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# Rare coding variants in GABA<sub>A</sub> receptor subunit encoding genes increase the risk for genetic generalized epilepsies

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## Summary

**Background** Generalized epilepsy with genetic etiology (GGE) is the most common type of inherited epilepsy characterized by absence, myoclonic and generalized tonic-clonic seizures typically occurring with generalized spike-and-wave discharges on electroencephalography. Despite a high concordance rate of 80% in monozygotic twins, the genetic background is still largely unknown.

**Methods** Individuals included in the study were clinically evaluated for GGE. Whole-exome sequencing (WES) was performed for the discovery case cohort, the first replication case cohort and for two independent control cohorts. A second replication case cohort underwent targeted next-generation sequencing of the 19 known genes encoding subunits of GABA<sub>A</sub> receptors and was compared to the respective GABA<sub>A</sub> receptor variants of a third independent control cohort. Functional investigations were performed using automated two-microelectrode voltage clamping in *Xenopus* oocytes.

**Findings** Statistical comparison of 152 familial index cases with GGE in the discovery cohort to 549 ethnically matched controls suggested an enrichment of rare missense variants in the ensemble of GABA<sub>A</sub> receptor encoding genes in cases. The enrichment for these genes could be replicated in a second WES cohort of 357 sporadic and familial GGE cases and 1485 independent controls. Comparison of these genes in a second independent replication cohort of 635 familial and sporadic GGE index cases, based on candidate-gene panel sequencing, to a third independent control cohort confirmed the overall enrichment of rare missense variants in cases. Functional studies for two selected genes (*GABRB2*, *GABRA5*) showed significant loss-of-function effects with reduced current amplitudes in five of seven tested variants compared to wild-type receptors.

**Interpretation** Our results suggest that functionally relevant variants in GABA<sub>A</sub> receptor subunit encoding genes constitute a significant risk factor for GGE. This conclusion is based on an enrichment of rare variants in those genes in three independent case-control datasets and physiological studies revealing a loss of function for tested variants which are supposed to favor a neuronal disinhibition which is a well-known mechanism in epilepsy.

**Funding** The study was supported by different national funding agencies in the frame of EuroEPINOMICS (a project of the European Science Foundation), by Epicure and EpiPGX (funded by the FP6 and FP7 programs of the European Commission), by Research Unit FOR2715 (funded by the DFG and the FNR), and by a couple of smaller grants from different bodies. More detailed information on the funding sources is given in the acknowledgements.

## **Research in context**

### **Evidence before this study**

It is commonly accepted that generalized epilepsies with genetic etiology (GGE) are a group of diseases with a complex inheritance, meaning that probably many common and rare genetic factors are involved in the etiology of the disease, the genetic background of which is still largely unknown. We searched the Pubmed database with the search terms “Exome sequencing ion channels OR exome sequencing genetic generalized epilepsy OR exome sequencing idiopathic generalized epilepsy”. At the time we conducted this study, there were only two studies which had performed targeted or whole-exome sequencing in smaller cohorts of subjects with GGE not finding significant differences in the burden of genetic variants in cases vs. controls. One study used a targeted Sanger sequencing approach in 237 ion channel genes in 152 cases vs. 139 normal controls. In the other study, whole exome sequencing was performed in 118 subjects with juvenile myoclonic epilepsy and absence epilepsy, two of the main sub-phenotypes of GGE, and 242 controls. In parallel with our study, the Epi4K and EP/GP consortia sequenced whole exomes of 640 subjects with GGE and 3877 controls which showed that the rate of ultra-rare deleterious variants in a group of established epilepsy genes was significantly increased; no single gene showed a mutational burden in GGE.

### **Added value of this study**

Compared to these studies, we found a difference between cases and controls in missense variants in a specific group of genes encoding all known GABA<sub>A</sub> receptors. We were able to reproduce our findings in two independent cohorts of cases in comparison to two independent control cohorts. We also demonstrate the functional relevance of some of the detected variants in two GABA<sub>A</sub> receptor subunit genes that were so far not known to be associated with GGE or epilepsy. Our study therefore provides strong evidence that variation in GABA<sub>A</sub> receptor encoding genes confers a genetic burden in GGE.

