ANOVA) and multiple corrections with the Dunnett post hoc test. Maximum GABA-evoked current amplitudes and ages at onset were compared by the Mann-Whitney test. The Fisher exact test was used to compare clinical features where appropriate, with a threshold of  $p < 0.05$ . The age at onset for epilepsy was compared with the Mann-Whitney test, and the Mantel-Cox test was used to account for patients without epilepsy. The severity index was compared with the Mantel-Cox test and a receiver operating characteristic (ROC) analysis with the Wilson-Brown test for significance.

## Standard Protocol Approvals, Registrations, and Patient Consents

The study was conducted according to the ethical principles for medical research outlined in the Declaration of Helsinki. The study was approved by the local ethics committee in the Zealand region of Denmark (number SJ-91) and by the Institutional Review Board at the Danish Epilepsy Centre, Filadelfia (EMN-2024-01998). Informed consent of individuals or their responsible relatives and approval by local ethical committees were obtained. Clinicians and caregivers filled anonymized data in an online questionnaire and standardized phenotyping sheets. The database was stored at the Danish Epilepsy Centre.

## Data Availability

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

## Results

### Genetic Landscape

We studied a cohort of 44 previously unpublished probands (19 male, 25 female) with presumed pathogenic *GABRG2* variants. The median age at the last follow-up was 6 years and 4 months (range: 8 months to 43 years). A total of 35 variants were identified (Table 1). These occurred de novo in 14 of 44 individuals (32%) and were inherited in 26 of 44 individuals

(59%) while segregation was not available for 4 of 44 individuals. Of the 35 variants, 17 were missense and 18 were null variants. Null variants included 10 nonsense variants, 4 large deletions resulting in missing exons, and 4 splice-site variants. The variants were absent from the population databases (gnomAD v4.0.0) except for I85K (allele count 1), T90M (allele count 4), R125C (allele count 2), and M199V (allele count 5). In silico tools predicted A106T and I344L to be tolerated while the remainder were predicted to be deleterious by most tools (Table 2 and eTable 1). When assessed using the American College of Medical Genetics and Genomics (ACMG) classification, the variants were predicted to be likely pathogenic or pathogenic in 35 of 44 individuals and variants of uncertain significance (VUS) in 9 of 44 individuals before functional analyses (Table 2).

### Structural Mapping

Missense and nonsense variants occurred at 27 different amino acid positions (Figure 1A). Recurrent variants included T90M, A106T, R136\*, R323Q, and R323W. Two different missense variants affected the same amino acid residue: A322D and A322T, as well as R323Q and R323W. Nonsense null variants are primarily located in the extracellular domain, apart from C283Lfs\* and Q398\*, located in the TM1 domain and M3-M4 intracellular loop. Among missense variants, 7 affected amino acid positions that are 100% conserved across all GABA<sub>A</sub>R subunits associated with epilepsy, another 7 positions are conserved among the 3  $\gamma$  subunits (Figure 1B), and 3 (A106T, M169T, and I344L) are not conserved. The missense variants are distributed along the  $\gamma 2$  subunit protein, with 9 in the extracellular domain and 8 in the transmembrane domain (Figure 1C). Of interest, 9 reside at the coupling region at the interface of the extracellular and transmembrane regions. This region mediates the conformational changes that convert the GABA-binding event to ion channel opening and is a region where both LoF and GoF variants are typically found.<sup>28,29</sup>

### Functional Analysis

To ensure uniform receptor populations, mutated  $\gamma 2^*$  subunits were expressed using pentameric concatenated cDNA constructs (Figure 2A). Functional analysis was performed using two-electrode voltage clamp electrophysiology. Receptor sensitivity to GABA is a critical property for effective mediation of synaptic inhibition. Therefore, changes to GABA sensitivity were evaluated by constructing GABA concentration-response relationships and deriving half-maximal GABA concentration ( $EC_{50}$ ) values from fitted curves (Figure 2, B and C). From these,  $\Delta \log EC_{50}$  values were obtained as the difference in  $\log EC_{50}$  values between mutant and wild-type receptors. A one-way ANOVA revealed significant differences between GABA sensitivities for wild-type and mutant receptors ( $F(15, 392) = 120$ ). Among the 17 mutations assessed, 9 ( $\gamma 2^{P83T}$ ,  $\gamma 2^{R125C}$ ,  $\gamma 2^{M169T}$ ,  $\gamma 2^{A322D}$ ,  $\gamma 2^{A322T}$ ,  $\gamma 2^{R323Q}$ ,  $\gamma 2^{R323W}$ ,  $\gamma 2^{L326H}$ , and  $\gamma 2^{P327L}$ ) decreased receptor GABA sensitivity by 2–5 folds consistent with a LoF effect ( $\Delta \log EC_{50} = -0.33$  to  $-0.70$ ; all  $p < 0.0001$ , Dunnett

**Table 1** Summary of Key Genetic and Phenotypic Characteristics of 44 Individuals With Likely Pathogenic/Pathogenic Variants or VUS in *GABRG2*

#	<i>GABRG2</i> variant	Inheritance	Epilepsy	Age at seizure onset (m)	Epilepsy diagnosis	DD/ID	Seizure free
<b>Null variants</b>							
1	p.(Asn71Thrfs*14)	De novo	Yes	10–12	FS+	No	Yes
2	p.(Lys133Serfs*26)	Mat	No	10–12	FS (GEFS+)	No	Yes
3	p.(Arg136*)	Mat (u)	No	37–60	FS	No	Yes
4	p.(Arg136*)	Pat (u)	Yes	10–12	DEE	Moderate	No
5	p.(Ala158Leufs*13)	Pat	Yes	13–18	GGE (GEFS+)	No	No
6	p.(Ile186Metfs*59)	Mat	Yes	13–18	FS+ (GEFS+)	No	Yes
7	p.(Trp222*)	Pat	No	7–9	FS (GEFS+)	No	Yes
8	p.(Arg224*)	Pat (u)	Yes	25–36	FS+ (GEFS+)	No	Yes
9	p.(Glu250*)	Mat	Yes	10–12	MAE (GEFS+)	No	Yes
10	p.(Cys283Leufs*16)	Mat	Yes	4–6	GGE (GEFS+)	No	Yes
11	p.(Gln398*)	Mat	No	25–36	FS (GEFS+)	No	No
12	Large deletion	Mat	No	7–9	FS (GEFS+)	No	Yes
13	Large deletion	Mat	Yes	13–18	FS+ (GEFS+)	No	No
14	Large deletion	Mat	Yes	13–18	FS+ (GEFS+)	No	Yes
15	Large deletion	Pat	Yes	25–36	GGE (GEFS+)	No	No
16	Splice-site variant	UK	Yes	4–6	FS+	No	No
17	Splice-site variant	Pat	No	4–6	FS (GEFS+)	No	Yes
18	Splice-site variant	Pat	Yes	13–18	FS+ (GEFS+)	No	Yes
19	Splice-site variant	De novo	Yes	19–24	FS+	No	Yes
<b>Missense LoF variants</b>							
20	p.(Pro83Thr), P83T	Mat	Yes	37–60	CAE	No	No
21	p.(Arg125Cys), R125C	Pat	Yes	13–18	FS+ (GEFS+)	Mild	Yes
22	p.(Met169Thr), M169T	UK	Yes	19–24	DEE	Profound	No
23	p.(Ala322Asp), A322D	Pat	Yes	13–18	FS+ (GEFS+)	No	Yes
24	p.(Ala322Thr), A322T	De novo	Yes	10–12	DEE	Mild	No
25	p.(Arg323Gln), R323Q	De novo	Yes	7–9	MAE	Mild	No
26	p.(Arg323Gln), R323Q	Mat	Yes	10–12	DEE	Moderate	No
27	p.(Arg323Gln), R323Q	De novo	Yes	7–9	FS+	Mild	Yes
28	p.(Arg323Trp), R323W	De novo	Yes	13–18	DEE	Moderate	Yes
29	p.(Arg323Trp), R323W	UK	Yes	7–9	Fo	No	No
30	p.(Leu326His), L326H	De novo	Yes	13–18	FS+	Mild	Yes
31	p.(Pro327Leu), P327L	Mat	Yes	13–18	MAE	Mild	No
<b>Missense GoF variants</b>							
32	p.(Ala106Thr), A106T	De novo	Yes	4–6	DEE	Profound	No
33	p.(Ala106Thr), A106T	De novo	Yes	0–3	DEE	Severe	No
34	p.(Ala106Thr), A106T	UK	Yes	0–3	DEE	Profound	No
35	p.(Ala106Thr), A106T	De novo	Yes	0–3	DEE	Profound	No
36	p.(Ser139Asn), S139N	De novo	No	/	/	Mild	Yes
37	p.(Leu285Ile), L285I	De novo	Yes	0–3	DEE	Severe	No

