Mutations in *GABRB3*

From febrile seizures to epileptic encephalopathies

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ABSTRACT

Objective: To examine the role of mutations in *GABRB3* encoding the β_3 subunit of the $GABA_A$ receptor in individual patients with epilepsy with regard to causality, the spectrum of genetic variants, their pathophysiology, and associated phenotypes.

Methods: We performed massive parallel sequencing of *GABRB3* in 416 patients with a range of epileptic encephalopathies and childhood-onset epilepsies and recruited additional patients with epilepsy with *GABRB3* mutations from other research and diagnostic programs.

Results: We identified 22 patients with heterozygous mutations in *GABRB3*, including 3 probands from multiplex families. The phenotypic spectrum of the mutation carriers ranged from simple febrile seizures, genetic epilepsies with febrile seizures plus, and epilepsy with myoclonic-atonic seizures to West syndrome and other types of severe, early-onset epileptic encephalopathies. Electrophysiologic analysis of 7 mutations in *Xenopus laevis* oocytes, using coexpression of wild-type or mutant β_3, together with α_5 and γ_{2s} subunits and an automated 2-microelectrode voltage-clamp system, revealed reduced GABA-induced current amplitudes or GABA sensitivity for 5 of 7 mutations.

Conclusions: Our results indicate that *GABRB3* mutations are associated with a broad phenotypic spectrum of epilepsies and that reduced receptor function causing GABAergic disinhibition represents the relevant disease mechanism. ***Neurology*® 2017;88:483–492**

GLOSSARY

DS = Dravet syndrome; **EE** = epileptic encephalopathies; **EOAE** = early-onset absence epilepsy; **ExAC** = Exome Aggregation Consortium; **FS** = febrile seizures; **GFS+** = genetic epilepsies with febrile seizures plus; **GGE** = genetic generalized epilepsies; **ID** = intellectual disability; **LGS** = Lennox-Gastaut syndrome; **MAE** = epilepsy with myoclonic atonic seizures; **WS** = West syndrome; **WT** = wild-type.

Disruption of GABAergic inhibition has been shown to cause epileptic seizures.[1] $GABA_A$ receptors are ligand-gated anion channels, and mutations in genes encoding different receptor subunits, e.g., *GABRG2, GABRA1*, *GABRD, GABRB2*, and *GABRB3*, have been associated with a wide spectrum of epilepsies from mild genetic generalized epilepsies (GGE)[2–6] to epileptic encephalopathies (EE).[7–10]

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Supplemental data at Neurology.org

Mutations in *GABRB3* encoding the β_3 subunit of the $GABA_A$ receptor have recently been reported in patients with severe epilepsies through large-scale studies.[7,9,11–14] However, the specific role of *GABRB3* pathogenic variants in the disease context has only recently been assessed in a single study for a small number of mutations,[15] and data on the functional consequences of most of the mutations are still lacking.

Here, we systematically analyze a large cohort of patients with various epilepsies for mutations in *GABRB3* and integrate clinical and genetic data from additional, unreported patients. We provide insight into the mutational landscape of *GABRB3*, including several recurrent mutations, and characterize the functional consequences of mutations for milder and more severe phenotypes using 2-microelectrode voltage clamping in *Xenopus laevis* oocytes.

METHODS

Patients. We screened a cohort of 416 patients sequentially referred for testing with various childhood-onset epilepsies for mutations in the *GABRB3* gene using a next-generation sequencing panel. Genomic DNA from blood was extracted with standard methods, and a next-generation sequencing panel screening method was applied that was based on the Ion Torrent PGM platform. Ion AmpliSeq (kit version 2.0) or Sureselect library building methods were used with subsequent clonal amplification and enrichment on an Ion OneTouch 2 system with the Ion PGM Template OT2 200 Kit, followed by sequencing on the Ion Torrent system with the Ion PGM 200 Sequencing Kit. In parallel, we ascertained additional, previously unreported patients through centers in Europe and the United States. The probands and their families underwent detailed clinical examinations, review of the medical files, MRIs, and EEG investigations. Seizures were diagnosed according to the International League Against Epilepsy, and epilepsy syndromes were established when possible.[16]

Mutation analysis. In 2 cases, mutations were identified with the gene panel mentioned above, which included targeted capture of all exons and at least 5 base pairs of flanking intronic sequence of *GABRB3*.

Patients with *GABRB3* mutations ascertained through collaborators were diagnosed through established diagnostic programs or research studies. Sanger sequencing was used to confirm all mutations and to perform segregation analysis.

Standard protocol approvals, registrations, and patient consents. The study was approved by the local ethics committees. All probands or, in case of minors, their parents or legal guardians gave informed consent.

Functional data. ***Mutagenesis and RNA preparation.*** We used the Quick Change kit (Stratagene, La Jolla, CA) to engineer 7 variants (p.V37G, p.R111*, p.T157M, p.Y184H, p.L256Q, p.Y302C, and p.R429Q) in cDNA encoding the $GABA_A$ receptor subunit β_3 (NM_000814.4, Origene Technologies, Rockville, MD). cRNAs were prepared with a custom laboratory protocol or the T7 mMessage mMachine kit from Ambion (Thermo Fisher Scientific, Waltham, MA).