### **Implications of all the available evidence**

The study by the Epi4K and EPGP consortia and our findings present the first evidence that there is a significant genetic burden in GGE compared to controls in ultra-rare variants in previously identified epilepsy genes or in the specific gene group of the most important inhibitory receptors in the mammalian brain.

## **Introduction**

In the recent past, gene discovery in monogenetic diseases, including familial and severe epilepsy syndromes, has been boosted by next generation sequencing yielding a steadily increasing number of disease-causing genetic defects. Unraveling the genetic origin of complex inherited disorders

has, however, been more difficult. GGE comprises common epilepsies with generalized absence, myoclonic and tonic-clonic seizures<sup>1</sup>. It has a high heritability, as has been shown in twin studies<sup>2</sup> and represents a kind of ‘prototype’ of genetic epilepsy with complex inheritance.

A few single nucleotide polymorphisms in genome-wide association studies and altered copy number variations have been the major common risk factors identified so far in GGE. These, however, only explain a small part of the high heritability. Single gene defects in larger families with autosomal dominantly inherited GGE have been identified as disease-causing, e.g. in *GABRA1* or *GABRG2* encoding subunits of GABA<sub>A</sub> receptors<sup>3-5</sup>, or in *SLC2A1* encoding the glucose transporter type 1<sup>6,7</sup>. Larger candidate-gene or whole-exome sequencing (WES) studies have not revealed a significant burden of mutations in single genes or groups of genes thus far<sup>8,9</sup>. Only recently, a study running in parallel to this study demonstrated mutational burdens of ultra-rare variants in gene-sets related to epilepsy<sup>10</sup>.

We set out to investigate the burden of genetic mutations in mainly familial GGE by first testing all genes in a hypothesis-free approach, and second hypothesis-driven sets of genes related to the disease and disease-relevant pathways. Subsequent steps were to replicate the findings in additional cohorts and to perform functional studies for selected variants. Our results suggest the presence of such a genetic burden in a gene set encoding the main inhibitory receptors in the mammalian brain in cases, replicate the finding in two independent GGE cohorts and prove the functional significance of some variants in two GABA<sub>A</sub> receptor genes by physiological investigations.

## Methods

### Participants

The discovery GGE exome sequencing case cohort included 152 subjects (after quality control (QC) of the exome sequencing data) with GGE from multiplex families which were collected by the Epicure and the EuroEPINOMICS-CoGIE consortia. All subjects were of European descent (Italian n=69, German n=51, Dutch n=11, Danish n=8, British n=6, Finnish n=4, Swedish n=2, Greek n=1). The cohort included 88 females (58%). The primary GGE-diagnoses were childhood absence epilepsy (CAE, n=68), juvenile absence epilepsy (JAE, n=16), juvenile myoclonic epilepsy (JME, n=37), GGE with generalized tonic-clonic seizures alone (EGTC, n=24), early-onset absence epilepsy (EOAE, defined as beginning below 3 years of age, n=4), epilepsy with myoclonic absences (EMA, n=1) and unclassified GGE (n=2) (see appendix table S1). The age of epilepsy onset ranged from 1.5 to 38 years with a median of 10 years and all subjects had a normal development without obvious developmental delay or intellectual disability, although most were not formally tested. We included the few cases with EOAE, EMA and unclassified GGE since these entities in our view are close to classical GGE. For EOAE it has been recently suggested by

a large study that it is likely genetically similar to classical CAE<sup>11</sup>, EMA may also have genetic overlaps with GGE<sup>12</sup> and we often find in family studies both well classified and unclassified GGE cases in the same pedigrees. The majority of cases (n=143, 94%) derived from multiplex families with at least two affected family members, thereof 76 families with three or more affected members. All cases had EEG changes consistent with GGE (see appendix, table S1).