Continued

**Table 1** Summary of Key Genetic and Phenotypic Characteristics of 44 Individuals With Likely Pathogenic/Pathogenic Variants or VUS in *GABRG2* (continued)

#	<i>GABRG2</i> variant	Inheritance	Epilepsy	Age at seizure onset (m)	Epilepsy diagnosis	DD/ID	Seizure free
<b>Missense variants with no functional changes</b>							
38	p.(Ile85Lys), I85K	Paternal	Yes	4–6	EMA (GEFS+)	No	Yes
39	p.(Thr90Met), T90M	De novo	Yes	0–3	FS+	No	Yes
40	p.(Thr90Met), T90M	Maternal	Yes	13–18	FS+ (GEFS+)	Moderate	No
41	p.(Thr90Met), T90M	Maternal	Yes	37–60	FS+ (GEFS+)	Moderate	No
42	p.(Val104Gly), V104G	De novo	Yes	13–18	CAE	No	Yes
43	p.(Met199Val), M199V	Paternal	Yes	>60	FS+ (GEFS+)	No	Yes
44	p.(Ile344Leu), I344L	Maternal	Yes	0–3	DEE	Profound	No

Abbreviations: CAE = childhood absence epilepsy; DD/ID = developmental delay and/or intellectual disability; DEE = developmental and epileptic encephalopathy; EMA = epilepsy with myoclonic absence; Fo = focal; FS = febrile seizure; FS+ = febrile seizures plus; GEFS+ = genetic epilepsy with febrile seizures plus; m = month; MAE = myoclonic atonic epilepsy; Mat = maternal; Pat = paternal; (u) = unaffected parent; UK = unknown; y = year.  
\*Protein termination.

post hoc test) (Figure 2D, eTable 1). Three mutations ( $\gamma 2^{A106T}$ ,  $\gamma 2^{S139N}$ , and  $\gamma 2^{L285I}$ ) exhibited a 2–5-fold increase in GABA sensitivity indicating GoF traits ( $\Delta \log EC_{50} = 0.33$  to 0.73;  $p < 0.0001$ , Dunnett post hoc test). The remaining 5 mutations ( $\gamma 2^{I85K}$ ,  $\gamma 2^{T90M}$ ,  $\gamma 2^{V104G}$ ,  $\gamma 2^{M199V}$ , and  $\gamma 2^{I344L}$ ) did not exhibit altered receptor sensitivity to GABA.

Another receptor property that may influence inhibitory neurotransmission is the maximum GABA-evoked current amplitude, a product of the efficacy of GABA activation and cell surface receptor expression. To assess this, maximum current amplitudes were evaluated by applying a saturating concentration of GABA (10 mM) and comparing wild-type and mutant receptor currents elicited on the same days. Among the 17 mutations assessed, 5 ( $\gamma 2^{P83T}$ ,  $\gamma 2^{R125C}$ ,  $\gamma 2^{M169T}$ ,  $\gamma 2^{L326H}$ , and  $\gamma 2^{P327L}$ ) showed a substantial reduction in maximum elicited current with amplitudes varying from 11% to 34% of the wild type ( $I_{max} = 0.11$ –0.34,  $p < 0.0001$ , Mann-Whitney test) (Figure 2D, eTable 1). Notably, all 5 mutations also displayed a significant reduction in GABA sensitivity.

### Reclassification Using ACMG Guidelines

Functional studies categorized 3 *GABRG2* variants, A106T, S139N, and L285I, as GoF because of increased GABA sensitivity. Nine variants were classified as LoF with 5, P83T, R125C, M169T, L326H, and P327L, displaying both impaired GABA sensitivity and reduced maximum currents while 4, A322D, A322T, R323Q, and R323W, displaying impaired GABA sensitivity alone. These 12 variants displayed significant functional alterations, providing pathogenic strong support. This reclassified 4 variants from VUS to likely pathogenic in 4 individuals (Table 2). For the remaining 8 variants in 14 individuals, the status remained or changed to pathogenic.

Conversely, functional analysis did not reveal significant alterations for 5 variants I85K, T90M, V104G, M199V, and I344L, providing strong benign support. These 5 variants seen in 7 individuals were classified as VUS, representing a change from likely pathogenic in 2 individuals. The 7 individuals harboring a VUS variant were excluded from our genotype-phenotype analysis, but their phenotypes are presented in Table 1 and eTable 1.

After reclassification, 30 pathogenic/likely pathogenic variants were detected in 37 individuals. Based on the functional properties of the variants and the electroclinical information, 3 groups were identified: (1) LoF null variants resulting in an aberrant or truncated protein; (2) LoF missense variants with impaired GABA sensitivity; (3) GoF missense variants that increased GABA sensitivity.

### Individuals Harboring Null Variants

Nineteen individuals (#1 to #19) carried null *GABRG2* variants with segregation analysis available for 18 (Table 1, eTable 1). The variants occurred de novo in 2 of 18 individuals (11%) and segregated with affected parents in 13 of 18 (72%) and unaffected parents in 3 of 18 (17%). Epilepsy was diagnosed in 13 of 19 individuals (68%) with a median onset of 14 months (range 5 months to 2 years and 4 months) (Figure 3). The remaining 6 of 19 (32%) only had FSs with a median onset of 10 months (range 6 months to 5 years). Among the 13 individuals with epilepsy, 8 (62%) had FS+ and 3 (23%) had GGE (#5, #10, and #15). The remaining 2 (#4 and #9) had either DEE or epilepsy with myoclonic-atonic seizures (MAEs). Common seizure types included tonic-clonic, focal, typical absences, atonic, and myoclonic (Figure 3). Most epilepsy syndromes fell within the GEFS+ spectrum, and based on familial history, 14 of 19 individuals (74%) were assigned a GEFS+ diagnosis.