Oocyte preparation and injection. The use of animals and all experimental procedures were approved by local authorities (Regierungspraesidium Tuebingen, Tuebingen, Germany). Oocytes were obtained from the Institute of Physiology I, Tuebingen and prepared as previously described.[10] Briefly, the procedure included treatment with collagenase (1 mg/mL of type CLS II collagenase, Biochrom KG, Berlin, Germany) in OR-2 solution (mmol/L: 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, and 5 Hepes, pH 7.6), followed by thorough washing and storing at 16°C in Barth solution [mmol/L: 88 NaCl, 2.4 $NaHCO_3$, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, and 5 Tris/HCl, pH 7.4 with NaOH] supplemented with 50 μg/mL gentamicin (Biochrom KG, Berlin, Germany). Equivalent amounts of cRNA were injected in parallel in oocytes from the same batch, plated in 96-well plates, and recorded at day 3 after injection.[10,17] The subunit combination was α_5, β_3, and γ_2s in a 1:1:2 ratio. All cRNA concentrations were adjusted to 2 μg/μL and 70 nL of the corresponding cRNA mixtures injected with the Robooinject system (Multi Channel Systems, Reutlingen, Germany).

Automated oocyte 2-microelectrode voltage clamp. $GABA_A$ receptor–mediated currents were recorded in oocytes at room temperature (20°C–22°C) with the Roboocyte2 system (Multi Channel Systems). Intracellular glass microelectrodes had a resistance of 0.3 to 1 MΩ when filled with 1 mol/L KCl/1.5 mol/L K-acetate. ND96 (mmol/L: 93.5 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, and 5 Hepes, pH 7.5) was used as the bath solution. Currents were sampled at 1 kHz, and the holding membrane potential was −70 mV.[10]

Electrophysiologic data analysis. Currents were analyzed with Roboocyte2+ (Multi Channel Systems), Microsoft Excel (Microsoft, Redmond, WA), and GraphPad Prism software (GraphPad Software, La Jolla, CA). Current amplitudes in response to 1 mmol/L GABA recorded on the same day were normalized to the mean value of the wild-type (WT) so that normalized data from different experiments could be pooled together. To obtain dose-response curves, the current response to each GABA concentration was normalized to the maximum response evoked by the highest GABA concentration (1 mmol/L), plotted against the respective concentration and for each cell fit to the following 4-parameter equation:

$$Y(X) = B_{max} \times X^h \Big/ \left(K_d^h + X^h\right)$$

where B_{max} is the maximum response to GABA, K_d is the concentration to achieve half-maximum response, and h is the Hill slope.

Data and statistical analyses. Data were tested for normal distribution with GraphPad Prism 6. Groups were compared with the use of one-way analysis of variance with the Tukey post hoc test. All data are presented as mean ± SEM.

RESULTS

Mutation analysis. In our screening cohort of 416 patients, we identified 2 patients (0.4%) with de novo *GABRB3* mutations and ascertained 20 additional patients through collaborators, yielding in total 22 patients with presumed pathogenic variants in *GABRB3* (table 1). Three patients have previously been reported (patients 3, 12, and 13).[9,18] Eighteen of 22 mutations were missense; 3 of 22 were truncating mutations; and one alteration was a partial duplication of exons 1 through 9.

