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Rare *GABRA3* variants are associated with epileptic seizures, encephalopathy and dysmorphic features

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Genetic epilepsies are caused by mutations in a range of different genes, many of them encoding ion channels, receptors or transporters. While the number of detected variants and genes increased dramatically in the recent years, pleiotropic effects have also been recognized, revealing that clinical syndromes with various degrees of severity arise from a single gene, a single mutation, or from different mutations showing similar functional defects. Accordingly, several genes coding for $GABA_A$ receptor subunits have been linked to a spectrum of benign to severe epileptic disorders and it was shown that a loss of function presents the major correlated pathomechanism. Here, we identified six variants in *GABRA3* encoding the α_3-subunit of the $GABA_A$ receptor. This gene is located on chromosome Xq28 and has not been previously associated with human disease. Five missense variants and one microduplication were detected in four families and two sporadic cases presenting with a range of epileptic seizure types, a varying degree of intellectual disability and developmental delay, sometimes with dysmorphic features or nystagmus. The variants co-segregated mostly but not completely with the phenotype in the families, indicating in some cases incomplete penetrance, involvement of other genes, or presence of phenocopies. Overall, males were more severely affected and there were three asymptomatic female mutation carriers compared to only one male without a clinical phenotype. X-chromosome inactivation studies could not explain the phenotypic variability in females. Three detected missense variants are localized in the extracellular GABA-binding NH_2-terminus, one in the M2-M3 linker and one in the M4 transmembrane segment of the α_3-subunit. Functional studies in *Xenopus laevis* oocytes revealed a variable but significant reduction of GABA-evoked anion currents for all mutants compared to wild-type receptors. The degree of current reduction correlated partially with the phenotype. The microduplication disrupted *GABRA3* expression in fibroblasts of the affected patient. In summary, our results reveal that rare loss-of-function variants in *GABRA3* increase the risk for a varying combination of epilepsy, intellectual disability/developmental delay and dysmorphic features, presenting in some pedigrees with an X-linked inheritance pattern.

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Abbreviation: GGE = genetic generalized epilepsy

Introduction

Genetic epilepsy has been commonly associated with alterations in genes coding for ion channels and receptors (Lerche *et al.*, 2013). Recent employment of next generation sequencing techniques, including exome sequencing and whole genome copy number variant analysis, corroborate such observations by identifying a number of novel ion channel and receptor gene variants in epilepsy patients (Veeramah *et al.*, 2012; Weckhuysen *et al.*, 2012; Epi4K Consortium *et al.*, 2013; Carvill *et al.*, 2014; Lemke *et al.*, 2014; Nava *et al.*, 2014; Schubert *et al.*, 2014; Weckhuysen and Korff, 2014; Larsen *et al.*, 2015; Syrbe *et al.*, 2015; Leu *et al.*, 2016; Papandreou *et al.*, 2016; Møller *et al.*, 2017). Apart from identifying novel disease genes, these findings reveal several additional developments. First, they expand the range of syndromes associated with some of the previously identified epilepsy genes. Second, they suggest that severe epileptic encephalopathies, encompassing both refractory seizures and developmental delay often represent sporadic cases, with *de novo* occurring mutations. Lastly, they point to an increasing number of genes and variants showing a pleiotropic effect, when distinct clinical syndromes with different degrees of severity arise from a single gene, a single variant, or from different variants showing similar functional defects (Claes *et al.*, 2001; Harkin *et al.*, 2007; Veeramah *et al.*, 2012; Weckhuysen *et al.*, 2012; Epi4K Consortium *et al.*, 2013; Suls *et al.*, 2013; Carvill *et al.*, 2014; Nava *et al.*, 2014; Weckhuysen and Korff, 2014; Blanchard *et al.*, 2015; Howell *et al.*, 2015; Syrbe *et al.*, 2015; Epi4K Consortium, 2016; Johannesen *et al.*, 2016; Janve *et al.*, 2017; Møller *et al.*, 2017; Shen *et al.*, 2017; Wolff *et al.*, 2017).

GABA is the main inhibitory neurotransmitter in the adult brain. Its action depends on two classes of proteins, ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors, which mediate fast and slow synaptic inhibition, respectively. Furthermore, both receptor types are involved in tonic inhibition. Nineteen different genes from eight different classes encode $GABA_A$ receptor subunits, which combine into heteropentamers to form postsynaptic ligand-gated anion channels. Pentamers usually contain two alpha and two beta subunits, which conjoin with a subunit

from one of the other classes. Subunit combination determines functional properties of the receptor as well as its spatiotemporal expression pattern (Fritschy and Panzanelli, 2014).

Presuming that neuronal hyperexcitability seen in epilepsy arises from a disturbed balance of inhibitory and excitatory neurotransmission in the brain, it is somewhat surprising that so far only four genes (*GABRA1*, *GABRB3*, *GABRG2* and *GABRD)* encoding the α_1-, γ_2-, β_3- and δ-subunits of $GABA_A$ receptors have been directly associated with epilepsy (Macdonald *et al.*, 2012). Initially, the correlated syndromes included childhood absence epilepsy, juvenile myoclonic epilepsy, and febrile seizures with or without epilepsy including generalized/genetic epilepsy with febrile seizures plus (GEFS +) (Baulac *et al.*, 2001; Wallace *et al.*, 2001; Cossette *et al.*, 2002; Harkin *et al.*, 2002; Kananura *et al.*, 2002; Maljevic *et al.*, 2006; Macdonald *et al.*, 2012). Recent studies extended this list to different forms of epileptic encephalopathies, including epilepsy with myoclonic-atonic seizures and Dravet syndrome (Carvill *et al.*, 2014; Epi4K Consortium, 2016; Johannesen *et al.*, 2016; Papandreou *et al.*, 2016; Janve *et al.*, 2017; Møller *et al.*, 2017; Shen *et al.*, 2017). The association of a single gene with a spectrum of partially overlapping epileptic phenotypes, from mild to severe ones has been mainly attributed to the brain sodium channel gene *SCN1A* and to *SLC2A1* encoding the GLUT1 transporter (Claes *et al.*, 2001; Harkin *et al.*, 2007; Leen *et al.*, 2010; Lerche *et al.*, 2013; Weckhuysen and Korff, 2014). However, a similar genotype–phenotype correlation pattern has recently also been reported for the *GABRA1*, *GABRB3* and *GABRG2* genes encoding α_1-, β_3- and γ_2-subunits of the $GABA_A$ receptor, respectively (Carvill *et al.*, 2014; Epi4K Consortium, 2016; Johannesen *et al.*, 2016; Kang and Macdonald, 2016; Møller *et al.*, 2017).

In this study, we identified several variants in the *GABRA3* gene, encoding the α_3-subunit of $GABA_A$ receptors, in families and sporadic cases affected either by variably severe epilepsy with encephalopathy, or by genetic generalized epilepsy (GGE), presenting a milder form of epilepsy with complex inheritance.

Materials and methods

Patients and cohorts

Parents or the legal guardian of each patient enrolled in this study signed an informed consent form for participation. The study was approved by the local ethics committees of each participating clinical centre. Genomic DNA of the individuals was extracted from peripheral blood according to standard procedures.

The analysed cohorts included: (i) a cohort from the Wolfson Medical Center, Holon, Israel wih 15 families with various forms of epilepsy; (ii) a cohort of 480 families with X-linked intellectual disability (XLID) collected by the EURO-MRX consortium and associated groups; (iii) a cohort of 600 cases undergoing diagnostic high-density array-comparative genomic hybridization (CGH) screening; and (iv) a cohort of 238 exome-sequenced cases with GGE collected by the EuroEPINOMICS consortium. Additionally, diagnostic services within the European epilepsy community were also included. Detailed description of the patient cohorts is provided in the Supplementary material.

Whole exome sequencing

Target enrichment

Whole exome sequencing and target enrichment were performed according to standard procedures at three different centres [Wolfson Medical Center (WMC), Holon, Israel; CeGaT GmbH, Tübingen, Germany; Cologne Center for Genomics (CCG), Cologne, Germany]. Samples were enriched with Agilent Sureselect Human All Exon v.2 (WMC), or v.5 (CeGaT) or v.6 (CCG) kit. Sequencing was carried out on Illumina HiSeq2000 or HiSeq2500 platforms as 100 bp paired-end runs (WMC, Cegat) or 76 bp paired-end runs (CCG).