**Table 2** ACMG Classification for 44 Individuals With *GABRG2* Variants

#	<i>GABRG2</i> variant	Population data	Computational/predictive data	Functional data	De novo data	Other data	Initial classification <sup>c</sup>	In vitro assay	Final classification <sup>c</sup>
<b>Null variants</b>									
1	Null variant	PM2	PVS1		PS2	PP4	Pathogenic	NA	Pathogenic
2-18	Null variant	PM2	PVS1			PP4	Pathogenic	NA	Pathogenic
19	Null variant	PM2	PVS1		PS2	PP4	Pathogenic	NA	Pathogenic
<b>Missense LoF variants</b>									
20	p.Pro83Thr	PM2	PP3	PP2		PP4	VUS	PS3	Likely pathogenic
21	p.Arg125Cys	2 <sup>a</sup>	PP3	PP2		PP4	VUS	PS3	Likely pathogenic
22	p.Met169Thr	PM2	PP3	PP2		PP4	VUS	PS3	Likely pathogenic
23	p.Ala322Asp	PM2	PP3	PP2, PM1 <sup>b</sup>		PP4	Likely pathogenic	PS3	Pathogenic
24	p.Ala322Thr	PM2	PP3	PP2, PM1 <sup>b</sup>	PS2	PP4	Pathogenic	PS3	Pathogenic
25	p.Arg323Gln	PM2	PP3	PP2, PM1 <sup>b</sup>	PS2	PP4	Pathogenic	PS3	Pathogenic
26	p.Arg323Gln	PM2	PP3	PP2, PM1 <sup>b</sup>		PP4	Likely pathogenic	PS3	Pathogenic
27	p.Arg323Gln	PM2	PP3	PP2, PM1 <sup>b</sup>	PS2	PP4	Pathogenic	PS3	Pathogenic
28	p.Arg323Trp	PM2	PP3	PP2, PM1 <sup>b</sup>	PS2	PP4	Pathogenic	PS3	Pathogenic
29	p.Arg323Trp	PM2	PP3	PP2, PM1 <sup>b</sup>		PP4	Likely pathogenic	PS3	Pathogenic
30	p.Leu326His	PM2	PP3	PP2, PM1 <sup>b</sup>	PS2	PP4	Pathogenic	PS3	Pathogenic
31	p.Pro327Leu	PM2	PP3	PP2, PM1 <sup>b</sup>		PP4	Pathogenic	PS3	Pathogenic
<b>Missense GoF variants</b>									
32	p.Ala106Thr	PM2	BP4	PP2	PS2	PP4	Likely pathogenic	PS3	Pathogenic
33	p.Ala106Thr	PM2	BP4	PP2	PS2	PP4	Likely pathogenic	PS3	Pathogenic
34	p.Ala106Thr	PM2	BP4	PP2		PP4	VUS	PS3	Likely pathogenic
35	p.Ala106Thr	PM2	BP4	PP2	PS2	PP4	Likely pathogenic	PS3	Pathogenic
36	p.Ser139Asn	PM2	PP3	PP2	PS2	PP4	Likely pathogenic	PS3	Pathogenic
37	p.Leu285Ile	PM2	PP3	PP2, PM1 <sup>b</sup>	PS2	PP4	Pathogenic	PS3	Pathogenic
<b>Missense variants with no functional change</b>									
38	p.Ile85Lys	1 <sup>a</sup>	PP3	PP2		PP4	VUS	BS3	VUS
39	p.Thr90Met	4 <sup>a</sup>	PP3	PP2	PS2	PP4	Likely pathogenic	BS3	VUS
40	p.Thr90Met	4 <sup>a</sup>	PP3	PP2		PP4	VUS	BS3	VUS
41	p.Thr90Met	4 <sup>a</sup>	PP3	PP2		PP4	VUS	BS3	VUS
42	p.Val104Gly	PM2	PP3	PP2	PS2	PP4	Likely pathogenic	BS3	VUS
43	p.Met199Val	5 <sup>a</sup>	PP3	PP2		PP4	VUS	BS3	VUS
44	p.Ile344Leu	PM2	BP4	PP2, PM1 <sup>b</sup>		PP4	VUS	BS3	VUS

Variants were classified according to the 2015 ACMG (American College of Medical Genetics and Genomics) guidelines. Population data information was obtained from gnomAD v4 (genome aggregation database). Computational predictions were obtained using 4 in silico tools: SIFT, PolyPhen-2, CADD v1.6, and AlphaMissense. Initial classification was performed before functional assessment while final classification includes functional assessment information.

<sup>a</sup> Number of alleles in the gnomAD database.

<sup>b</sup> PM1 data were obtained from the Missense Tolerance Ratio viewer where residues are in the transmembrane region.

<sup>c</sup> Where contradictory evidence was identified, variants were classified on a descending hierarchy of evidence.

Interictal EEGs were available in 15 of 19 individuals (79%) and showed normal background activity in 13 of 15. Generalized or focal epileptiform abnormalities were observed in 6 of 13 (46%) and 4 of 13 (31%), respectively. One individual (#15) had myoclonic seizures associated with generalized spike-wave discharges on ictal EEG (Figure 4). Brain MRI studies were performed in 13 of 19 individuals (68%) and were unremarkable.

Moderate DD/ID was reported for the individual with DEE (#4) while the remaining 18 of 19 (95%) were considered normal (Figure 5A). All individuals acquired language skills, although #4 experienced severe language delay. Two individuals (#9 and #14) exhibited psychiatric features including ADHD and hyperactivity, and another 2 (#2 and #5) exhibited short attention span or concentration difficulties. All demonstrated normal motor skills and could walk independently.

At the last follow-up, 13 of 19 individuals (68%) were seizure free while 6 of 19 (32%) had persistent seizures ranging from daily to rare (Figure 5A). Two individuals with FSs (#11, #17) were never treated with ASMs and became seizure free with age. The remaining 17 were treated with ASMs with a very good response (seizure freedom) in 11 of 17 (65%) and a partially good response in 5 of 17 (29%) while epilepsy was not controlled in 1 (#16). Valproate was used in 11 of 17 individuals (65%), resulting in seizure freedom as monotherapy in 5 and as add-on treatment in another. GABAergic ASMs, such as benzodiazepines and phenobarbital, improved seizure frequency in 3 of 4 individuals (75%), and ketogenic diet reduced seizure frequency in 1 (#15).

### Individuals Harboring Missense LoF Variants

Twelve individuals (#20 to #31) carried *GABRG2* missense variants causing LoF with segregation information available for 10 of 12 individuals (Table 1, eTable 1). The variants occurred de novo in 5 of 10 (50%) and were inherited from affected parents in 5 of 10 (50%). All 12 individuals had epilepsy with a median seizure onset at 15 months (range 7 months to 3 years and 6 months) (Figure 3). Four (33%) (#21, #23, #27, and #30) had FS+, with 2 assigned a GEFS+ diagnosis based on familial history (#21 and #23). Of the remaining individuals, 4 of 12 (33%) had a DEE (#22, #24, #26, and #28), 2 had MAE (#25 and #31), and 1 each had focal epilepsy (#29) and childhood absence epilepsy (#20). Notably, the 4 individuals with DEE experienced generalized (tonic-clonic, myoclonic, atonic, absences) and focal seizures (Figure 3). Four individuals (31%) (#21, #22, #26, #28) had at least one episode of convulsive or nonconvulsive status epilepticus.

EEG studies were available for all 12 individuals and showed focal/multifocal (frontal, temporal, posterior, central) or generalized epileptiform abnormalities with normal background activity in 9 of 12 (75%) (Figure 4). Two individuals (#30, #31) had normal EEGs while the remaining (#22) showed an encephalopathic pattern of diffuse slowing with

multifocal epileptiform discharges. Brain MRI, performed in 10 of 12 individuals (83%), revealed a temporal arachnoid cyst in 2 (#24, #25), left mesial temporal sclerosis in 1 (#26), and thinning of the corpus callosum with a right frontal gliotic focus in 1 (#27). The remaining 6 of 10 MRI scans (60%) were unremarkable.

DD/ID was observed in 9 of 12 individuals (75%), with mild/moderate severity in 8 of 9 and severe/profound severity in 1 of 9 (#22) (Figure 5A). The remaining 3 (#20, #23, and #29) had normal intellect. All individuals acquired the ability to communicate albeit with a language delay in 5 of 12 (42%). Eight individuals (67%) exhibited behavioral and/or psychiatric features including ADHD, hyperactivity, attention deficit, and autism spectrum disorder (ASD). Normal motor skills were observed in 7 of 12 (58%) while 5 of 12 (42%) experienced various degrees of motor skill issues and one was wheelchair bound. Mild hypotonia was reported in 1 individual (#25).

At the last follow-up, 5 of 12 individuals (42%) were seizure free, including 1 (#30) with FS+ who was never treated with ASMs (Figure 5A). Eleven individuals were treated with ASMs. Valproate was administered in 9 of 11 (82%), achieving partial seizure control as monotherapy or as add-on treatment in 4 of 9 (44%). Other ASMs targeting GABAergic neurotransmission were tried in 3 of 11 individuals (27%), and clobazam improved seizure frequency in 1 (#26), whereas it was not tolerated in another (#25). Phenobarbital and vigabatrin were ineffective in 1 (#28) while ketogenic diet was effective in another (#25).