Table 1 Phenotypic features of the 22 novel patients with *GABRB3* mutations

Patient	Epilepsy syndrome	Seizure onset	Seizure types/fsens	Seizure outcome/ age at offset	Intellectual disability	EEG	Additional features	MRI	cDNA*/protein position detected by (1), (2)
Familial epilepsies with febrile and generalized seizures (n = 2)									
1 (female)	EOAE,[a] Fam1	9 mo	FS, A, GTCS, fsens+	Sz-free/4 y	Normal intellect	GSW, PSW	None	None	c.110T>G, p.V37G, paternal, (1)
2 (male)	GEFS+,[a] Fam2	6 mo	FS, dyscognitive, GTCS, fsens+	Sz-free/10 y	Normal intellect	PSW, R frontal	None	Normal	c.1286G>A, p.R429Q, maternal, (1)
Focal/multifocal/ unclassifiable epilepsies (n = 4)									
3 (male)	Focal epilepsy	7 mo	Focal, unclassified, fsens+	Rare Sz	Mild, plateau at 7 mo	Focal IED	Mild ataxia, hypotonia	Normal	c.905A>G, p.Y302C, de novo (2)
4 (female)	Focal epilepsy	15 mo	Focal clonic, focal, myoclonic, fsens+	Sz-free/2 y	Mild	Multifocal, focal IED, +diffuse SW activated by sleep	Ataxia, hypotonia	Normal	c.902C>T, p.P301L, de novo (2)
5 (female)	Unclassified	4 mo	SE, clonic, hypomotor, GTCS, fsens−	Sz-free/3 y	Moderate	Multifocal	Autistic features	Bifrontal heterotopia	c.695G>A, p.R232Q, de novo (1)
6 (male)	Unclassified	11 mo	A, EM, unclassified, fsens+	Sz-free for 2 y/ rare seizures	Moderate	Multifocal	None	Normal	c.695G>A, p.R232Q, de novo (2)
EE with mild to severe intellectual disability (n = 16)									
7 (male)	MAE	36 mo	M, MA, GTCS, fsens−	Sz-free/4 y	Moderate, plateau at 3 y	GSW, PSW	None	Normal	c.8delG, p.Gly3fs*26, unknown (1)
8 (male)	MAE	9 mo	FS, GTCS, MA, fsens+	Daily Sz	Mild, stagnation at 16 mo	GSW	Mild ataxia	Normal	c.227C>G, p.S76C, de novo (1)
9 (male)	MAE	12 mo	FS, MA, GTCS, fsens+	Sz-free/4 y	Severe, 24 mo regression	Multifocal	Autism	Normal	c.331C>T, p.R111*, maternal (1)
10 (female)	MAE	12 mo	MA, fsens+	Sz-free/18 mo	Mild	PSW	Behavioral issues (aggression)	Normal	c.425G>T, p.R142L, maternal (mosaic: 10%–20%) (1)
11 (male)	MAE	14 mo	MA, GTCS, fsens+	GTCS in clusters	Mild	GSW	Normal	Normal	c.550T>C, p.Y184H, de novo (1)
12 (male)	DS-like,[a] Fam3	8 mo	FS, GTCS, Abs, M, A, fsens+	Weekly Sz	Mild, delays at 2.5 y	Bilateral SW	ADHD	Normal	c.470C>T, p.T157M, maternal, (2)
13 (male)	EE/WS	1 d	Focal, GTCS, IS, tonic, fsens-	Daily Sz	Severe, 3 mo stagnation	Hypsarrhythmia, GSSW	Hypotonia, dyskinesia	Hypomyelination	c.767T>A, p.L256Q, de novo (1)
14 (male)	EE/WS	4 mo	Dyscognitive, clonic, IS, fsens-	Sz-free/1 y	Moderate	Multifocal, burst sup	Hypotonia	Hypomyelination	Exon 1–9 duplication, de novo (1)
15 (male)	EE/WS	8 mo	IS, tonic, FS, GTCS, fsens−	Rare Sz	Moderate, regression (12 m)	Multifocal	Autism, ataxia, tremor	Normal	c.205G>A, p.A69T, transcript: ENST00000541819.2 unknown (1)
16 (female)	EE/LGS	17 mo	A, tonic, dyscognitive, GTCS, fsens−	Frequent Sz	Severe	Multifocal	Strabismus, hyperactivity and aggression	Normal	c.905A>G, p.Y302C, de novo (1)
17 (female)	EOEE	2.5 mo	Focal, clonic, migrating, fsens−	Weekly Sz	Severe	Multifocal	Acquired microcephaly (HC < −2 SD), hypotonia, quadriplegia	Severe, diffuse brain atrophy	c.372A>C, p.L124F, de novo (1)

Continued

Table 1 Continued

Patient	Epilepsy syndrome	Seizure onset	Seizure types/fsens	Seizure outcome/ age at offset	Intellectual disability	EEG	Additional features	MRI	cDNA*/protein position detected by (1), (2)
18 (male)	EOEE	3 mo	Focal, tonic, fsens−	Rare tonic Sz	Severe	Multifocal	Acquired microcephaly (HC < −3 SD), quadriplegia	Hypoplastic cerebellum	c.761C>T, p.S254F, de novo (1)
19 (female)	EOEE	2.5 mo	Hypomotor, M, fsens+	Rare Sz	Severe	Multifocal	Hypotonia	Atrophy, hypomyelination, thin CC	c.554C>T, p.T185I, de novo (2)
20 (male)	EOEE	5 mo	A, tonic, SE, fsens NA	Weekly Sz	Severe	Multifocal	Hypotonia	Volume loss, brainstem atrophy	c.380A>G, p.K127R, de novo (1)
21 (male)	Focal epilepsy/ EE	11 mo	Dyscognitive, GTCS, unclassified, A, fsens NA	Frequent Sz up to 300 Sz/d	Severe	Multifocal	Autistic features, hand stereotypies, Rett-like	Normal	c.758C>T, p.P253L, (mosaic: 20%), de novo (1)
22 (male)	EE/LGS	11 mo	Febrile GTCS, dyscognitive, Abs-like, tonic, fsens NA	Daily	Severe, regression (5 y)	Multifocal	Self-injury episodes, hand stereotypies, hyperventilation episodes	Normal	c.694C>T, pArg232*, paternal (1)
Family members with *GABRB3* mutations (n = 9)									
Fam1, I.2 (male)	FS+	<5 y, 14 y	FS, GTCS	Multiple FSs, single GTCS Sz-free	Normal intellect	NA	None	Not done	c.110T>G, p.V37G, paternal
Fam1, II.2 (female)	FS	9 mo	FS	6 FS, Sz-free	Normal intellect	NA	None	Not done	c.110T>G, p.V37G, paternal
Fam2, I.1 (female)	No Sz	—	—	No Sz	Normal intellect	NA	None	Not done	c.1286G>A, p.R429Q, maternal
Fam2, II.2 (male)	FS+	2 y, 5 y	FS, GTCS	Sz-free	Normal intellect	Occipital sharp-slow waves (9 y)	None	Not done	c.1286G>A, p.R429Q, maternal
Fam3, I.1 (female)	FS	NA	FS	Multiple FSs, Sz-free	Normal intellect	NA	None	Not done	c.470C>T, p.T157M,
Fam3, II.2 (female)	GEFS+	First year of life	FS, GTCS, Abs	Sz-free	Normal intellect	NA	None	Not done	c.470C>T, p.T157M, maternal
Fam3, II.3 (female)	FS	First year of life	FS	Multiple FSs, Sz-free	Normal intellect	NA	None	Not done	c.470C>T, p.T157M, maternal
Fam3 III.1 (male)	GEFS+	First year of life	FS, GTCS	Sz-free	Normal intellect	Normal	None	Not done	c.470C>T, p.T157M, maternal
Fam3, III.5 (female)	DS-like	5 mo	FS, febrile SE, GTCS, Abs, M, fsens+	Weekly to monthly Sz	Mild delay since age 1.5 y	Normal	Hyperactivity, aggressiveness	Not done	c.470C>T, p.T157M, maternal