The replication case cohort 1 consisted of 357 GGE cases (after QC) that were collected in six European countries (Belgium n=5, Germany n=174, Ireland n=22, Italy n=23, Netherlands n=61, and UK n=72) by the EpiPGX consortium. The cohort included 225 females (63%) and 132 males (37%). GGE diagnosis included CAE (n=55), JAE (n=28), JME (n=157), EGTC (n=19), and unclassified GGE (n=98). 92 cases (26%) derived from multiplex families with at least two affected members. 131 cases were sporadic, for the remaining 134 cases, familial history was not known. Age of epilepsy onset ranged from 0 to 49 years with a median of 13 years. All cases had EEG changes consistent with GGE (appendix, table S2).

Two independent control cohorts for the case discovery and the replication cohort 1 were obtained from two independently sequenced cohorts from the Rotterdam study<sup>13,14</sup> which were matched for ethnicity and sex (see appendix). All the control samples were at least 55 years old or older and were checked for several neurological conditions at baseline. As GGE is a disease with typical onset from childhood to adolescence, it is unlikely that people from this older control cohort could still develop GGE.

For the GABA<sub>A</sub> receptor panel cohort (replication cohort 2), individuals were collected by referral from neurologists or paediatricians in Quebec, Canada, and in Europe by Epicure or CoGIE partners. The replication cohort 2 consisted of 631 subjects (after QC) with GGE that were collected from Canada (n=290) and five European countries (Germany n=153, Denmark n=58, Belgium n=71, Netherlands n=58, and Finland n=1). They included 390 females (62%) and 241 males (38%). Subjects were diagnosed with CAE (n=109), JAE (n=92), JME (n=189), EGTC (n=104), or unclassified absence epilepsy (n=137) not otherwise specified according to ILAE definitions<sup>1</sup> (appendix table S3). 154 cases were familial with at least 2 affected family members, for 51 there was a positive family history of epilepsy but only one affected member was available, and the remaining 426 cases were sporadic. All cases had EEG changes consistent with GGE. A third independent set of controls was obtained from the UK10K project consortium<sup>15</sup>. A full list of the investigators who contributed to the generation of the UK10K data is available from [www.UK10K.org](http://www.UK10K.org). Funding for UK10K was provided by the Wellcome Trust under award WT091310 (EGAS00001000101,129,130,131,242,306). Data transfer agreements were made between the CRCHUM and the appropriate instances. A total of 639 ethnically matched individuals were selected from the exome control cohort (324 females and 315 males).

The diagnosis of GGE in all case cohorts was based on detailed clinical interview, a full neurological examination and respective EEGs. Written informed consent was obtained from all subjects or their respective relatives and the study was approved by the local Ethical Committees. One affected individual of each family was selected for sequencing.

## Procedures

For the discovery stage, paired-end whole-exome sequencing (WES) of cases and controls was performed with the Illumina HiSeq 2000 using the EZ Human Exome Library v2.0 kit (NimbleGen, Madison, WI). Cases and controls were sequenced at different locations, cases at the Cologne Center for Genomics, the controls in Rotterdam<sup>13</sup>. Sequencing adapters were trimmed and samples with <30X mean depth or <70% total exome coverage at 20X mean depth of coverage were excluded from further analysis. Variant calling was performed by using the GATK<sup>16</sup> best practices pipeline with the GRCh37 human reference genome (see appendix).

The replication case cohort 1 was paired-end whole-exome sequenced at deCODE genetics (Iceland) on the Illumina HiSeq 2500 using the Nextera Rapid Capture Expanded Exome kit (Illumina). A second set of Rotterdam control samples was sequenced again in Rotterdam<sup>14</sup> using the EZ Human Exome Library kit (NimbleGen, Madison, WI).

For all WES samples, we applied standard procedures for assessing potential population stratification for the European population as well as a relatedness check (appendix and figures S1-3). To exclude low quality variants, we performed an additional filtering based on quality metrics of individual genotypes, using read depth and genotype quality as the filtering criteria. We excluded any variant position with mean depth of <10 in either cases or controls. For all WES samples the same exome regions file from the EZ Human Exome Library v2.0 kit was used. For the WES analysis, only samples with more than 30X mean coverage or more than 70% of the exome intervals covered by at least 20x mean coverage were included for the analysis (appendix).