Data processing

Adaptors were removed with Skewer 0.1.1161 (Cegat) or cutadapt (CCG). The data preprocessing and variant calling was performed following the GATK (DePristo *et al.*, 2011) best practice or the Varbank pipeline v.2.3 developed by the Cologne Center of Genomics, University Cologne. For alignment of the reads, we used bwa-mem (Li and Durbin, 2009) with default parameters and hg19 as reference. The sam file format was converted to bam files with samtools (Li *et al.*, 2009). The subsequent steps by means of sorting bam files, marking duplicated reads and addition of read groups was performed with default parameters using picard tools (https://github.com/broadinstitute/picard). In order to recalibrate base quality scores and do local realignment, GATK version 3.2 was used. Variant filtering was done using GATK best practice (for SNV: FS > 60.0, QD < 2.0, MQ < 40.0, ReadPosRankSum < −8.0, MQRankSum < −12.5, DP < 10.0, GQ_MEAN < 20.0, VQSLOD < 0, ABHet > 0.75 or < 0.25; For Indel: QD > 2.0, FS > 200, ReadPosRankSum < −20.0, DP < 10.0).

Variant annotation

The variants were annotated using ANNOVAR (Wang *et al.*, 2010) (WMC, CeGaT, CCG) or additionally by in-house software (CCG). RefSeq, dbNSFP30a (Liu *et al.*, 2011, 2016), caddgt20 (Kircher *et al.*, 2014), ClinVar (20150330), HGMD (1000 Genomes Project Consortium *et al.*, 2012), 1000 Genomes, dbSNP (Sherry *et al.*, 2001), ExAC (Lek *et al.*, 2016) (release 0.3) and the EVS (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) were used. Non-coding variants and variants with minor allele frequency (MAF) <0.01 were filtered out. In addition, variants were filtered against an in-house database to exclude pipeline-related artefacts (MAF < 0.02). Only variants with a coverage $\geqslant 10\times$ were analysed. All genes were filtered for the expression in brain and variants not being located in the repeat regions (Reumers *et al.*, 2011).

Furthermore, we filtered variants to be deleterious when one of the following criteria was fulfilled: the variant had to be (i) predicted by 5 of 11 missense prediction scores (SIFT,

Polyphen_HDiv, Polyphen_HVar, LRT, MutationTaster, MutationAssessor, FATHMM, MetaSVM, MetaLR, VEST3, Provean); (ii) scored in caddgt20; (iii) pathogenic in ClinVar; (iv) in HGMD; and (v) highly conserved in two conservation tools (GERP++RS $\geqslant 3$, PhyloP $\geqslant 0.95$ and Siphy $\geqslant 10$). Nonsense, frameshift and splicing variants were included *per se*.

X-chromosome exome sequencing

To identify the disease-causing mutations in families with X-linked intellectual disability we performed X-chromosome exome sequencing using DNA of the index patients and analysed the data, as previously described (Hu *et al.*, 2014, 2016).

Diagnostic panels

After referral for routine diagnostic exome sequencing, exomes of a child and their parents (Families 4 and 5) were enriched using the Agilent SureSelect XT Human All Exon kit V5 (Family 5) and V6 (Family 4) and sequenced in rapid 2×100 bp run mode on the HiSeq2500 sequencing system (Illumina) at a mean target depth of $100\times$. The target was defined as all coding exons of UCSC and Ensembl $\pm$ 20 bp intron flanks. At this depth >95% of the target is covered at least $15\times$. Reads were aligned to hg19 using BWA (BWA-MEM v0.7.5a) and variants were called using the GATK haplotype caller (v2.7-2 and v3.4.46). Detected variants were annotated, filtered and prioritized using the Bench NGS Lab platform (Agilent-Cartagenia). Analysis was based on a tiered analysis approach. In the first tier known intellectual disability genes were analysed. This first tier did not lead to a diagnosis. The second tier, which filters for *de novo* variants, resulted in the detection of the *de novo GABRA3* variants. The last tier, which filters for recessive variants, did not result in additional candidates.

Microduplication analysis

Array-CGH was performed on 44k slide (Agilent) as previously described (Coppola *et al.*, 2010). Assays showing a DLRS (derivative of log ratio spread) score >0.3 were excluded. Detection of gains and losses was performed using the ADM-2 algorithm with a moving average of 500 kb and a threshold of 6.0. To further define genomic breakpoints on Patient II-2 of Family 3, a high-density 244K microarray was used according to the same protocol. To assess the effect of the microduplication on *GABRA3* transcription we amplified *GABRA3* exons 1–2 and 8–9 by RT-PCR using retrotranscribed RNA from fibroblasts of the proband and of a sex-matched control (iScript™ cDNA synthesis kit, Bio-Rad Laboratories).

Validation of *GABRA3* variants and segregation analysis

For validation of the *GABRA3* variants identified in this study and for segregation analysis we used gene-specific primers flanking the exons that harbour the mutations and determined genotypes for all available family members by conventional Sanger sequencing of specific PCR products. Primer sequences are available upon request.

X-inactivation test

To assess the inactivation status of chromosome X in heterozygous female carriers of *GABRA3* variants we used the human androgen receptor (HUMARA) assay as described (Gibson *et al.*, 2005).

Functional investigations

Mutagenesis and RNA preparation

We used the QuikChange® kit (Stratagene) to engineer five missense mutations, p.T166M, p.Q242L, p.T336M, p.Y474C and p.G47R in the $GABA_A$ receptor α_3-subunit cDNA (NM_000808) cloned in the pcDNA3 (kind gift from Dr Steven Petrou, Melbourne). All mutations were confirmed and additional changes excluded by Sanger sequencing. Primers are available upon request. cRNA was prepared using the T7 RNA polymerase kit from Roche.

Oocyte preparation and injection

The use of animals and all experimental procedures were approved by local authorities (Regierungspräsidium Tübingen, Tübingen, Germany). Oocytes were obtained from the Institute of Physiology I, Tübingen, or purchased from EcoCyte Bioscience. Preparation of oocytes for recordings included treatment with collagenase (1 mg/ml of type CLS II collagenase, Biochrom) in OR-2 solution (in mM: 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 (in mM: 88 NaCl, 2.4 $NaHCO_3$, 1 KCl, 0.41 $CaCl_2$, 0.82 $MgSO_4$ and 5 Tris/HCl, pH 7.4 with NaOH) supplemented with 50 µg/ml gentamicin (Biochrom). To compare current amplitudes of wild-type and mutant channels, the same amounts of cRNA were injected on the same day using the same batch of oocytes plated in 96 well-plates and measured in parallel at Days 1–3 after injection. The combination used was $\alpha_3\beta_2\gamma_2$s in a 1:1:2 ratio. All cRNA concentrations were adjusted to 2 µg/µl and 70 nl of the corresponding cRNA was injected using Roboinject® (Multi Channel Systems).

Automated oocyte two-microelectrode voltage clamp

GABA-evoked currents in oocytes were recorded at room temperature (20–22°C) using Roboocyte2® (Multi Channel Systems). Pre-pulled and prepositioned intracellular glass microelectrodes had a resistance of 0.3–1 MΩ when filled with 1 M KCl/ 1.5 M KAc. The bath solution was ND96 (in mM: 93.5 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES, pH 7.5). Currents were sampled at 1 kHz. GABA-evoked currents were used to analyse the activation of the wild-type using automated two-microelectrode voltage clamping. Different GABA concentrations (in µM: 1, 3, 10, 40, 100, 300, 1000) diluted in ND96 solution were applied for 15 s to activate the channels. The holding potential was −70 mV.

Electrophysiological data analysis

The amplitude of the GABA-induced currents was analysed using Roboocyte2+ (Multi Channel Systems), Clampfit (pClamp 8.2, Axon Instruments), Microsoft Excel (Microsoft, Redmond, WA) and Graphpad Prism software (GraphPad Software). Current response for each GABA concentration

was normalized to the maximum response evoked by the highest GABA concentration. The normalized GABA responses of each cell were fitted to the four-parameter logistic equation:

$$Y = min + \frac{(max - min)}{1 + 10^{((\mathrm{LogEC50} - X) * \mathrm{nH})}} \quad (1)$$

with max and min being the maximum and minimum evoked responses and X the corresponding GABA concentration. The EC_{50} value is the concentration of the agonist at which half of the maximum response is achieved while the nH presents the Hill coefficient, which determines the steepness of the dose response curve. EC_{50} values were determined for each oocyte and the averaged values for wild-type and each mutation are shown as mean ± standard error of the mean (SEM). Current amplitudes recorded in parallel on the same experimental day in response to 1 mM GABA application from oocytes expressing wild-type or mutant channels, were normalized to the mean value of the wild-type response.