Abbreviations: A = atonic; Abs = absence seizure; ADHD = attention-deficit/hyperactivity disorder; CC = corpus callosum; DS = Dravet syndrome; dyscognitive = focal dyscognitive seizure; EE = epileptic encephalopathies; EM = eyelid myoclonia; EOAE = early-onset absence epilepsy; EOEE = early-onset epileptic encephalopathy; Fam = family; FS = febrile seizures; FS+ = febrile seizures plus; fsens+/− = fever sensitivity positive/negative; GSSW = generalized synchronized spike wave; GSW = generalized spike wave; GTCS = generalized tonic-clonic seizure; IED = interictal epileptic discharges; IS = infantile spasm; LGS = Lennox-Gastaut syndrome; M = myoclonic; MA = myoclonic atonic; MAE = myoclonic atonic epilepsy; NA = not applicable; PSW = poly spike wave; SE = status epilepticus; SW = spike wave; Sz = seizure; WS = West syndrome; *Transcript = NM_000814.4.

cDNA*/protein position detected by next-generation sequencing panel diagnostic (1) or whole-exome sequencing (2).

[a] Family history (see figure 1B).

Concerning the inheritance mode, 14 of 22 mutations occurred de novo, including in one patient who was mosaic; 3 of 22 mutations segregated within the family in a dominant fashion; 1 of 22 was inherited from an unaffected mother who was mosaic for the mutation; and 2 of 22 were inherited from an unaffected parent. Complete segregation analysis could not be performed for 2 of 22 mutations, including one recurrent mutation that occurred de novo in 2 other patients in this study.

All missense mutations were predicted to be damaging by 1 or 2 prediction tools (Polyphen2 or SIFT; table e-1 at Neurology.org). Twenty-one of 22 mutations were not present in the Exome Aggregation Consortium (ExAC) database, whereas one mutation (p.A69T) found in an alternative *GABRB3* transcript was observed in 2 of 14,132 chromosomes. The protein positions of the different *GABRB3* mutations are shown in figure 1A.

Overall mutational landscape. Mining the available literature and databases, we were able to identify 16 additional previously reported *GABRB3* cases (table e-1).[7,9,11,12,14,19] The majority of the published mutations occurred de novo and were associated with EE. From the complete dataset of 38 patients with *GABRB3* mutations, 5 sites with recurring mutations at identical amino acid positions emerged: p.D120N (2x), p.K127R (2x), p.R232Q (2x)/p.R232*(1x), p.Y302C (3x), and p.A305V/p.A305T (1x each) (figure 1A).

Phenotypic analysis. ***Index patients.*** The phenotypic spectrum in our study varied from genetic epilepsies with febrile seizures plus (GEFS+) and early-onset absence epilepsy (EOAE) to multifocal epilepsy, EE within the Dravet syndrome (DS) spectrum, epilepsy with myoclonic atonic seizures (MAE), West syndrome (WS), Lennox-Gastaut syndrome (LGS), and other unclassifiable types of EE. The median age at seizure onset was 8.5 months (range 1 day–36 months). Fever-associated seizures were reported in 11 of 19 patients for whom data were available (2 of 2 with GGE, 3 of 4 with unclassified epilepsies, 1 of 1 with DS-like EE, 4 of 5 with MAE, and 1 of 7 with other EE/EOEE).

Sixteen of 22 patients (73%) had EE, including DS-like EE (n = 1), MAE (n = 5), WS (n = 3), LGS (n = 2), and unclassifiable EE/EOEE (n = 5). These patients had various seizure types, including infantile spasms, focal dyscognitive seizures, tonic seizures, myoclonic seizures, atonic seizures, myoclonic-atonic seizures, and generalized tonic-clonic seizures. Four of 22 patients had focal or unclassifiable epilepsy with onset between 4 and 15 months and various seizure types. One patient (patient 4) with focal epilepsy had focal EEG abnormalities, which were activated by sleep and became bilaterally diffuse. Three of 4 patients with focal/unclassifiable epilepsy (patients 4–6) became seizure-free between 2 and 3 years of age. However, patient 6 had single seizures after he was tapered off medication. The last patient (patient 3) in this group had rare seizures. Two of 22 patients had generalized epilepsies well controlled on antiepileptic medication, EOAE (patient 1) and GEFS+ (patient 2) with offset at age 4 and 10 years, respectively.