For replication case cohort 2, a total of 19 genes encoding for all known subunits of GABA<sub>A</sub> receptors were selected for deep sequencing (*GABRA1*, *GABRA2*, *GABRA3*, *GABRA4*, *GABRA5*, *GABRA6*, *GABRB1*, *GABRB2*, *GABRB3*, *GABRD*, *GABRE*, *GABRG1*, *GABRG2*, *GABRG3*, *GABRP*, *GABRQ*, *GABRR1*, *GABRR2*, *GABRR3*, altogether referred to as *GABRX* herein). Exon targets were generated based on RefSeq, representing 184 exons from 19 genes. Primer design was made using the Primer3 oligonucleotide design tool and in silico PCR tool for validating the specificity of each amplicon. Target regions were enriched by PCR using the 48.48 Access Array Integrated Fluidic Circuit (IFC) (Fluidigm, San Francisco, CA). In the final assay, 185 amplicons targeted the protein-coding sequence of 19 *GABRX* genes with an overhang at exon boundaries in order to capture splice site variants. *GABRX* exon-specific primers with Fluidigm tags were tested along with materials and reagents as recommended in the Access Array System User Guide

(Fluidigm, South San Francisco, CA). Finally, *GABRR3* had to be dropped because of QC reasons having not enough good quality reads covering this gene. After quality trimming the reads were mapped against the GRCh37 human reference genome using the GATK<sup>161514</sup> suite and the MUGQIC pipelines ([https://bitbucket.org/mugqic/mugqic\\_pipelines](https://bitbucket.org/mugqic/mugqic_pipelines)). Data from the control cohort were processed using the same pipelines. Coverage comparisons were made to keep bases covered in at least 95% of the subjects as well as the control cohort.

RefSeq gene annotation information was used for the classification into missense and synonymous variants and to filter for rare (allele frequency smaller than 0.5%) variants using the ExAC database<sup>17</sup> (for details see appendix).

### **Population stratification**

We applied principal-components analysis (PCA) to assess potential population substructure separately for each case-control cohort, using the implementation in Eigenstrat<sup>18</sup>. Population outliers were defined as SD of >3 based on the first 10 PC and excluded from further analysis (appendix).

### **Statistical analysis**

Due to the limited sample size, single-gene collapsing analysis for the discovery stage was performed using Combined and Multivariate Collapsing<sup>19</sup>(CMC) method for collapsing and combining rare variants together with a two-sided Fisher's exact test, as implemented in the Exact CMC method in *rvtests*<sup>20</sup> (appendix). P-values for single-gene collapsing tests were corrected for multiple testing by use of the Bonferroni method (as implemented in the R function *p.adjust*) for 18,668 protein-coding genes. For all three stages, gene-set collapsing tests were performed using the regression-based two-sided SKAT-O test method<sup>21</sup>, as implemented in *rvtests*<sup>20</sup>. For the two WES cohorts, SKAT-O was used and we included sex and the first 10 PC from the Eigenstrat analysis as covariates to account for possible gender and population substructure effects. Gene-set collapsing tests were applied separately to missense and to synonymous variants of specific sets of candidate genes. Seven different disease- and process-specific gene sets were constructed based on their relation to GGE together with a control gene set not related to GGE. A description of the gene-set construction is given in the appendix and the gene sets are given in the appendix in table S5. In order to control the family-wise error rate, we applied Holm's correction for multiple testing 14 hypotheses, namely seven gene sets combined with two sets of variant type (missense and synonymous), in the discovery cohort, while correction was done for only two hypotheses in each of the two replication cohorts, since only the GABA<sub>A</sub> receptor gene set was carried forward the replication (appendix). The odds ratio (OR) for a given gene-set was determined by comparing the presence of qualifying rare (nonsynonymous or synonymous) variants in all genes within each gene-set between cases and controls.

## Functional analysis

Functional experiments were performed using automated two-microelectrode voltage clamping in *Xenopus* oocytes. All methods for functional studies have been described previously<sup>21,22</sup> (see appendix).

## Role of the funding source

The funders of the study had no role in the study design, data collection, data analysis, data interpretation or writing of the report. The authors had full access to the data in the study and had final responsibility for the decision to submit for publication.