Western blot analysis

Injected *Xenopus* oocytes expressing either wild-type or mutant $\alpha_3\beta_2\gamma_{2s}$ receptors were lysed in a buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Triton^{TM} X-100 and 10% glycerol with cOmplete protease inhibitors (Roche). Water-injected oocytes were used as a control. Protein concentration was measured (DC Protein Assay, Bio-Rad) and 25 µg of protein was separated using SDS-PAGE on 8% polyacrylamide gels. After the transfer onto nitrocellulose membrane (Protran®, Whatman), protein detections were performed using a rabbit polyclonal antibody against the $GABA_A$ receptor α_3-subunit (1:250; HPA000839 Sigma) and against actin (1:1000; Sigma A3853) as a loading control. Quantification of signals was performed using ImageJ software (NIH). Expression levels were normalized to actin and pooled from four different experiments.

Statistical analysis

Data were tested for normal distribution using GraphPad Prism 6 (GraphPad Software). Groups were compared using one-way ANOVA with Dunnett's *post hoc* test for normally distributed data or one-way ANOVA on ranks with Dunn's *post hoc* test for not normally distributed data. All data are presented as mean ± SEM. Statistical differences are indicated in the figure legends using the following symbols: $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

Results

The study was initiated by recruitment of 15 families with various forms of epilepsy (Supplementary material), including Family 1 of Israeli Jewish origin, in which the disease status of affected individuals was indicative of an X-linked inheritance. Two males in this pedigree (Fig. 1A) were severely affected with pharmacoresistant epileptic encephalopathies with infantile or childhood onset, featuring infantile spasms, tonic and generalized tonic-clonic seizures, moderate-to-severe intellectual disability and developmental delay

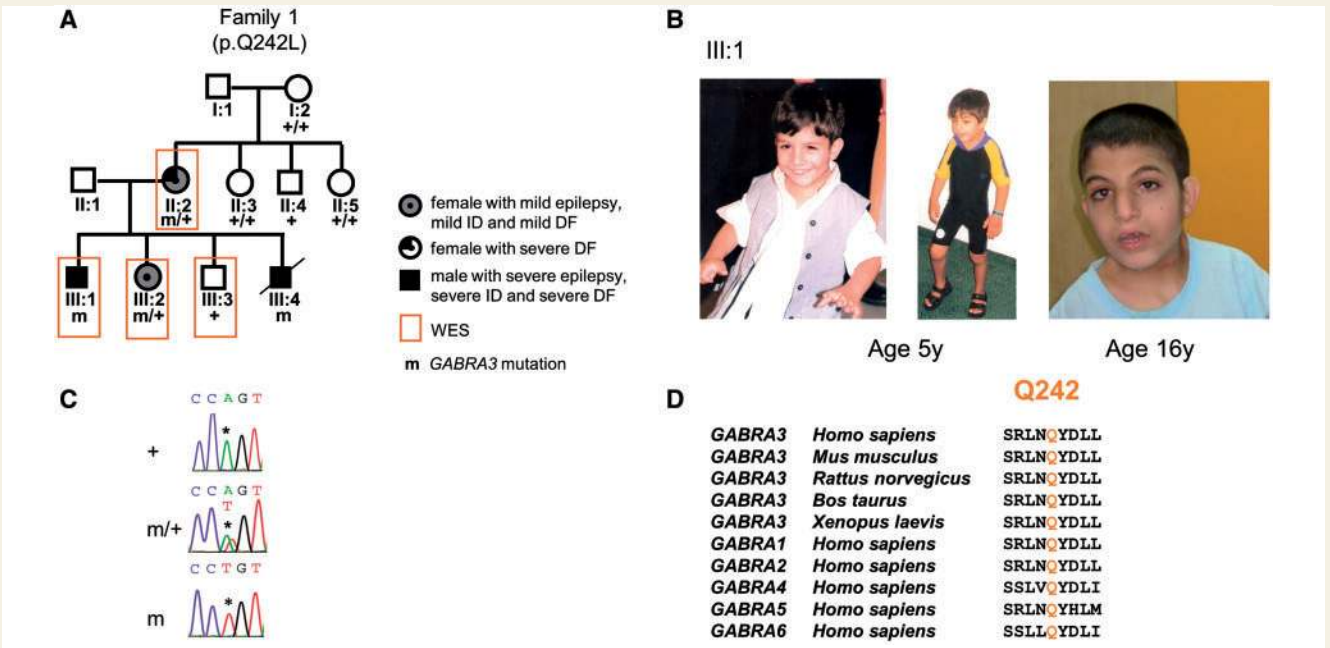

Figure 1 Novel *GABRA3* pathogenic variant associated with severe epileptic encephalopathy. (**A**) Pedigree of Family 1 and co-segregation of the genomic A>T exchange with the disorder. (**B**) Photographs of Patient III-1 from Family 1, carrying the p.Q242L mutation, at 5 and 16 years of age showing micrognathia, sloping shoulders and short neck. (**C**) Sanger sequence chromatograms from three individuals of Family 1. (**D**) p.Q242L amino acid exchange and evolutionary conservation of the mutated amino acid. DF = dysmorphic features; ID = intellectual disability; WES = whole exome sequencing.

with speech problems. In contrast, two affected females had a much milder phenotype, which included well treatable generalized tonic-clonic seizures and mild learning disability. All of them had micrognathia, short stature and further dysmorphic features (Fig. 1B), as well as nystagmus. Additionally, cleft palate was present in all but Patient III-2 (Table 1 and Supplementary material). Whole exome sequencing in Patient III-1 of this family (Fig. 1A) revealed a variant in *GABRA3* as the most plausible disease causing change identified on chromosome X (c.725A>T, p.Q242L, Fig. 1C) (Supplementary material). The p.Q242 is a highly conserved amino acid (Fig. 1D). The variant is predicted to be deleterious according to PolyPhen-2, SIFT and MutationTaster, and is found neither in the ExAC (exome aggregation consortium) database nor in gnomAD (genome aggregation database) (Supplementary Table 1). Direct sequencing in the whole family revealed perfect co-segregation showing that all affected individuals (mother, two affected sons, and daughter) carry the same p.Q242L variant, whereas the healthy son, grandmother, maternal aunts and uncle do not carry this variant (Fig. 1A). To exclude further disease-associated variants, we performed exome sequencing of the remaining three affected individuals and the healthy brother. Among the variants shared by the affected, but not the healthy individual, we found no particular changes in genes that had been previously associated with dysmorphic features, intellectual disability or epilepsy (Supplementary Table 2). We thus assume that the p.Q242L variant is pathogenic and mainly responsible for all clinical features observed in this family.

To further assess the role of *GABRA3* in epilepsy and intellectual disability, we selected three clinical cohorts according to the main clinical features of Family 1: a cohort of 480 families with X-linked intellectual disability (XLID) collected by the EURO-MRX consortium and associated groups, a cohort of 600 cases undergoing diagnostic high-density array-CGH screening, and an exome-sequenced cohort of 238 cases with idiopathic/genetic generalized epilepsy (IGE/GGE) collected by the EuroEPINOMICS consortium. Additionally, diagnostic services in our European epilepsy community were included in our search for pathogenic variants in *GABRA3*.

X-chromosome exome resequencing of index patients from 480 families with XLID, performed as previously described (Hu *et al.*, 2014, 2016), identified a likely deleterious *GABRA3* missense variant in Family 2, corroborating the findings for Family 1. Segregation analysis performed using conventional Sanger sequencing revealed the presence of the variant in four males who are first degree cousins (Fig. 2A). Their maternal uncle (Patient II:1), who has intellectual disability, is not a mutation carrier and also presented with a different haplotype. Moreover, four mildly affected females from this family carry this variant (Fig. 2A). The index proband (Patient III:10, Fig. 2A and B) and his brother (Patient III:13) have mild intellectual disability and delayed speech. Both suffered from generalized tonic-clonic seizures and their mother (Patient II:6) suffered from absences. Their sister (Patient III:12) carries the variant and has learning problems. The two other male mutation carriers in another branch of the family have different phenotypes—one of them has intellectual disability (Patient III:7) and the other is unaffected (Individual III:6). Their mother, who is a mutation carrier, suffered from absence seizures. Another affected male in the family (Patient III:4) also has intellectual disability but was unavailable for molecular testing. Recent clinical re-evaluation of the proband and his brother revealed dysmorphic features similar to those seen in affected individuals of Family 1 (e.g. nystagmus, micrognathia, arched palate) (Table 1 and Supplementary material). However, some of the observed traits are specific for Family 2 (Table 1, Fig. 2B and Supplementary material). Opposite to the short stature and overall present dysmorphic features seen in Family 1, affected Family 2 members present with a tall and thin stature and more pronounced dysmorphisms in males. The absence of the cleft palate in Family 2 members is another distinctive feature. The detected *GABRA3* variant c.497C>T, p.T166M (Fig. 2C) affects a highly conserved amino acid in the N-terminal sequence of the $GABA_A$ receptor α_3-subunit (Fig. 2D) and is predicted to be deleterious by *in silico* analysis. While it is not present in ExAC, one case has now been reported in gnomAD with a frequency of 5.61×10^{-6} (Supplementary Table 1). To verify that no additional pathogenic autosomal variants were present in this family, whole exome sequencing was performed on Individuals II:1. III:3 III:6, III:7, III:10, III:12, and III:13 with no other genes emerging as directly related to the disease phenotype or explaining the inheritance pattern (Supplementary Table 3).