The 2 patients with GGE had normal cognitive skills, in contrast to patients with focal/unclassifiable epilepsy and EE, who presented with mild to severe intellectual disability (ID). Behavioral and psychiatric disturbances, including attention-deficit/hyperactivity disorder, autistic features, agitation, hyperactivity, and aggression, were reported in 8 of the 22 patients (36%).

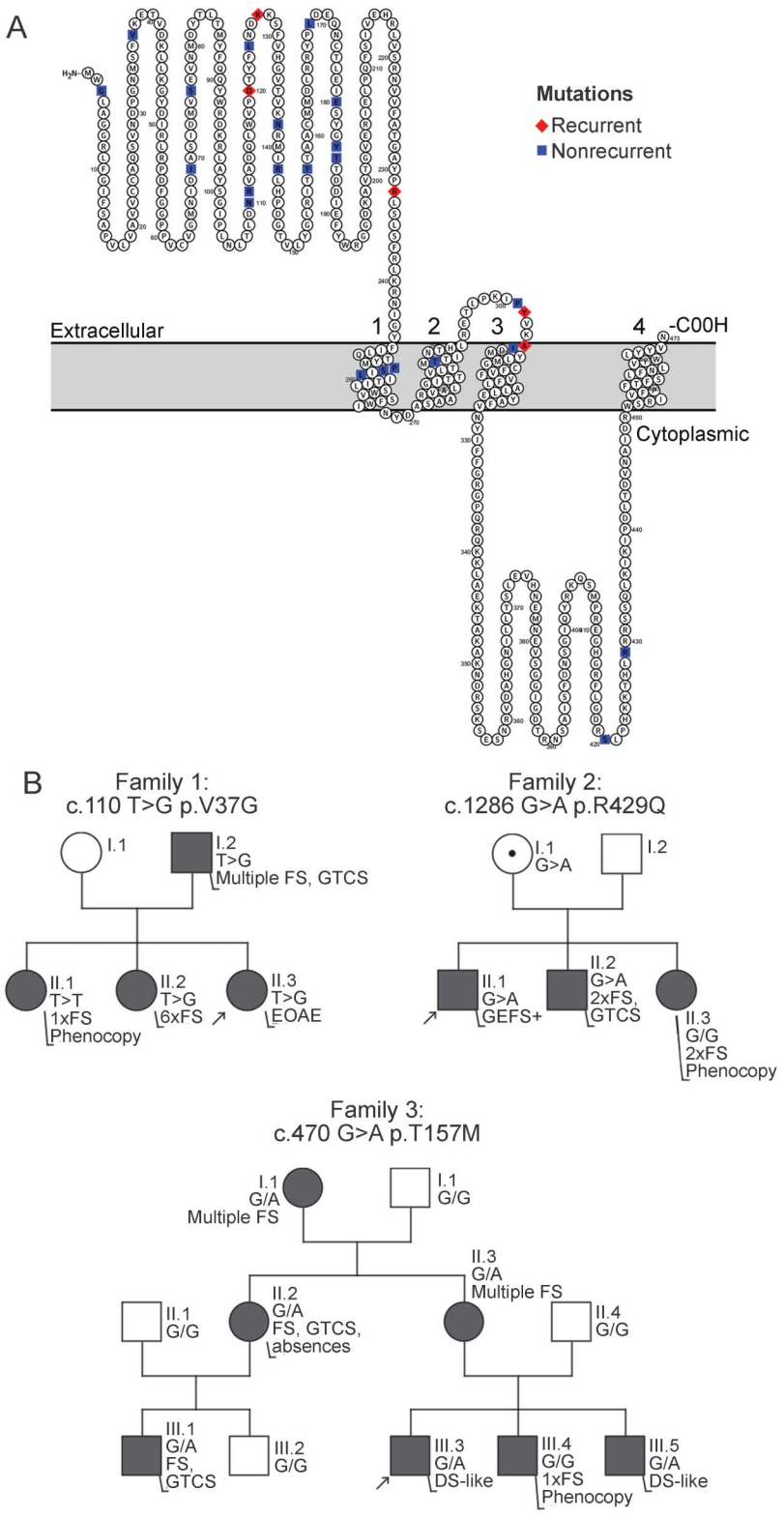
Seven of 22 patients (32%) had MRI abnormalities, including bilateral frontal heterotopia with suspected polymicrogyria (patient 5), hypomyelination (patients 13 and 14), severe diffuse brain atrophy (patient 17), hypoplastic cerebellum (patient 18), generalized volume loss with thin corpus callosum (patient 19), or prominent cortical and subcortical volume loss with brainstem atrophy (patient 20). However, no clear pattern typical for *GABRB3* mutations emerged from these observations. Except for one patient (patient 5), all MRI abnormalities were detected in patients of the EE subgroup.