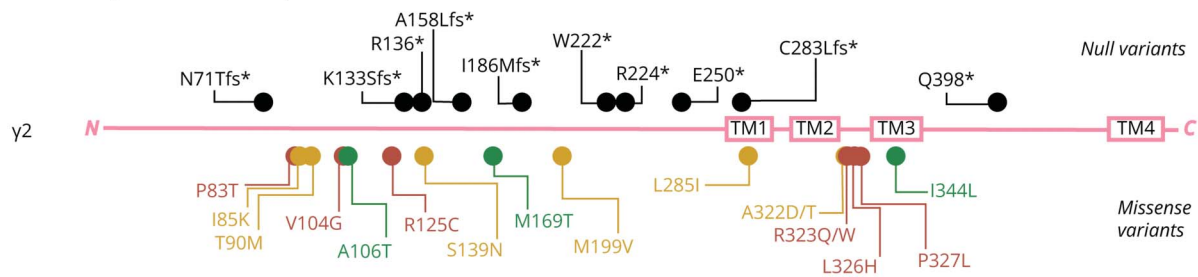
### Individuals Harboring GoF Variants

Six individuals (#32 to #37) had GoF variants, with 4 carrying the A106T variant and 1 each having the S139N and L285I variants (Table 1, eTable 1). Segregation analysis was available for 5 of 6, and all carried a de novo variant. Epilepsy was diagnosed in 5 of 6 individuals (83%), with 1 (#36) never experiencing seizures (Figure 3). All 5 had a DEE with a median seizure onset at 2 months (range 1 month–3.5 months). Seizure types included focal (including focal impaired awareness), gelastic (#33) with right frontocentral onset (Figure 4), migrating focal (#37), and focal to bilateral tonic-clonic (#33 and #34) (Figure 3). Of interest, gelastic seizures have not previously been reported in individuals with GABA<sub>A</sub>R variants. Two individuals (#32 and #35) had bilateral tonic-clonic seizures, and 1 had asymmetric tonic seizures (#35). Episodes of convulsive status epilepticus were reported in 1 individual (#34). All 5 individuals with a DEE were drug-resistant, although some showed seizure frequency improvement with valproate (#32, #33, and #35) and ketogenic diet (#34).

Interictal EEG data for all 6 individuals showed diffuse slowing and frequent multifocal or generalized epileptiform discharges (Figure 4). EEG in 1 (#36) showed asynchronous sharp waves over the left occipital and the right frontal areas.

**Figure 1** Structural Location of *GABRG2* Variants

**A.** Linear  $\gamma 2$  amino acid sequence and variant locations

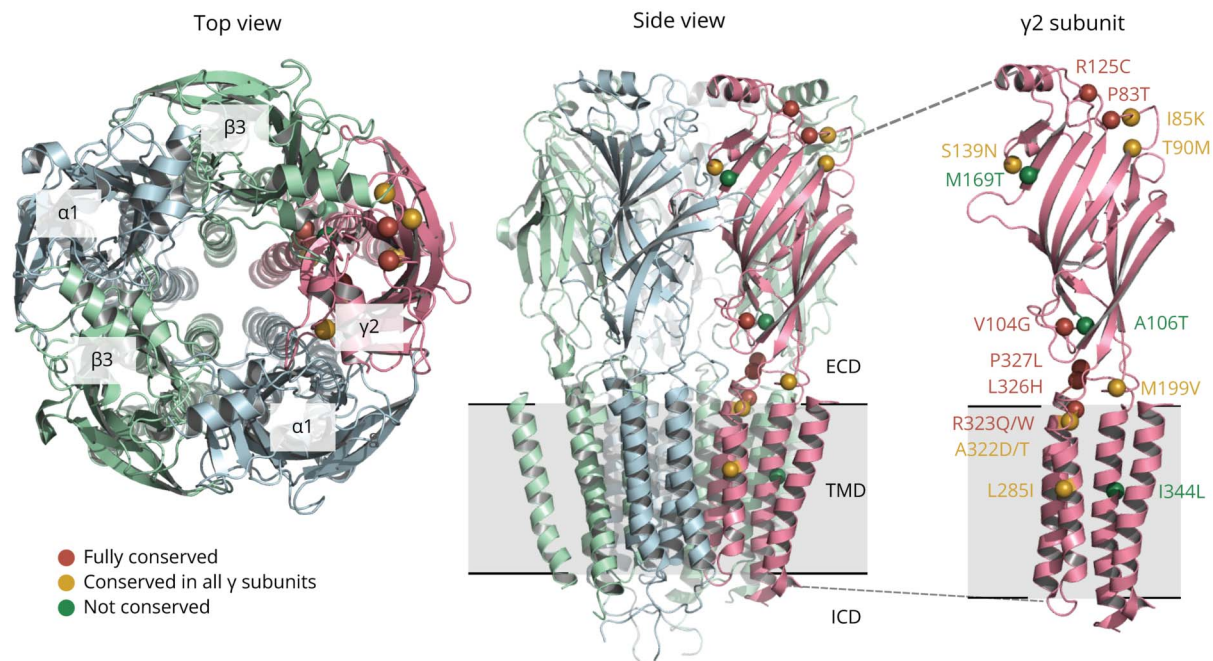


**B.** Sequence alignment of  $\alpha 1$ ,  $\beta 3$ , and  $\gamma 1$ -3 at missense variant locations

	83	85	90	104	106	125	139	169	199	285	322	327	344																																									
$\gamma 2$	R	P	D	I	G	V	K	P	T	L	P	V	N	A	I	D	R	R	L	K	L	N	S	N	M	P	N	R	M	L	R	I	F	P	M	D	E	C	T	L	I	V	I	A	R	K	S	L	P	C	F	I	F	V
$\alpha 1$	R	P	G	L	G	E	R	V	T	E	P	V	S	D	H	D	E	R	L	K	L	N	N	L	M	P	N	K	L	L	R	I	F	P	M	D	A	C	I	M	T	V	S	A	R	N	S	L	P	C	Y	A	F	V
$\beta 3$	R	P	D	F	G	G	P	P	V	C	M	V	S	E	V	D	K	R	L	A	L	D	N	R	V	K	N	R	M	I	R	L	Y	P	L	D	E	S	I	L	I	T	H	L	R	E	T	L	P	C	F	V	F	V
$\gamma 1$	R	P	D	I	G	V	R	P	T	V	P	V	D	P	I	D	S	R	L	K	L	N	S	N	M	P	N	R	L	L	R	I	F	P	M	D	E	C	I	L	T	V	I	A	R	K	S	L	P	C	F	I	F	V
$\gamma 3$	R	P	D	I	G	I	K	P	T	V	P	V	S	S	I	D	S	R	L	R	L	N	S	N	M	P	N	Q	L	L	R	I	F	P	M	D	E	C	I	L	T	V	I	A	R	K	S	L	P	C	F	L	F	V

■ Fully conserved  
■ Conserved in all  $\gamma$  subunits  
■ Not conserved

**C.** Structure of the  $\alpha 1\beta 3\gamma 2$  receptor and location of missense  $\gamma 2$  variants



(A) 2D representation of the amino acid sequence of the  $GABA_A$   $\gamma 2$  subunit peptide with the location of null variants and missense variants. Transmembrane regions are represented as open boxes. (B) Sequence alignments of  $\gamma 1$ -3 with  $\alpha 1$  and  $\beta 3$  subunits in the immediate vicinity of missense variants with the indicated color scheme. (C) 3D representation of the  $\alpha 1\beta 3\gamma 2$   $GABA_A$  R (pdb:6hup) with the location of assessed missense  $\gamma 2$  variants displayed as spheres. Red spheres represent variants with complete sequence identity across all main  $GABA_A$  receptor subunits, yellow spheres with sequence identity across  $\gamma 1$ -3 subunits, and green spheres with limited sequence identity across  $GABA_A$  R subunits.  $GABA_A$  =  $\gamma$ -aminobutyric acid type A.