We further aimed to identify copy number variants involving *GABRA3* by analysing a cohort of patients affected by epilepsy and intellectual disability ($n = 103$), only epilepsy ($n = 198$) or intellectual disability without epilepsy ($n = 299$), who underwent diagnostic high-density array-CGH screening. An intragenic microduplication encompassing exons 1–3 of *GABRA3* (chrX:152277607-152451201, GRCh38/hg38; arr[hg38]Xq28(152.277.607-152.451.201) x2) was identified in a male affected by pharmacoresistant epilepsy with weekly generalized seizures, generalized spike- and polyspike-wave discharges in EEG, but without any dysmorphic signs or nystagmus (Family 3) (Fig. 3A, C, Table 1 and Supplementary material). The duplication was inherited from a healthy mother. No deletions or duplications affecting *GABRA3* were identified in a cohort of 273 in-house controls. In addition, no copy number variants encompassing the coding region of *GABRA3* have been reported in the database of genomic variants (DGV database). We demonstrated that the rearrangement disrupts the expression of the gene in cultured fibroblasts from the patient, providing additional evidence for a pathogenic loss-of-function *GABRA3* variant (Fig. 3B). Furthermore, whole exome sequencing was performed in this family to exclude any other potentially pathogenic variants, in particular those

Table 1 Clinical characteristics of affected members in families with identified *GABRA3* alterations

Individual	Sex, age at last follow-up	Seizures (onset/ end in y)	Type of seizures	EEG	Treatment	Additional symptoms (morphological features)	Behavioural symptoms and development	*GABRA3* variant	X-chromosome inactivation
Family F1 (Israel)									
II-2	F	21	Tonic-clonic	Slowing of background EEG activity	CBZ	Cleft palate, nystagmus, microretrognathia, synophrys	Mild developmental delay, learning disabilities	c.725A>T p.Q242L	0.32 versus 0.68
III-1	M, 18	3	Infantile spasms, Lennox-Gastaut	Right parietal spikes at the age of 7 m	ACTH, VIG, CLZ, LEV, VPA, RFM	Cleft palate, nystagmus, micrognathia, sloping shoulders, short neck	Severe intellectual disability	c.725A>T p.Q242L	
III-2	F, 14	7	Tonic-clonic	Short bursts of spikes and multi spikes. Slowing of background EEG activity	LTG, VPA	Retrognathia, sloping shoulders, fine nystagmus	Mild learning disability	c.725A>T p.Q242L	0.38 versus 0.62
III-3	M							No	
III-4	M	3	Epileptic spasms and tonic seizures	Slowing over the left centro-parietal region; beta activity; polymorphic delta waves and spike and wave complexes over the left fronto-central region	LEV, CLB, CBZ, LTG	Cleft palate, micrognathia, short neck, nystagmus, sloping shoulders	Global developmental delay, walking at age 3 y, speech starting at age 6 y. Moderate intellectual disability	c.725A>T p.Q242L	
Family F2 (Poland)									
II-1	M, -	ND	ND	ND	ND	ND	Intellectual disability	No	
II-2	F, -	5 / 14	Generalized epilepsy	ND	ND	ND	Learning difficulties	Likely, not tested	
II-4	F, -	In adulthood/ in adulthood	Absence seizure	ND	ND	ND	ND	c.497C>T p.T166M	0.68 versus 0.32
II-6	F, 46	In childhood/ in childhood	Absence seizures	ND	No treatment	Micrognathia, long fingers, big low set ears, small mouth, height 172 cm	Learning difficulties	c.497C>T p.T166M	0.47 versus 0.53
II-7	M, -					Height 176 cm		No	
III-2	F, 25	Absent	Absent	ND	No treatment	Absent, height 155 cm	Absent	No	
III-3	F, 19	Absent	Absent	ND	No treatment	Small mouth, micrognathia, height 160 cm	Learning difficulties	c.497C>T p.T166M	
III-4	M, -	ND	ND	ND	ND	ND	Moderate intellectual disability	Not tested	
III-5	M, -	Absent	Absent	ND	No treatment	Absent	Absent	Not tested	
III-6	M, 25	Absent	Absent	ND	No treatment	ND	Unaffected	c.497C>T p.T166M	

(continued)

Table 1 Continued

Individual	Sex, age at last follow-up	Seizures (onset/ end in y)	Type of seizures	EEG	Treatment	Additional symptoms (morphological features)	Behavioural symptoms and development	*GABRA3* variant	X-chromosome inactivation
III-7	M, 21	Absent	Absent	ND	No treatment	Elongated skull, long neck, narrow and narrowly spaced palpebral fissures, sharply ended and long nose, arched palate, large protruding ears, sloping shoulders, long fingers, second and third toes – small syndactyly	Mild intellectual disability, learning difficulties, hyperactivity, disturbances of visual-motor integration, speech defect	c.497C>T p.T166M	
III-9	F, 25	Absent	Absent	ND	No treatment	Absent	Absent	No	
III-10	M, 23	17/17	Absence seizure	Generalized spike waves	No treatment	Horizontal nystagmus, micrognathia, elongated skull, arched palate, small mouth, long fingers, big low set ears, high stature (190 cm)	Delayed speech; mild intellectual disability	c.497C>T p.T166M	
III-11	F, -	Absent	Absent	ND	No treatment	Absent	Absent	No	
III-12	F, 19	Absent	Absent	ND	No treatment	Small mouth, micrognathia, height 169 cm	Learning difficulties	c.497C>T p.T166M	0.48 versus 0.52
III-13	M, 21	12/12	Absence seizures	Generalized spike waves	Valporate	Horizontal nystagmus, micrognathia, elongated skull, arched palate, small mouth, long fingers, big low set ears, high stature (182 cm)	Mild intellectual disability; delayed speech, starting at age 3 y	c.497C>T p.T166M	
Family F3 (Italy)									
I-2	F					No	Normal	Intragenic GABRA3 duplication	0.50 versus 0.50
II-1	F		Febrile seizure					No	
II-2	M	3	Generalized tonic-clonic seizures	Generalized spike and polispike and wave complexes	VPA, LEV, CLZ, CLB, ETX, PHB, FBM	No	Borderline intellectual functioning	Intragenic GABRA3 duplication	
Family F4									
II-1	F	3	Partial complex, tonic-clonic	Frontal/central/ parasagittal focus	VPA, LEV, CBZ, CLB, diphantoine, LZP, OCB, FLX	No	Speech defect, IQ 72, anxiety, generalized fear, afraid to walk	c.1421A>G pY474C	
Family F5									
II-1	F, 12	2	Complex partial complex, with tonic component	Central focus	Multidrug, neurovalgus stimulator, ketogenic diet	Microtia and strabismus surgery	Delayed language development, IQ earlier 65-70, now 50; autism like behaviour	c.1421A>G pY474C	Not performed
Family F6 (France)									
II-1	M						Speech starting at age 6; autism spectrum disorder	c.139G>A p.G47R	

(continued)

Table 1 Continued

Individual	Sex, age at last follow-up	Seizures (onset/ end in y)	Type of seizures	EEG	Treatment	Additional symptoms (morphological features)	Behavioural symptoms and development	*GABRA3* variant	X-chromosome inactivation
II-2	M						Behavioural disturbances, agitation, aggressiveness, stereotypies and hypersensitivity to noise (ASD?)	No	
II-3	F							No	
II-4	M						Delayed language development	No	
Family F7 (Italian IGE pool)									
I-1	M		Generalized tonic-clonic	Generalized spike and wave complexes				No	
I-2	F							c.1007C>T p.T336M	0.59 versus 0.41
II-1	F		Generalized tonic-clonic	Generalized spike and wave complexes				No	
II-2	F	8	Generalized tonic-clonic	Generalized spike and wave complexes	Valporate	No		c.1007C>T p.T336M	0.49 versus 0.51

CBZ = carbamazepine; CLB = clobazam; CLZ = clonazepam; ETX = etosuccimide; FBM = felbamate; FLX = fluoxetine; LEV = levetiracetam; LTG = lamotrigine; LZP = lorazepam; ND = not determined; OCB = oxcarbazepine; PHB = phenobarbital; RFM = rufinamide; VIG = vigabatrin; VPA = valproic acid.

occurring *de novo,* which could explain the observed severe phenotype. However, no other genetic defect likely to contribute to the disease phenotype could be detected (Supplementary Table 4).