## Results

We first performed WES in a cohort of 238 independent, mainly familial cases of classical forms of GGE, i.e. childhood or juvenile absence epilepsy (CAE/JAE), juvenile myoclonic epilepsy (JME) and epilepsy with generalized tonic-clonic seizures on awakening (EGTCA), collected by the Epicure and EuroEPINOMICS-CoGIE consortia. As controls, we used ethnically and sex matched (appendix figure S1) population control individuals from the Rotterdam Study<sup>13</sup>, that underwent WES using the same enrichment and sequencing procedures, albeit with a somewhat lower coverage than in the GGE cohort. After quality control (QC) and population outlier removal, the final dataset consisted of 152 unrelated GGE and 549 unrelated control samples. To adjust for the different coverage between case and control samples, we considered only variants with an average read depth of >10 both in case and in control samples (see appendix, figure S2). From the total of 701 samples, 204,023 exonic and splice site variants were called. The mean exonic transition/transversion ratio equaled 3.46, indicating good data quality. Rare variants (MAF<0.005) were classified as missense (Nonsyn) and silent (Syn) variants. 93,893 Nonsyn and 55,170 Syn variants constituted the analysis data set (see appendix, table S4). First, we tested hypothesis-free all individual RefSeq genes separately for association but could not identify any single gene enriched for any variant type (appendix). Therefore, we next applied an independent hypothesis-driven analysis by testing the enrichment of rare variants in seven gene sets related to epilepsy and its underlying molecular processes. These gene sets represented (i) all voltage-gated cation channels, (ii) all excitatory postsynaptic receptors, (iii) all GABA<sub>A</sub> receptors as the main inhibitory postsynaptic receptors, (iv) more broadly the GABAergic pathway (since such genes have been associated specifically with generalized epilepsies), and genes associated (v) with generalized epilepsies, (vi) with epileptic encephalopathies, (vii) with focal epilepsies (appendix table S4). We tested separately for each variant type; silent variants were expected to show no difference between cases and controls. We found an enrichment for missense variants in a set of GABA<sub>A</sub> receptor genes which was significant when correcting for multiple comparisons in the

seven gene sets for both nonsyn and syn (so 14 altogether) by use of the two-sided SKAT-O test (19 genes,  $p_{\text{Nonsyn}}=0.019$ ,  $\text{OR}=2.40$ ,  $95\% \text{ CI}=[1.41,4.10]$ ) (figure 1). However, the GABA gene set did not reach study-wide significance when also correcting for all single genes tested. None of the other gene sets showed a significantly increased burden of rare variants. Synonymous variants, used as a negative control, did not show a significant enrichment in any of the gene sets (appendix, tables S8 and S9).

To replicate the finding for the GABA<sub>A</sub> receptor encoding genes, we first used the replication case cohort 1 collected by the EpiPGX consortium, consisting of 724 individuals with GGE from six European countries. They were mainly sporadic ( $n=268$ ) or of unknown familial history ( $n=265$ ) and diagnosed with classical forms of GGE (appendix, table S2). For the analysis of this cohort, an independent matched subset of control samples from the Rotterdam Study<sup>12</sup> was used. After applying the same QC steps as applied to the discovery cohort, the final dataset consisted of 357 unrelated GGE and 1485 unrelated control samples<sup>14</sup>. We confirmed the significant enrichment of rare missense variants in GABA<sub>A</sub> receptor genes in cases compared to controls after multiple-testing correction for two sets of variants (nonsyn and syn;  $p_{\text{Nonsyn}}=0.016$ ,  $\text{OR}=1.46$ ,  $95\% \text{ CI}=[1.05,2.03]$ ) by use of the SKAT-O test (appendix table S8 and S9). Synonymous variants showed again no significant enrichment.