Brain MRI scans revealed calcification in both cerebral hemispheres in 1 individual (#34) and diffuse atrophy in another (#35), whereas it was unremarkable in the remaining individuals.

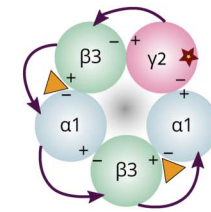
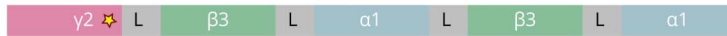
All individuals exhibited DD/ID, mild in the one without epilepsy (#36) and severe/profound without language

acquisition in those with DEE (Figure 5A). Individual #36 had a language delay and ASD along with features not seen in the rest of the cohort such as obesity, coarse facial features, and tall stature. Three individuals (#32, #34, and #37) did not acquire the ability to walk while 2 could walk with support (#35) or short distances without support (#33). Only individual #36 was ambulant, though clumsy, and hypotonia was

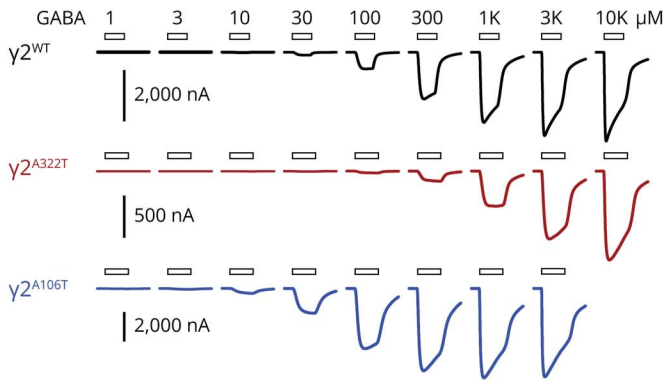
**Figure 2** Functional Analysis of  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub>Rs Containing  $\gamma 2$  Mutations

**A. Concatenated receptors**

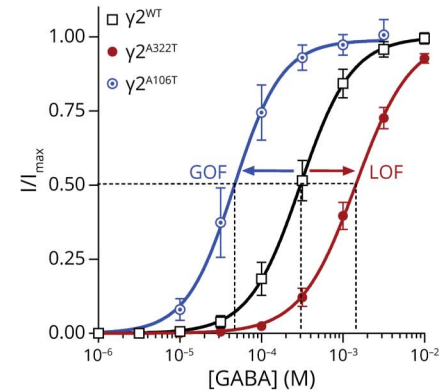
cDNA construct for expression of  $\alpha 1\beta 3\gamma 2$  GABA<sub>A</sub> receptors with  $\gamma 2$  subunit mutations



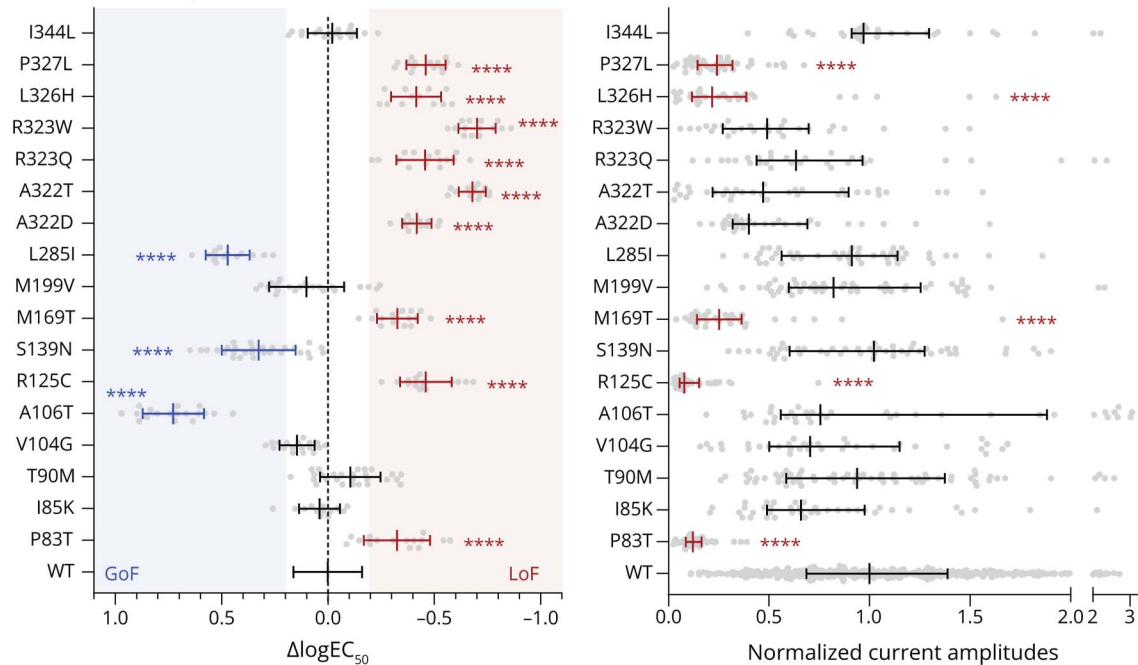
**B. Representative traces for receptors with  $\gamma 2$  mutations**



**C. Concentration-response curves**

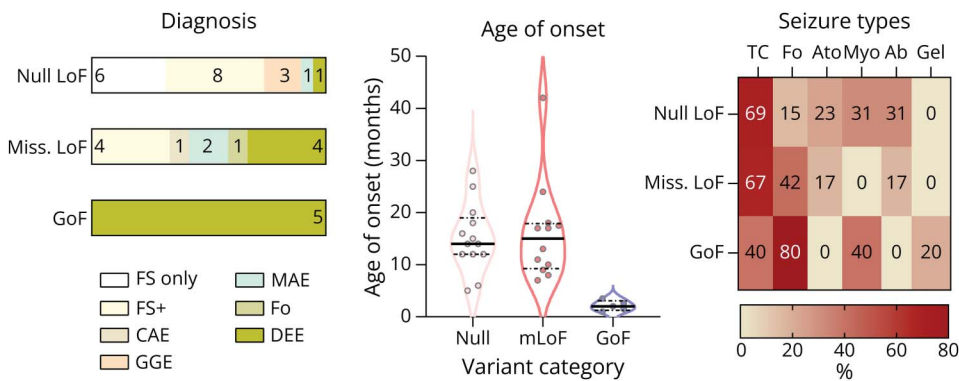


**D. GABA sensitivity and maximal evoked currents**



(A) The concatenated pentameric  $\gamma 2$ - $\beta 3$ - $\alpha 1$ - $\beta 3$ - $\alpha 1$  cDNA construct used for functional analysis is illustrated with 4 linkers (L, gray) and resulting expressed fusion protein viewed from the extracellular side. A yellow star indicates that  $\gamma 2$  subunit mutations are entered in the 1st subunit position. (B) Representative traces depict concentration-response relationships for the wild-type  $\alpha 1\beta 3\gamma 2$  receptor and receptors containing the  $\gamma 2^{A322T}$  and  $\gamma 2^{A106T}$  mutations. Bars above traces designate the 25-second application time and GABA concentrations. (C) Normalized GABA concentration-response relationships were plotted as a function of the GABA concentration, and the Hill equation was fitted to each data set by nonlinear regression (eTable 1). Data points are presented as mean  $\pm$  SD for  $n = 12$ –13 independent experiments. Dotted lines indicate the concentrations that lead to half-maximal activation ( $EC_{50}$ ) for each receptor type. Arrows indicate whether a variant caused increased (GoF) or a decreased (LoF) GABA sensitivity. (D, left) The difference in GABA sensitivity between wild-type and 17 mutated receptors was calculated from the logarithmic conversion of  $EC_{50}$  values ( $\Delta \log EC_{50}$ ) on each experimental day. Final  $\Delta \log EC_{50}$  data sets for each variant contain data from  $n = 12$ –29 independent experiments and are presented as mean  $\pm$  SD with individual data points (eTable 1). Blue indicates mutations with significantly increased sensitivity to GABA, red indicates variants with significantly decreased GABA sensitivity, and gray indicates mutations with no significant change. Significance was determined by one-way ANOVA ( $F(17, 460) = 120$ ;  $p < 0.0001$ ) with the Dunnett post hoc test ( $****p < 0.0001$ ). The transparent blue and red areas indicate the typical areas wherein mutated receptors show significance. (D, right) Normalized maximal GABA-evoked current amplitudes are presented as median with interquartile ranges (IQRs) for  $n = 388$  (WT) or  $n = 27$ –65 ( $\gamma 2$  mutations) experiments. Red indicates mutations causing LoF while black indicates mutations with no significant change. Significance was determined using the Mann-Whitney test ( $****p < 0.0001$ ).