While the genetic analysis of these three families provided evidence for the co-segregation with the complex disease phenotype, we further explored the role of *de novo* occurring *GABRA3* variants. Diagnostic trio-based whole exome sequencing revealed a recurrent *de novo* variant, p.Y474C, c.1421A>G, in two sporadic females (Families 4 and 5) (Fig. 4A) presenting with partial seizures and mild-to-moderate intellectual disability. This variant affects transmembrane segment 4 of the receptor subunit and the replaced residue is highly conserved (Fig. 4B). The variant is not present in any of the control databases.

One more *GABRA3* variant (c.139G >A, p.G47R) was identified in the X-chromosome exome-sequencing study (Family 6) (Table 1 and Fig. 5A). The male proband presented with autism spectrum disorder and severe learning disabilities, but no epileptic seizures. His brother, who does not carry the variant, is affected with a very similar phenotype (Table 1 and Fig. 5A). This variant is found at a somewhat less conserved part of the N-terminus of the α_3-subunit (Fig. 5B). It has been identified in one male individual (allele frequency of 1.177×10^{-5} in ExAC) and in two individuals in gnomAD (allele frequency 1.163×10^{-5}) (Supplementary Table 1). Furthermore, a different gene, *SLC7A3*, has been proposed to cause the phenotype in both brothers of this family (Nava *et al.*, 2015).

We further searched for pathogenic variants in *GABRA3* in a cohort of 238 independent families with classical GGE syndromes that underwent whole exome sequencing. In a female proband (Family 7, Fig. 5C), suffering from GGE with generalized tonic-clonic seizures (EGTCS), we identified one further missense variant (c.1007C >T, p.T336M) affecting a conserved threonine in the extracellular loop between transmembrane segments 2 and 3 (Fig. 5D). Her unaffected mother also carried the variant, in contrast to her affected sister (suffering from classical childhood absence epilepsy) and her affected father (experiencing few generalized tonic-clonic seizures). Moreover, in this family there were no signs of additional dysmorphic features as observed in the first two families (Supplementary material and Table 1).

Finally, to test for a significant enrichment of *GABRA3* variants in our cohorts compared to controls, we compared the allele frequency of the non-synonymous *GABRA3* variants in our patient cohorts of the known size—excluding the diagnostic services—(4/733) with missense, nonsense and splice site variants reported in the ExAC Browser (84/87765). This calculation showed a significant enrichment of *GABRA3* variants in the patient cohort using Fisher's exact test ($P < 0.01$).

Three out of the five missense variants are localized at the N-terminus of the α_3-subunit (Fig. 6A), including the largely co-segregating variants from Families 1 (p.Q242L) and 2 (p.T166M) as well as the one found in the boy with

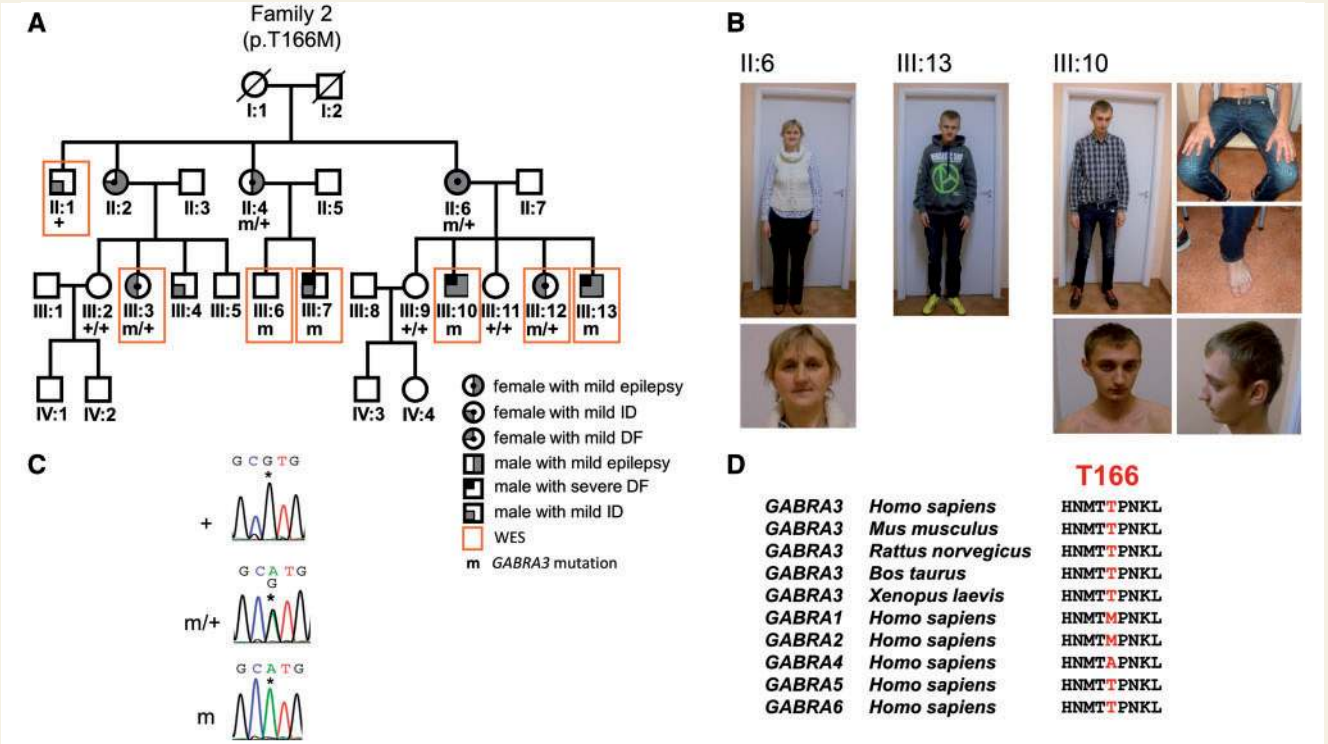

Figure 2 Novel *GABRA3* likely pathogenic variant associated with severe epilepsy. (**A**) Pedigree of Family 2 with co-segregation of the p.T166M variant. (**B**) photographs of Patients II-6, III-10 and III-13 from Family 2, carrying the p.T166M variant. Micrognathia, elongated skull, small mouth, long fingers, big low set ears and high stature can be observed. (**C**) Sanger sequence chromatograms showing the genomic G > A exchange. (**D**) p.T166M amino acid exchange and the evolutionary conservation of the mutated amino acid. DF = dysmorphic features; ID = intellectual disability; WES = whole exome sequencing.

autism spectrum disorder (Family 6, p.G47R). The GGE-associated variant p.T336M affects the extracellular loop between transmembrane segments M2 and M3, whereas the *de novo* variant p.Y474C lies within the transmembrane segment M4 (Fig. 6A). We used *Xenopus laevis* oocytes and an automated two-microelectrode voltage clamp technique to assess the functional consequences of all identified variants. They were introduced in the cDNA encoding the human isoform of the $GABA_A$ receptor α_3-subunit. After *in vitro* transcription, cRNAs encoding wild-type or mutant were co-injected with wild-type β_2- and γ_{2s}-subunits into oocytes and GABA-evoked ionic currents were recorded. We first investigated the effects of a high GABA concentration of 1 mM, closely mimicking physiological conditions when GABA is released into the synaptic cleft (Roth and Draguhn, 2012). This screening experiment revealed that all mutations, except p.G47R, led to a strong, statistically significant ($^{***}P < 0.0001$ ANOVA on ranks, Dunn's *post hoc* test) reduction of GABA-evoked currents compared to the wild-type (Fig. 6B and C). For p.Q242L, the currents were reduced by $85 \pm 3\%$, for p.T166M by $75 \pm 3\%$, for p.Y474C by $68 \pm 9\%$, for p.G47R by $46 \pm 10\%$ and for p.T336M by $91 \pm 2\%$ (Fig. 6B and C). Further investigation showed diminished responses to different GABA concentrations in the whole concentration range (Fig. 6D). Interestingly, the GABA sensitivity was increased for p.Q242L, p.Y474C and p.T336M variants compared to the wild-type (EC_{50} of 25 ± 2; 22 ± 7; 38 ± 4 and $96 \pm 3\,\mu M$ for mutants and wild-type, respectively). However, this shift was not predicted to compensate for the pronounced decline of current amplitudes (Fig. 6D). Western blot analysis of oocytes injected with wild-type or mutant $\alpha_3\beta_2\gamma_{2s}$ receptors performed using an anti-α_3-subunit antibody revealed a statistically significant decrease in the total amount of protein only for p.T166M compared to the wild-type (Fig. 6E and F) ($^{*}P < 0.05$, ANOVA on ranks, Dunn's *post hoc* test).