Family members. The 2 individuals with GGE (patient 1/family 1, patient 2/family 2) and the patient with a DS-like phenotype (patient 12/family 3; e-supplement) were index patients of multiplex families (table 1 and figure 1B). In total, we identified 9 family members carrying pathogenic *GABBR3* variants, including one unaffected carrier. Seven of the 8 affected family members became seizure-free and had a normal intellect. The sister of patient 12 was classified as DS-like (e-supplement), whereas the remaining affected family members of family 3 had GEFS+ or simple FS (table 1). The 2 sibs in family 3 with a DS-like phenotype have previously tested negative for mutations in other DS-associated genes, including *SCN1A*, *GABRG2*, and *GABRA1*. Phenocopies presenting with simple FS were observed in each family.

Functional data. To cover the described phenotypic spectrum, a set of 7 mutations was assorted for functional analysis using 2-microelectrode voltage clamp experiments in *Xenopus laevis* oocytes (figure 2A). The spectrum included mutations associated with familial GEFS+/DS-like (p.R429Q and p.T157M), familial GEFS+ and EOAE (p.V37G), MAE (p.Y184H, p.R111*), WS (p.L256Q), and focal epilepsy/EE/LGS (p.Y302C).

Recordings of different mutations were performed in parallel with the WT in every batch of oocytes.

Figure 1 ***GABRB3* mutations in epilepsy and pedigrees of *GABRB3* multiplex families**



(A) The amino acid locations of the identified *GABRB3* missense mutations. (B) Pedigrees of multiplex families carrying *GABRB3* mutations. DS = Dravet syndrome; EOAE = early-onset absence epilepsy; FS = febrile seizures; GEFS+ = generalized epilepsy with febrile seizures plus; GTCS = generalized tonic-clonic seizure.

Current amplitudes obtained after application of 1 mmol/L GABA showed tentatively but not significantly smaller currents for p.R429Q and p.T157M. In contrast, strongly and significantly reduced current amplitudes were found for p.V37G, p.Y184H, p.L256Q, p.R111*, and p.Y302C (figure 2B).

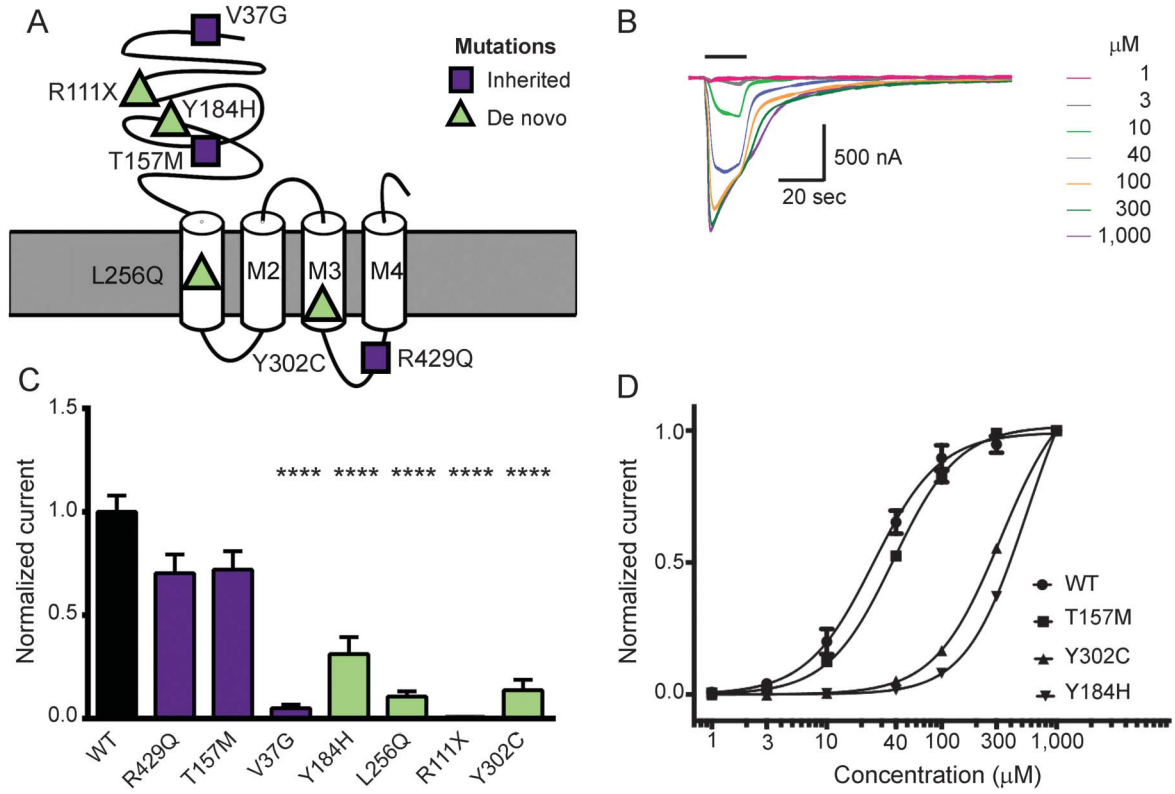
To investigate whether a change in the GABA sensitivity of the mutated receptors could be an additional underlying pathophysiologic mechanism, we recorded concentration curves for WT and the mutations with current amplitudes large enough for this type of recording, including p.T157M, the recurring mutation p.Y302C, and p.Y184H. Whereas the dose-response curve obtained for p.T157M revealed no significant difference compared to the WT, the 2 remaining mutations caused a pronounced rightward shift, indicating a significantly reduced GABA sensitivity. This shift appears to explain, at least in part, the pronounced reduction of current amplitudes observed at 1 mmol/L GABA for the 2 mutations because dose-response curves did not saturate at 1 mmol/L GABA.