**Figure 3** Epilepsy Features for Individuals With Null LoF, Missense LoF and GoF *GABRG2* Variants



Epilepsy diagnosis percentages are presented as horizontal slices for individuals carrying null LoF ( $n = 19$ ), missense LoF ( $n = 12$ ), and GoF ( $n = 5$ ) variants. Age at seizure onset is presented as a violin plot for individuals diagnosed with epilepsy (not FFS) for the 3 groups of variants. Null signifies null LoF variants, and mLoF signifies missense LoF variants. Median values are indicated by solid lines and IQRs by dotted lines. Most common seizure types are presented as a heat map for the 3 groups of variants. The percentage of individuals with each seizure type is indicated by numbers inside the rectangles. Seizure types: Ab = typical absences; Ato = atonic; Fo = focal; Gel = gelastic; GoF = gain-of-function; LoF = loss-of-function; Myo = myoclonic; and TC = tonic-clonic.

observed in all 6. Chorea and orofacial dystonia were noted in 1 individual (#34).

### Comparison of GoF vs LoF and Missense vs Null LoF Subgroups

Compared with all individuals with LoF variants, those with GoF variants exhibited an earlier age at seizure onset (median 2 [interquartile range, IQR: 1.3–3.1] vs 14 [IQR: 11–18] months;  $p < 0.0001$ , Mann-Whitney test) and a different epilepsy risk profile ( $p = 0.032$ , Mantel-Cox test). Furthermore, GoF variants were associated with higher prevalence of DD/ID (100% GoF vs 32% LoF; odds ratio (OR) = not determined (ND);  $p = 0.0034$ , Fisher exact test), language issues (100% vs 26%; OR = ND;  $p = 0.0013$ ), psychiatric issues (100% vs 40%; OR = ND;  $p = 0.019$ ), motor skill issues (100% vs 16%; OR = ND;  $p = 0.00020$ ), hypotonia (100% vs 6.7%; OR = ND;  $p = 0.034$ ), and de novo variants (100% vs 25%; OR = ND;  $p = 0.033$ ) (Figure 5B). While epilepsy prevalence was similar (83% vs 81%; OR = 1.2 [95% CI 0.16–16];  $p = 1.0$ ), individuals with GoF variants were more likely to have ongoing seizures (83% vs 42%; OR = 6.9 [95% CI 0.91–85];  $p = 0.090$ ).

Seizure onset age was similar for individuals with missense vs null LoF variants (median 14 [IQR: 12–19] vs 15 [IQR: 9.3–18] months;  $p = 0.90$ , Mann-Whitney test), but those with missense LoF variants had a more severe epilepsy risk profile ( $p = 0.042$ , Mantel-Cox test). This is primarily because risk analysis includes all individuals in the null variant subgroup, that is, also those who only have FFSs (no epilepsy). Missense LoF variants were more likely to be inherited (50% vs 11%; OR = 8.0 [95% CI 1.3–46];  $p = 0.063$ , Fisher exact test) and associated with epilepsy (100% vs 68%; OR = ND;  $p = 0.059$ ) (Figure 5B). These individuals also had a higher prevalence of DD/ID (75% vs 5.2%; OR = 54 [5–610];  $p < 0.0001$ ), psychiatric issues (67% vs 22%; OR = 7.0 [1.5–35];  $p = 0.024$ ), and motor skill issues (42% vs 0%; OR =

ND;  $p = 0.0047$ ). No noticeable differences were noted for seizure freedom, language issues, and hypotonia.

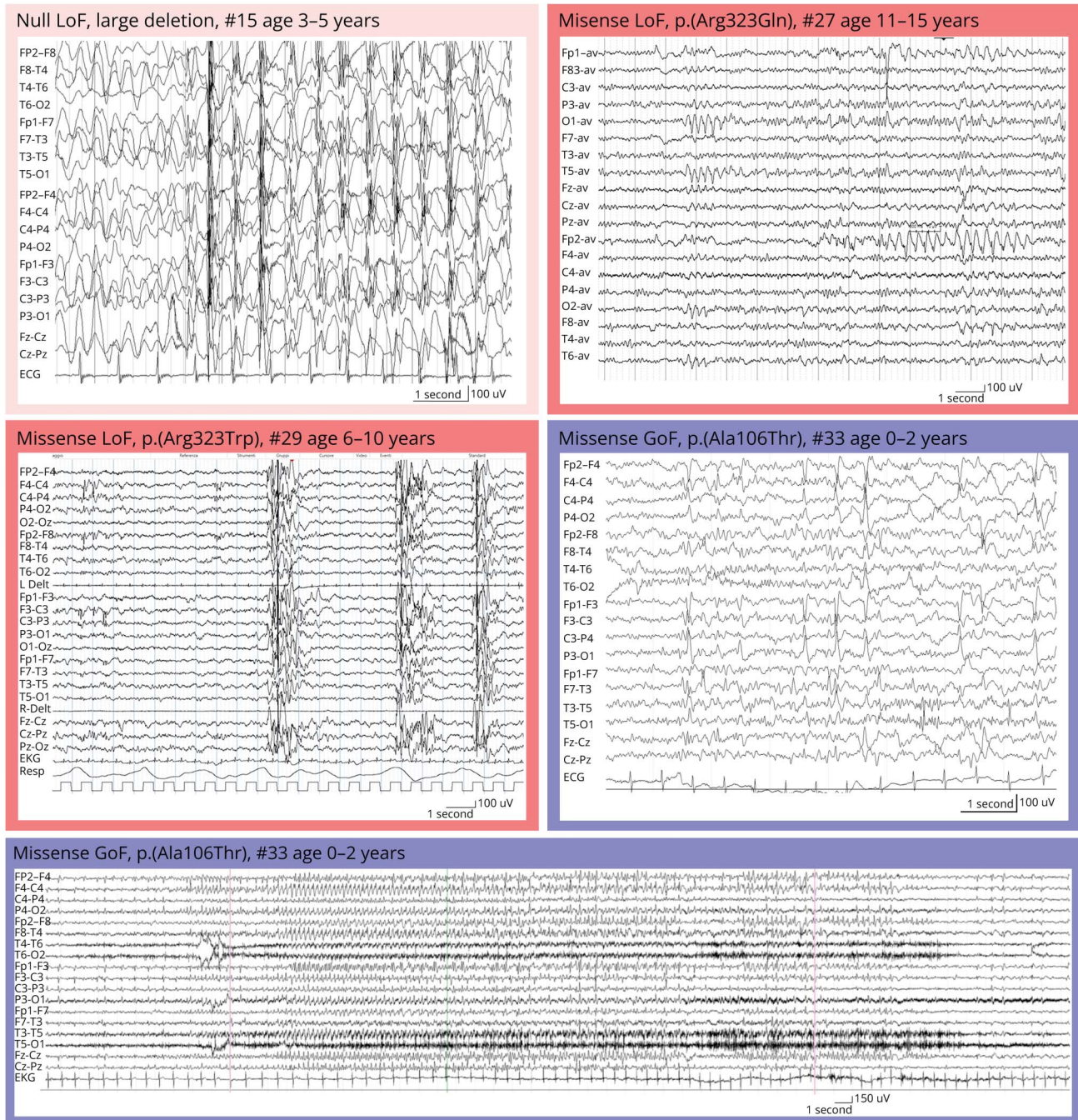
### Cumulative Severity Scores Reveal Strong Genotype-Phenotype Associations