Discussion

Our results suggest that *GABRA3* is a new gene associated with epilepsy and related disorders. In total, this study identified five missense variants and a microduplication within this gene. We provide strong genetic evidence for the pathogenicity of the fully co-segregating variant detected in Family 1. Family 2 is more complex, and the variant detected in this family, which largely co-segregated with epilepsy-related phenotypes in male and female carriers, is also found in an unaffected male, and one male affected only with intellectual disability. Especially Family 1 supports the pattern of X-linked inheritance, with males

being more severely affected than females. This inheritance pattern also fits Family 3, since the boy in this family carrying a duplication with deleterious effects on *GABRA3* expression also presented with severe epileptic encephalopathies in contrast to his unaffected mother. The detection of a recurrent *de novo* variant associated with a severe epileptic encephalopathies phenotype in females corroborates a role of this gene in epilepsy and related disorders. Moreover, *in vitro* analysis revealed that the severely affected individuals carried variants with a large functional deficit corresponding to at least a 70% reduction of GABA-evoked currents.

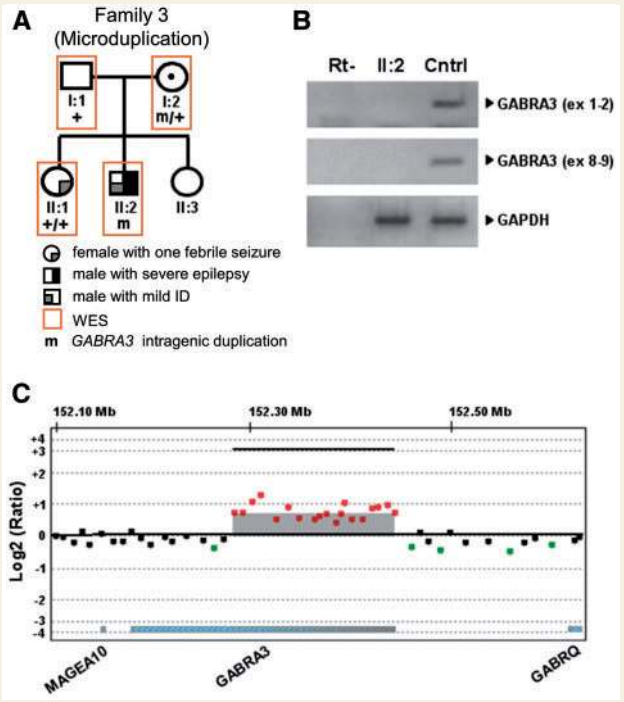

Figure 3 *GABRA3* microduplication. (**A**) Pedigree of Family 3 revealing the co-segregation of the *GABRA3* microduplication. (**B**) RT-PCR showing that retro-transcribed *GABRA3* RNA (exons 1–2 and exons 8–9) from cultured fibroblasts of the proband could not be detected; *GAPDH* was used as a housekeeping gene control. (**C**) Plot of the Log2 intensity ratios showing amplified signals for probes spanning the 5' genomic region of *GABRA3*. The gene is displayed in the reverse strand. ID = intellectual disability; WES = whole exome sequencing.

We also report two families in which *GABRA3* cannot be regarded as an obvious disease gene, but might act as a risk factor contributing to the development of disease in some mutation carriers. This is seen both in the patient with autism spectrum disorder (Family 6) and in the female with mild epilepsy (Family 7 with GGE). In both families, clearly affected individuals do not carry *GABRA3* variants, suggesting a dominant role of a different gene. In Family 6, this gene has already been suggested (Nava *et al.*, 2015). Moreover, the *GABRA3* variant detected in Family 6 showed a milder functional defect. The p.T336M amino acid exchange, which did show a very severe loss of function, was only found in two females within the GGE family, the unaffected mother and one of the affected sisters, but not in the other affected sister and the affected father. The incomplete co-segregation, as well as the fact that female mutation carriers from other families do not present with classical GGE syndromes, suggest that further factors or variants are involved in the pathogenesis within this family. However, these could not be identified by exome sequencing of the whole family (Supplementary Table 3).

The detected variants are associated with a large spectrum of neuropsychiatric symptoms, ranging from severe epileptic encephalopathies with dysmorphic features and nystagmus to relatively mild intellectual disability without epilepsy and one unaffected individual among the male mutation carriers. In females, the phenotypic spectrum extends

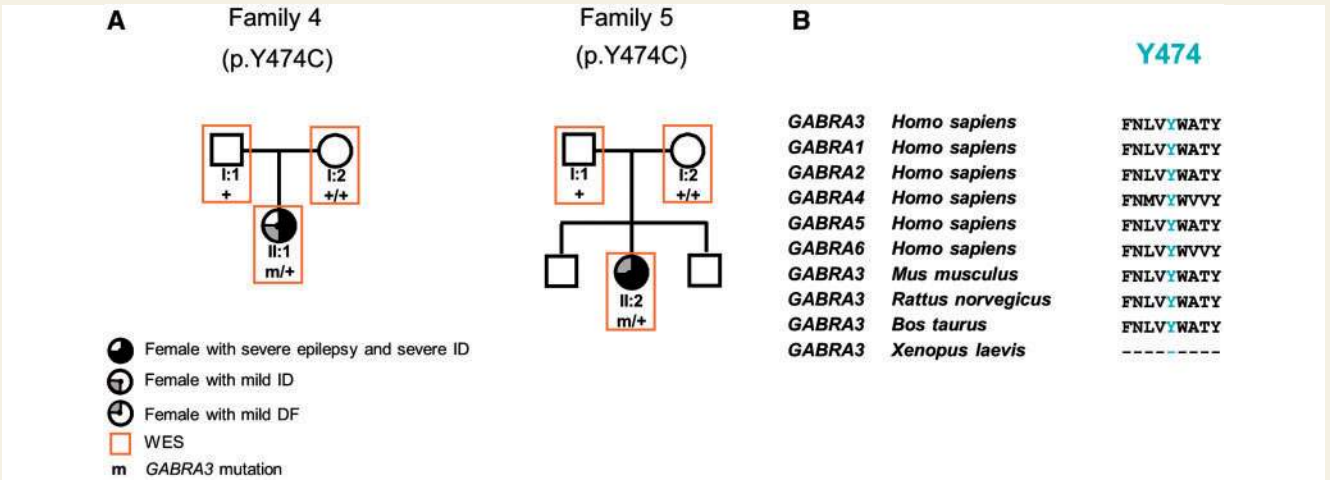

Figure 4 *Recurrent de novo GABRA3* variant identified in two unrelated girls. (**A**) Pedigrees of Families 4 and 5 showing the affected patients. (**B**) p.Y474C amino acid exchange and the evolutionary conservation of the mutated amino acid. DF = dysmorphic features; ID = intellectual disability; WES = whole exome sequencing.