DISCUSSION Even though individual mutations in *GABRB3* have recently been reported in patients with severe epilepsies,[7,11,12,14] the role of *GABRB3* in human epilepsies in terms of causality and associated phenotypes has remained largely unclear. In this study, we describe a comprehensive cohort of patients carrying *GABRB3* mutations, delineate the mutational and phenotypic spectrum, and demonstrate loss of protein function as the causative disease mechanism in severe cases.

We found a wide phenotypic spectrum associated with *GABRB3* mutations, ranging from FS/GEFS+ and EOAE to unclassified focal epilepsies, MAE, DS-like EE, WS, LGS, and other types of EE. Among the group of patients with EE, the cognitive impairment was more severe and the epilepsy more refractory compared to the group of patients with GEFS+, EOAE, and unclassified focal epilepsy. Within the EE group, patients with MAE had a less severe outcome; 4 of 5 MAE patients became seizure-free, and most of them had mild ID.

We also tested 7 *GABRB3* mutations functionally. For 2 mutations (family 2, patient 2: p.R429Q; family 3, patient 12: p.T157M), we did not find significant changes compared to WT receptors. Such a finding is not unusual for the functional screening in *Xenopus* oocytes, which are not perfectly suited to detect minor abnormalities. Even though the severity of the phenotypes within families was variable, the overall familial phenotype was milder for both mutations compared to patients carrying de novo mutations. Furthermore, the phenotypes are compatible with the known GEFS+ spectrum, including FS, FS+, GEFS+, MAE, and DS, which is also well known from families carrying

Figure 2 Functional analysis of *GABRB3* mutations using *Xenopus laevis* oocytes



(A) Schematic representation of the β3 subunit of the $GABA_A$ receptor, including the predicted positions of the mutated amino acids. (B) Examples of current responses to application of increasing GABA concentrations (μmol/L: 1, 3, 10, 40, 100, 300, and 1,000) recorded from *Xenopus* oocytes expressing wild-type (WT) $\alpha_5\beta_3\gamma_{2s}$ receptors. (C) Normalized current response to 1 mmol/L GABA application for WT (n = 120), R429Q (n = 41), T157M (n = 39), V37G (n = 29), Y184H (n = 36), L256Q (n = 31), R111X (n = 31), and Y302C (n = 34). ****$p < 0.0001$, one-way analysis of variance with the Tukey multiple-comparisons test. (D) Dose-response curve for $\alpha_5\beta_3\gamma_{2s}$ WT (n = 11), T157M (n = 6), Y302C (n = 3), and Y184H (n = 7) receptors recorded on application of different GABA concentrations (as in A) and normalized to the maximal response (1,000 μmol/L) for each cell. EC_{50} values were 25.3, 38.0, 326.4, and 552.3 μmol/L with the 95% confidence intervals ranging from 20.7 to 29.9, 35.4 to 40.6, 293.8 to 359.0, and 433.9 to 670.7 μmol/L for WT, T157M, Y302C, and Y184H, respectively.

mutations in other GEFS+ genes, e.g., *SCN1A* or *GABRG2*.[20,21] Moreover, these mutations are not found in the general population, are evolutionarily conserved, and are predicted to be damaging by bioinformatic programs. In contrast, the third mutation (family 1, patient 1: p.V37G) identified in a family with GGE was found to cause a significant loss-of-function effect. These discrepancies between the genotypes and phenotypes with a severe loss of function in a family with a mild phenotype (family 1) and a virtual lack of *GABRB3* impairment in a family with a more severe phenotype (family 3) suggest that factors other than the *GABRB3* variant must be contributing to the disease phenotype. Possible reasons include the overall genetic background, i.e., a complex combination of different genetic variations, or more specific genetic factors with larger detrimental or protective effects. Both phenomena have been described in mouse models,[22,23] but their role in human epilepsy is not understood.

The remaining 4 mutations that were investigated functionally showed a clear loss of function, including a strong reduction in GABA-evoked current amplitudes and, as demonstrated for p.Y302C and p.Y184H, in GABA sensitivity. This is in line with previous findings in other $GABA_A$ receptor subunits causing similar syndromes.[2,4,5,24,25] Accordingly, our results suggest GABAergic disinhibition as a major disease mechanism in genetic epilepsies due to pathogenic *GABRB3* variants. This is corroborated in a recent study in which 4 EE-associated *GABRB3* mutations showed loss of function with a possible genotype-phenotype correlation.[26] Future studies will clarify whether these findings may extend to a number of novel mutations reported in our publication.