To further assess subgroup differences, a severity scale was used to score the severity of incidence or comorbidity across the 8 phenotypic categories presented in Figure 5B and age at seizure onset (details in Methods and eTable 1). The GoF variant group had a higher median severity score of 13.5 (IQR: 11–14) compared with 3.5 (IQR: 2–6) for the LoF group ( $p < 0.0001$ , Mann-Whitney test). ROC analysis showed that the severity index was an excellent predictor of the GoF or LoF genotype, with an area under the curve of 0.97 (95% CI 0.89–1.0) ( $p = 0.00037$ , Wilson-Brown test) (Figure 5C). In addition, missense LoF variants were associated with more severe outcomes, with a median severity score of 5.5 (IQR: 4.1–7.0) compared with 3 (IQR: 2.0–3.5) for null variants ( $p < 0.0001$ , Mann-Whitney test). ROC analysis confirmed the severity as an excellent predictor of the missense or null LoF genotype, with an area under the curve of 0.90 (95% CI 0.79–1.0) ( $p = 0.00022$ ).

## Discussion

*GABRG2* variants are often inherited, and previous studies have documented both missense and null variants within large family pedigrees.<sup>7,39</sup> In this study, our objective was to provide a broader description of the phenotypic and genotypic spectrum of *GABRG2*-related disease, offering insights into the expected outcomes for novel variants. To achieve this, we established inclusion criteria that encompassed only previously unreported probands. From analysis of 44 cases, we identified 3 groups of pathogenic *GABRG2* variants, each associated with increasing phenotypic severity: (i) null (LoF) variants, (ii) missense LoF variants, and (iii) missense GoF variants.

**Figure 4** Representative EEGs From Individuals Carrying LoF or GoF Variants



Slow background activity, with generalized high-amplitude 3–4-Hz activity/spike and slow waves, was observed in an individual (#15) with a null LoF variant. Preserved background activity with asynchronous runs of spike-wave discharges in the left temporo-occipital and right anterior regions was observed in an individual (#27) harboring a missense LoF variant. Bilateral spike abnormalities in bilateral frontocentral regions, with right predominance and diffuse burst of irregular spike-wave discharges, were observed in an individual (#29) with a missense LoF variant. Disorganized background activity with intermixed multifocal epileptiform abnormalities (spikes and sharp waves) was observed in an individual (#33) with a missense GoF variant. Ictal EEG of a gelastic seizure in individual #33 shows that onset of the ictal discharge is in the right frontocentral region with bilateral spreading. GoF = gain-of-function; LoF = loss-of-function.

Individuals with null and missense LoF variants in *GABRG2* exhibit similar median seizure onset age of 14–15 months. These findings align with the previously reported median age of 11 months for individuals with *GABRB3* LoF variants.<sup>28</sup> However, contrary to previous findings that suggested that *GABRG2* null variants lead to more severe phenotypes than

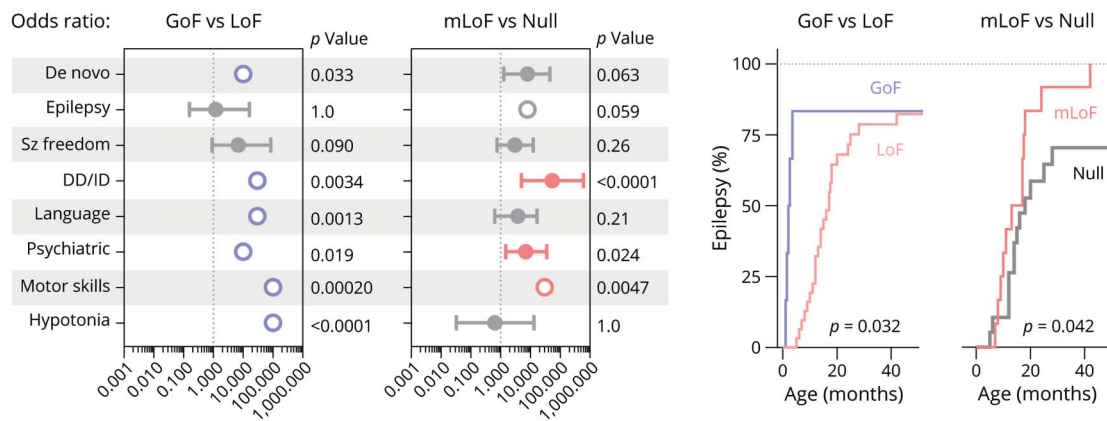
missense variants,<sup>12</sup> our observations indicate the opposite. Approximately 30% of individuals with null variants did not progress beyond simple FSs, and the remaining cases generally fell within the GEFS+ spectrum (Figure 3). By contrast, DEE and focal seizures were commonly observed in individuals with missense LoF variants. In addition, individuals with

**Figure 5** Genotype-Phenotype Associations for Individuals With Null LoF, Missense LoF, and GoF *GABRG2* Variants

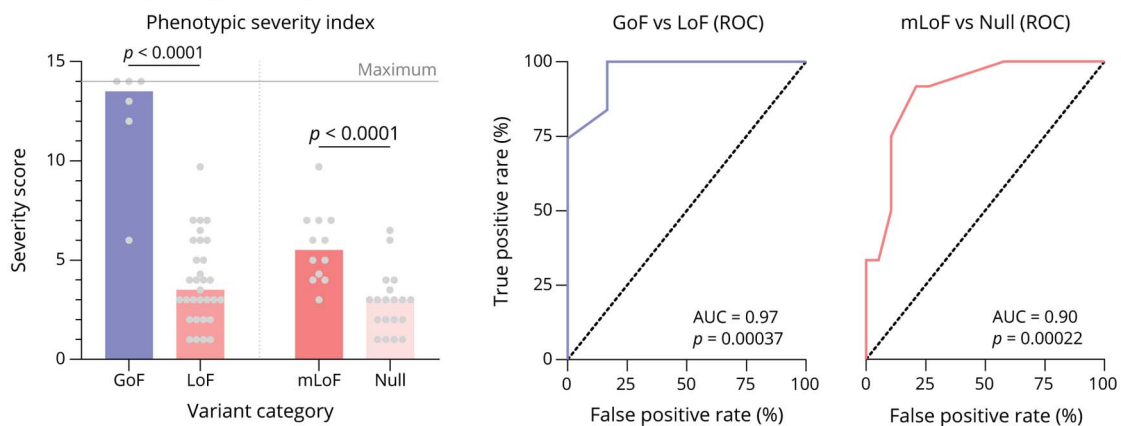
**A. Genotype-phenotype associations**



**B. Subcohort comparisons**



**C. Cumulative phenotypic severity**



(A) Phenotypic feature percentages are presented as horizontal slices for individuals carrying null LoF ( $n = 19$ ), missense LoF ( $n = 12$ ), and GoF ( $n = 6$ ) variants where available. The number of individuals with each feature is indicated, and the legend defines the meaning of the color coding for each feature. All displayed clinical information is extracted from eTable 1. (B) Odds ratio (OR) analyses of phenotype-genotype associations are presented, with the center circle denoting the OR with 95% CI. Light blue indicates significant enrichment in individuals with GoF variants. Light red indicates significant enrichment in individuals with missense LoF (mLoF) variants. Gray indicates no significant difference ( $p > 0.05$ ) between compared subgroups. Open circles without CIs indicate data where 1 category contains 0 or 100% of individuals, which means that the OR and CI cannot be determined. Statistical analyses were performed using the two-sided Fisher exact test with indicated  $p$  values. Statistics for epilepsy incidence were performed using the Mantel-Cox test with indicated  $p$  values. (C) As described in the methods, the cumulative phenotypic severities for individuals with the indicated subgroup of variants were calculated by assigning scores to each of the 9 phenotypic features depicted in Figure 5B. The maximum possible severity score of 14 is indicated by a gray line. Statistical analysis comparing the subgroups was performed using the Mann-Whitney test with indicated  $p$  values. A ROC curve comparing the true-positive and false-positive rates at different values of the severity index is shown comparing GoF and LoF variants and null and mLoF variants. The area under the curve is indicated, and statistical analysis comparing the subgroups was performed using the Wilson-Brown test with indicated  $p$  values. GoF = gain-of-function; LoF = loss-of-function.

null variants almost exclusively showed no DD/ID, psychiatric issues, and motor skill issues while most of those with missense LoF variants experienced DD/ID, psychiatric issues, and/or motor skill issues (Figure 5, A and B). A holistic approach to scoring phenotypic severity revealed that individuals with missense LoF variants received a median severity index score almost twice as high as that for individuals with null LoF variants (Figure 5C). Thus, despite overlapping features, individuals with null variants generally exhibit less severe phenotypes than those with missense LoF variants.