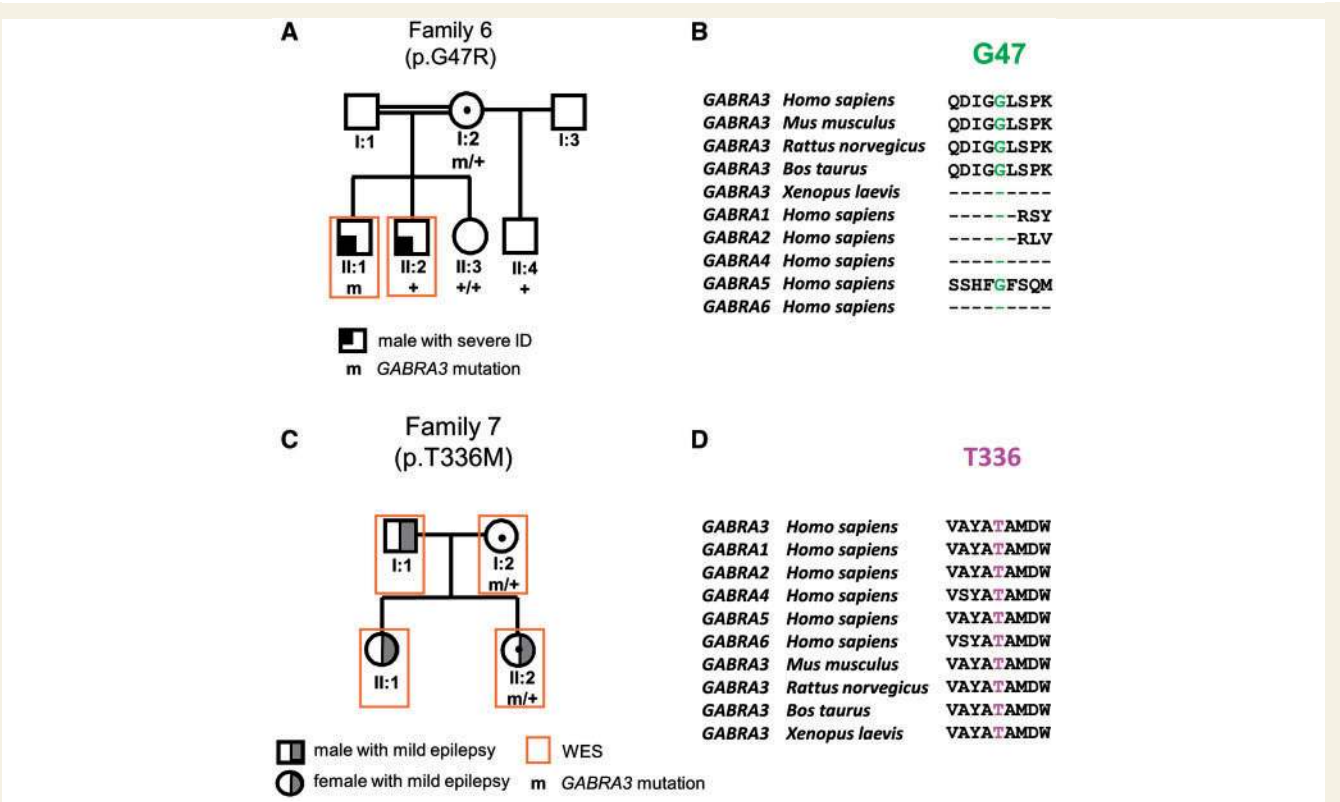

Figure 5 *GABRA3* variants identified in Family 6 with autism-like disorder and in Family 7 with GGE. (**A**) Family 6 pedigree showing the presence of the p.G47R α_3 variant in the index case who presented with speech delay, autism spectrum disorder or behavioural disturbances and his mother. (**B**) Alignment depicting the evolutionary conservation of the p.G47 amino acid. (**C**) Family 7 pedigree showing the co-segregation of the p.T336M variant with the phenotype in this GGE family. (**D**) Alignment depicting the evolutionary conservation of the p.T336 amino acid. ID = intellectual disability; WES = whole exome sequencing.

from normal, i.e. without detectable clinical signs for epilepsy, intellectual disability or dysmorphisms, to mildly affected with well treatable seizures, moderate developmental delay and similar dysmorphic features and nystagmus as in males. This spectrum is further extended to the severe end in one of the *de novo* mutation carriers presenting with pharmacoresistant seizures in addition to moderate intellectual disability. We hypothesized that variable X-inactivation may contribute to the large phenotypic variability in females, but an X-inactivation test performed in leucocyte DNA from eight mutation carriers (affected $n = 4$, unaffected $n = 4$) did not identify any correlation with the affection status (Table 1). However, the X-inactivation pattern in the nervous system can differ from that seen in blood, as reported for Rett syndrome (Gibson *et al.*, 2005), so that we cannot exclude that X-inactivation plays a role in the phenotypic variability observed in female mutation carriers.

A broad phenotypic spectrum is well known from other epilepsy genes including those coding for different $GABA_A$ receptor subunits (Harkin *et al.*, 2007; Carvill *et al.*, 2014; Epi4K Consortium, 2016; Johannesen *et al.*, 2016; Papandreou *et al.*, 2016; Møller *et al.*, 2017). Our results suggest that a combination of the severity of the mutation-induced $GABA_A$ receptor dysfunction and the 'genetic background', i.e. so far unknown modifying genetic factors that could not be identified in our exome sequencing studies, are responsible for the observed clinical phenotypic variability. The latter may play a role for example in the two different branches of Family 2 presenting males with large differences in clinical severity.

Some *GABRA* genes have further been linked to severe forms of epilepsy, such as Dravet syndrome (*GABRA1*) (Carvill *et al.*, 2014; Johannesen *et al.*, 2016), or Lennox–Gastaut syndrome (*GABRB3*) (Epi4K Consortium *et al.*, 2013). Genetic studies have shown that many of the most severe epileptic encephalopathies are caused by *de novo* mutations (Claes *et al.*, 2001; Harkin *et al.*, 2002; Kalscheuer *et al.*, 2003; Tao *et al.*, 2004; Veeramah *et al.*, 2012; Weckhuysen *et al.*, 2012; Epi4K Consortium *et al.*, 2013; Suls *et al.*, 2013; Carvill *et al.*, 2014; Nava *et al.*, 2014; Blanchard *et al.*, 2015; Syrbe *et al.*, 2015; Epi4K Consortium, 2016; Johannesen *et al.*, 2016; Shen *et al.*, 2017), which is confirmed by our findings in one of the *de novo* mutation carriers. However, our results also indicate that mildly affected female carriers can transfer severe mutations to their children, which can result in devastating epileptic encephalopathies and intellectual disability in