We identified several recurrent mutations and observed a wide range of phenotypic variability for mutation carriers. For example, p.Y302C was observed in 2 patients in this study. Patient 3 had focal epilepsy with onset at 7 months of age, rare seizures, and mild ID. In contrast, patient 16 had intractable EE starting at the age of 17 months, which evolved into LGS with severe ID. The mutation has previously been reported in a patient with onset of focal epilepsy starting at the age of 10 months that

evolved into LGS with severe ID. Furthermore, p. R232Q was observed in 2 individuals (patients 5 and 6) with moderate ID and treatable multifocal epilepsy with onset at 4 and 11 months of age, respectively. Patient 5 had autistic features and bifrontal heterotopias on MRI, features that were not present in patient 6, indicating that the phenotypic variability of single mutations may also include the presence of brain malformations. These results indicate a considerable pleiotropy even for single mutations. We were not able to identify a straightforward genotype-phenotype correlation, as previously described for other genes associated with genetic epilepsy.[15,27]

Of note, we found 5 patients with a phenotype compatible with MAE, forming a separate subgroup within the spectrum of *GABRB3*-related phenotypes. A genetic etiology for MAE has been suggested for decades, and several studies have shown that MAE can occur in GEFS+ families with *SCN1A* or *GABRG2* mutations.[6,13,28–33] Furthermore, mutations in *SLC2A1* have been found in a subset of patients with MAE and paroxysmal exertional dyskinesia/motor dysfunction.[34,35] Recently, we described mutations in *SLC6A1,* encoding one of the major GABA transporters, in several probands with MAE[36]; in addition, de novo mutations in *GABRG2* and *CHD2* have been reported.[37] The present study adds *GABRB3* to the growing list of genes associated with MAE.

For some of the *GABRB3* variants identified in our cohort, the evidence for pathogenicity is limited, and further validating studies are required to understand their significance. This limitation applies to all variants found in familial cases, given that none of the families were large enough to generate significant linkage. In addition, the fact that 2 of the familial variants did not show a significant change in function in *Xenopus* oocytes further complicates the interpretation. Interestingly, all families contained phenocopies. While this phenomenon is well known in familial fever-related epilepsies, it does not support our current evidence of the pathogenicity of the variants. Two individuals in the ExAC database were found to carry the predicted p.A69T variant, which we detected in our EE group. While there is no clear consensus in the field on how very-low-frequency ExAC mutations should be interpreted, the apparent presence of these variants in presumably unaffected individuals casts some doubt on the role of the variant in the etiology of the patient's disease. Furthermore, 2 truncating variants were inherited from unaffected parents, suggesting that the loss of one allele in these patients either is not associated with the disease or does not show full penetrance. The fact that *GABRB3* truncations have not been reported in the ExAC database argues for the latter hypothesis. It has also been shown that the epileptic phenotype observed in mouse models lacking one allele of a $GABA_A$ receptor subunit gene may be milder compared to missense mutation carriers,[38] although this may depend on the functional interactions of the truncated protein and the remaining WT subunits.[39] Lastly, we cannot completely exclude that the parents carry these mutations in a mosaic state, as seen in the mother of patient 9, even though this is not obvious from the blood DNA analysis.[40]

This study demonstrates that *GABRB3* mutations are associated with a phenotypic spectrum ranging from simple FSs, GEFS+, and EOAE to MAE, EE within the DS spectrum, and other severe EE, defining a novel genetic entity within the GEFS+ and EOEE spectrum. *GABRB3* mutations cause reduced receptor function, predicting impairment of GABA-mediated inhibition as disease mechanism.

AUTHOR CONTRIBUTIONS

R.S.M. and T.V.W. conceived and designed the study, collected and analyzed data, and wrote the manuscript. I.H. collected and analyzed data and edited the manuscript. C.M. provided inclusion of patients and analysis of phenotypic data. K.M.J. collected and analyzed data. E.H.B,.U.V., I.B., I.T., T.T., G.K., and L.L.F. provided inclusion of patients and analysis of phenotypic data. G.L. and J.d.B. collected and analyzed data. S.B. provided inclusion of patients and analysis of phenotypic data. N.C. collected and analyzed data. N.H., J.J., M.S., C.B., S.S., and M.N. provided inclusion of patients and analysis of phenotypic data. C.T.M., L.H.G.L., S.V., and M.P. collected and analyzed data. S.v.S., S.H., and H.D. provided inclusion of patients and analysis of phenotypic data. Y.M. collected and analyzed data. K.M., H.H., and K.L.v.G. provided inclusion of patients and analysis of phenotypic data. H.A.D., N.T., H.C.M., and G.R. collected and analyzed data. R.G. provided inclusion of patients and analysis of phenotypic data. J.R.L. collected and analyzed data. H.L. collected and analyzed data and edited the manuscript. H.M. and S.M. conceived and designed the study, collected and analyzed data, and edited the manuscript.

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DISCLOSURE

R. Møller, T. Wuttke, and I. Helbig report no disclosures relevant to the manuscript. C. Marini reports being associate editor of epileptic disorders; receiving speaker honoraria at VII Congresso Nazionale Simmesn–Firenze, December 16–18, 2015, from the Swedish Orphan Biovitrum; serving as a member of the editorial board of *Epilepsia* (2007–2012); being associate editor of epileptic disorders (2014), Italian Minister of Health, Research Program Section (RF- 2009-1525669; title of the project: Clinical and Genetic Study of Early Onset Epilepsies of Unknown Aetiology; duration, 2011–2013); and receiving a research grant from CARIPLO Foundation,

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