Null variants signify haploinsufficiency, potentially leading to an insufficient number of receptors in the synapse. By contrast, missense variants might result in the correct quantity of receptors, but with half exhibiting reduced or no functionality for the *GABRG2* gene. Therefore, although both null and missense LoF variants fall under the LoF category, they can still differ. Our findings indicate that having malfunctioning receptors in the synapse is more problematic than having an absence of receptors.

Three missense variants, A106T, S139N, and L285I, were identified as increasing GABA sensitivity consistent with a GoF molecular phenotype (Figure 2D). One individual (#36) harboring the S139N variant had ASD, mild DD/ID, and global hypotonia but no seizures. Five individuals carrying the A106T and L285I variants experienced early seizure onset, with a median of 2 months, and exhibited the most severe phenotypes within the cohort. These phenotypes closely resembled the pattern observed in individuals with GoF *GABRB3* variants, including an early infantile onset, drug-resistant seizures, and severe DD/ID.<sup>28</sup> The degree of phenotypic severity was highlighted by a median index score of 13.5 of 14 possible (Figure 5C), compared with 5.5 for the missense LoF.

The change in GABA sensitivity at receptors containing S139N was lower than that observed for A106T and L285I, which may explain the difference in phenotypic severity and lack of epilepsy in #36. In recent studies, some individuals with GoF *GABRA1*<sup>24</sup> and *GABRB2*<sup>27</sup> variants showing a small increase in GABA sensitivity likewise did not experience epilepsy, which may suggest that the magnitude of increase in GABA sensitivity is an important factor for epilepsy. Finally, previous reports have suggested that the A106T variant reduces GABA sensitivity,<sup>14</sup> but our functional studies contrast this finding and align with the observations for GoF phenotypes in *GABA<sub>A</sub>* receptors.

Two recurrent missense variants causing either LoF or GoF were observed in 3 or more unrelated individuals. This provides an opportunity to investigate clinical differences between individuals with the same variant but different genetic backgrounds. Individuals carrying the R323Q variant exhibited a range of phenotypes: one had MAE (#25, de novo), one had DEE (#26, inherited from a mother with mild phenotype), and one had FS+ associated with mild ID (#27, de novo). Notably, this recurrent variant has been reported in 13 families showing a broad phenotypic spectrum that includes

Dravet syndrome,<sup>40</sup> GEFS+ with mild ID,<sup>41</sup> GEFS+ with normal cognition,<sup>42</sup> self-limited epilepsy with centrotemporal spikes,<sup>19</sup> and early infantile epileptic encephalopathy with severe ID.<sup>14</sup> By contrast, the 4 individuals with the A106T variants displayed similar phenotypes falling within the DEE syndrome classification and exhibiting severe-profound DD/ID. Of interest, the A106T variant has been reported in 7 unrelated individuals, all showing a concordant phenotype. These individuals experienced seizure onset ranging from the first day of life to 4.5 months along with DEE syndrome and severe DD/ID.<sup>13,14</sup> Generally, it might be expected that the more severe the consequence of a variant on neuronal networks, the more consistent the clinical phenotype will be. For the recurrent variants in this study, the phenotypic spectrum associated with the LoF *GABRG2* variant leads to a wider range of clinical presentations compared with the GoF variant. This supports the notion that GoF variants cause more severe clinical phenotypes. However, larger studies involving additional recurrent variants are needed to clarify how much of the variation in clinical severity is due to the level of molecular dysfunction and how much is influenced by polygenic factors related to the genetic background or external environment.

Most protein-truncating variants in this study occur in the extracellular domain, resulting in the complete loss of the transmembrane domain essential for *GABA<sub>A</sub>R* function (Figure 1A). The Q398\* variant, however, resides in the TM3-TM4 intracellular loop and leads to a partial loss of the loop and the last transmembrane helix. Of interest, the location of truncating variants has been associated with varying levels of subunit aggregation and the potential for dominant negative effects. In fact, the Q398\* variant (previously referred to as per mature peptide, Q351\*, or by an alternative transcript Q390\*) has been suggested to exhibit such dominant negative effects.<sup>43</sup> In our cohort, the individual (#11) carrying the Q398\* variant only experienced FSs at 30 months of age without DD/ID or any other concerns. The variant was inherited from #11's mother, who was diagnosed with Dravet syndrome (patient V-21 in reference 7). In addition, #11's sister had epilepsy with eyelid myoclonia and ID (patient III-6, family M in reference 44). However, other close family members also had FSs or FSs with MAE despite not carrying the variant. As previously concluded,<sup>7</sup> factors beyond the variant itself contribute to the phenotypic variability within this family. Thus, #11's phenotype aligns with that of others carrying null variants in this cohort, and neither #11 nor the family history supports the notion that more severe phenotypes arise solely from dominant negative suppression effects of Q398\*.

Five primarily inherited variants, I85K, T90M, V104G, M199V, and I344L, showed no alterations in receptor function. Of these, T90M and M199V have been reported over 4 times in gnomAD, suggesting that they may be benign polymorphisms. While the remaining 3 variants may also be benign, the current data set cannot definitively exclude a potential pathogenic role. Of interest, the *in silico* prediction tools poorly anticipated the pathogenicity of these variants.

Only the I344L variant was correctly predicted as unlikely to be pathogenic while the remaining variants were predicted to be damaging, contradicting the functional studies and population database information. Furthermore, the A106T GoF variant was predicted as one of the least likely to be damaging, yet it causes the most severe phenotypes. Overall, the prediction tools resulted in discordant predictions for 29% of variants. These findings underscore the importance of assessing functional data for all *GABRG2* variants to both accurately establish their pathogenicity and determine whether the variant is a LoF or a GoF.

This study has several limitations. It relied on retrospective data from medical records and patient interviews, which may introduce variability. The limited number of participants also constrained our ability to develop a predictive model for the severity index, particularly for GoF variants. Despite the clear divergence in severity indices, a larger future cohort is needed for more robust predictive algorithms. In addition, *in vitro* expression systems do not fully capture the complexity of neuronal processes, potentially overlooking other factors influencing receptor function in a neuronal context.

In this study, we established associations between the phenotypic spectra of individuals with genetic variants in *GABRG2* and the functional consequences of these variants on GABA<sub>A</sub> receptor activity. The most severe epilepsy phenotype was observed in individuals with GoF variants, emphasizing *GABRG2*'s role as a cause of severe DEEs. In addition, LoF variants were associated with a milder phenotype in individuals with null variants compared with those with missense variants. This indicates that haploinsufficiency leads to mild or even benign (unaffected) phenotypes distinguishing GABA<sub>A</sub> receptors from other ion channels. For instance, *SCN1A* LoF variants are often associated with the GEFS+ spectrum while haploinsufficiency is linked to the more severe end of the spectrum such as Dravet syndrome.

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