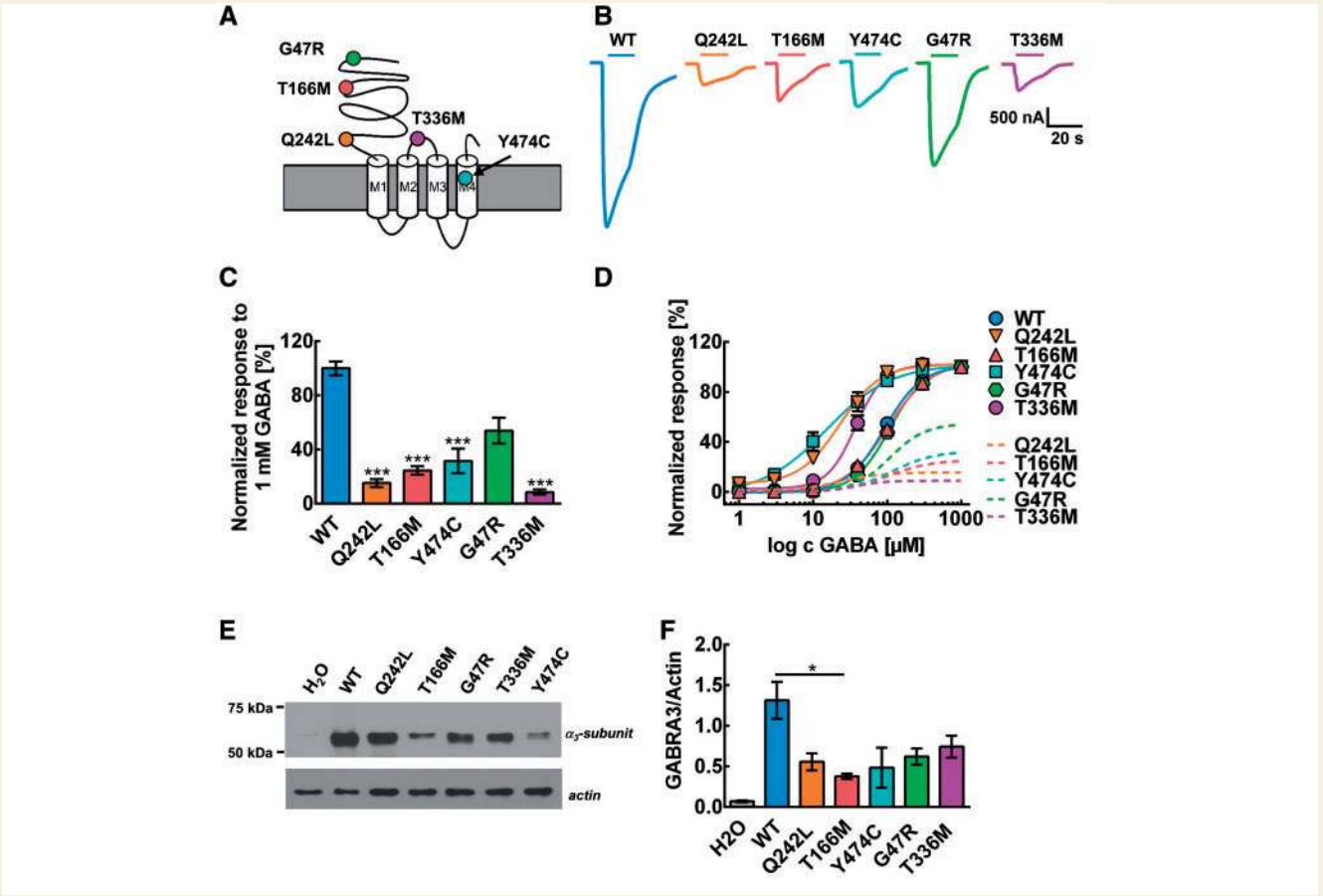

Figure 6 Functional analysis of the identified *GABRA3* variants in *Xenopus laevis* oocytes. (**A**) Schematic representation of the α_3-subunit of the $GABA_A$ receptor including the predicted positions of all mutated amino acids. (**B**) Representative examples of current responses to application of 1 mM GABA recorded from *Xenopus* oocytes expressing $\alpha_3\beta_2\gamma_{2s}$ receptors comprising wild-type or mutant (p.Q242L, p.T166M, p.Y474C, p.G47R, p.T336M) α_3-subunits. (**C**) Normalized current response to 1 mM GABA application for wild-type ($n = 137$), p.Q242L ($n = 29$), p.T166M ($n = 37$), p.Y474C ($n = 22$), p.G47R ($n = 10$) and p.T336M ($n = 28$) mutations; $^{***}P < 0.0001$ ANOVA on ranks, Dunn's *post hoc* test. The reduction in current amplitude was 85 ± 3% for p.Q242L; 75 ± 3% p.T166M; 68 ± 9% for p.Y474C; 46 ± 10% for p.G47R and 91 ± 2% for p.T336M in comparison with the wild-type. (**D**) Dose-response curve for $\alpha_3\beta_2\gamma_{2s}$ wild-type ($n = 77$), p.Q242L ($n = 16$), p.T166M ($n = 25$), p.Y474C ($n = 9$), p.G47R ($n = 19$) and p.T336M ($n = 14$) receptors recorded upon application of different GABA concentrations (in µM: 1, 3, 10, 40, 100, 300 and 1000) and normalized to the maximal response (1000 µM) for each cell. Statistically significant differences between the EC_{50} values were verified by ANOVA on ranks and Dunn's *post hoc* test ($^{***}P < 0.0001$ for p.Q242L, p.Y474C and p.T336M). The predicted dose-response curves, calculated from the current amplitude ratio of the mutant versus wild-type response to the application of 1 mM GABA (see **C**) are shown as dashed lines for all mutations. (**E**) Western blot analysis of whole cell lysates from *Xenopus* oocytes injected with cRNA encoding for $\alpha_3\beta_2\gamma_{2s}$ wild-type or mutated subunits. All lysates show a band of the expected size for the α_3-subunit protein (55 kDa). (**F**) Quantification of western blots revealing a significant reduction of the α_3-subunit signal for the T166M mutant ($n = 4$). $^{*}P < 0.05$ using ANOVA on ranks with Dunn's *post hoc* test.

the sons and therefore constitutes an important aspect of genetic counseling in these families with X-linked-like inheritance.

Reduced amplitudes of GABA-evoked currents were observed for all five detected variants. This loss-of-function effect has been a common feature of all $GABA_A$ receptor mutations associated with epilepsy and proposed to impair the $GABA_A$ receptor-mediated inhibition leading to an increased neuronal hyperexcitability and seizures (Baulac *et al.*, 2001; Wallace *et al.*, 2001; Harkin *et al.*, 2002; Maljevic *et al.*, 2006; Johannesen *et al.*, 2016; Møller *et al.*, 2017). The suggested molecular mechanisms include gating defects, reduced GABA sensitivity, or reduced surface expression due to protein misfolding, impaired assembly or trafficking defects (Wallace *et al.*, 2001; Harkin *et al.*, 2002; Maljevic *et al.*, 2006; Macdonald *et al.*, 2012; Johannesen *et al.*, 2016; Kang and Macdonald, 2016; Møller *et al.*, 2017). Messenger RNA and protein degradation of defective $GABA_A$ receptor subunits have also been shown (Gallagher *et al.*, 2005; Maljevic *et al.*, 2006; Kang and Macdonald, 2016). In our study, only one (p.T166M) of the five *GABRA3* variants showed a significantly lower amount of protein in total lysates obtained from injected *Xenopus* oocytes, indicating that at least for

this variant a less stable protein is the cause for the observed reduced current amplitudes.

Interestingly, three of the analysed five variants showed an increased GABA sensitivity. Since the amplitudes of GABA-evoked anion currents carried by the respective mutant receptors were strongly diminished, we estimated the combined effect of the amplitude reduction and the shift in GABA-sensitivity, which revealed that the effect of this shift may only have a minor gain-of-function effect at low GABA concentrations. At higher GABA concentrations, such as 1 mM, which is considered to represent the GABA concentration in the synaptic cleft (Roth and Draguhn, 2012), a severe loss-of-function remained obvious. However, this repeatedly identified functional alteration may suggest that other complex mechanisms, possibly including pre- or extrasynaptic processes may underlie pathology associated with the α_3-subunit variants. The role of the α_3-subunit in tonic inhibition has been demonstrated in principal cells of the amygdala (Marowsky *et al.*, 2012). Furthermore, the fact that $GABA_A$ receptors containing α_3-subunits show a higher affinity for GABA compared to those containing α_1-subunits (Keramidas and Harrison, 2010) might also indicate their extrasynaptic role.

In the rodent brain, the α_3-subunit of the $GABA_A$ receptor has a broad distribution and is specifically expressed in the thalamus, being the only α-subunit expressed in the nucleus reticularis thalami (nRT) (Pirker *et al.*, 2000; Hörtnagl *et al.*, 2013). Knockout mice lacking this subunit do not show spontaneous seizures. On the contrary, they show an enhanced intra nucleus reticularis thalami inhibition and a reduced susceptibility to pharmacologically induced seizures, probably due to a strong compensatory mechanism increasing the expression of a different α-subunit (Schofield *et al.*, 2009). However, in a mouse model in which the benzodiazepine binding site of the α_3-subunit is disrupted, an increase in spike and wave discharges, a characteristic of absence seizures, was found (Christian *et al.*, 2013). This suggests that an impaired function of the α_3-subunit may lead to epileptic seizures, which are not detected in knockout animals because of an adaptive process or compensatory mechanism.

Dysmorphic features are observed in the two families with the most severe phenotypes in most affected mutation carriers and partially overlap between the two families. Also one of the *de novo* mutation carriers showed a (different) dysmorphism. How a subunit of $GABA_A$ receptors may be linked to changes in morphology is not clear, although it has been reported that homozygous *Gabrb3* knockout mice exhibit a cleft palate in about half of the cases and the other half have feeding difficulties as neonates (DeLorey *et al.*, 1998). The observed nystagmus may be attributed to a particular inhibitory function of $GABA_A$ receptor α_3-subunits in the brainstem or cerebellum. Expression of *GABRA3* in these structures has been shown using specific antibodies against this subunit (Pirker *et al.*, 2000).

In summary, we have detected *GABRA3* variants in patients with a spectrum of neuropsychiatric disorders and demonstrated in our *in vitro* assay that they cause loss-of-function effects. The data obtained from whole exome sequencing in the affected families suggest that *GABRA3* variants present the major underlying genetic component of the observed disease phenotype in three and a contributing factor in two families. Detection of a recurrent *de novo* variant associated with a severe clinical picture corroborates these findings suggesting *GABRA3* as a new epilepsy gene.

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Supplementary material

Supplementary material is available at *Brain* online.

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