For a second replication cohort, we designed a targeted enrichment panel comprising all 19 GABA<sub>A</sub> receptor genes. All genes were sequenced in an independent cohort of 631 cases with familial or sporadic GGE (appendix, table S3). *GABRR3* was excluded for QC reasons. We obtained control samples from the UK10K project (<https://www.uk10k.org/>) and selected 639 gender matched individuals after sample QC. Additional variant QC reduced the number of individuals to 583 cases and 635 controls in the final sample set. We found a significant enrichment of rare missense variants for the GABA<sub>A</sub> receptor genes in cases compared to controls ( $p_{\text{Nonsyn}}=0.027$ ,  $\text{OR}=1.46$ ,  $95\% \text{ CI}=[1.02,2.08]$ ) by use of a SKAT-O test and after correction for two sets of variants (Nonsyn, Syn). No significant enrichment was observed for synonymous variants. Thus, we can conclude that enrichment of rare missense variants in GABA<sub>A</sub> receptor genes is reproducibly present in individuals with GGE when compared to controls. All detected case-only variants are given in the appendix, tables S8 and S9. Case-only rare missense variants were found in all GABA<sub>A</sub> receptor genes except in *GABRR3* (table S9).

The combination of two  $\alpha_1$ -, two  $\beta_2$ - and one  $\gamma_2$ -subunit (genes *GABRA1*, *GABRB2*, *GABRG2*) represents the most common form of a functional GABA<sub>A</sub> receptor in the brain<sup>24</sup>, and variants in *GABRA1* and *GABRG2* have been shown to play an important role in familial GGE, febrile seizures and EE<sup>4,5,22,23,25–27</sup>. It is important to note that the enrichment of missense variants in GABA<sub>A</sub> receptor genes was not driven by variants in those known epilepsy genes. The signal was no longer significant when reducing the analysis only to those two genes (table S9). Instead, the qualifying

variants were evenly distributed over all GABA<sub>A</sub> receptor encoding genes. The  $\alpha_5$  subunit (gene *GABRA5*) is supposed to mediate extrasynaptic tonic inhibition<sup>28</sup>, and tonic inhibition has been described to be altered in genetic mouse models of epilepsy<sup>29,30</sup>. *GABRB2* and *GABRA5* have not previously been associated with GGE, although *GABRB2* mutations were described recently in patients with intellectual disability and epilepsy<sup>31–33</sup>.

For functional studies, we selected seven missense variants in *GABRB2* and *GABRA5* (appendix, table S10) for electrophysiological studies in *Xenopus* oocytes (appendix). Those were selected early during the course of the study, before the final QC steps were performed, so that two of these variants do not belong to the final set of variants yielding a significant burden compared to controls (table S10). Five of these variants were selected since they co-segregated with the phenotype of available members in nuclear families. Another variant (p.R3S) was found in three different French-Canadian pedigrees, so we hypothesized whether this could be a more common causal variant in a specific population (figures 2a and 3a). The last variant, p.P453L, did not co-segregate, but was selected as another variant in *GABRA5* which is localized in a different protein region (the C-terminus) than the other variants. All missense variants were predicted to be deleterious by at least three out of seven missense prediction tools and were highly conserved (table S10). Three of these variants were consistently of ultra-low frequency in the European population in different public databases (dbGAP, 1000G, ExAC; appendix, table S10). The localization of the variants in the GABA<sub>A</sub> subunits is shown in figures 2b and 3b. After application of 1 mM GABA, we observed a significant reduction in current amplitudes of GABA<sub>A</sub> receptors containing either p.K221R or p.V316I variants in the  $\beta_2$ -subunit, and p.M1I, p.S238N, or p.E243K in the  $\alpha_5$ -subunit, in comparison to respective compositions of WT receptors. No significant reductions were observed for p.R3S in the  $\beta_2$ - and for p.P453L in the  $\alpha_5$ -subunit (figures 2c, 2d, 3c, 3d). The GABA sensitivity was studied by applying different GABA concentrations with no significant changes observed for receptors containing any of the studied variants (figures 2e, 3e). Thus, five out of seven variants suggest a loss of receptor function predicting postsynaptic or extrasynaptic neuronal disinhibition.

All variants showing significantly reduced current amplitudes co-segregated with the disease phenotype in family members that were available for testing (figures 2a, 3a), corroborating their contribution to the disease phenotypes. In two families, we observed co-segregating variants in two different GABA<sub>A</sub> receptor subunits: p.V316I in the  $\beta_2$ - and p.M1I in the  $\alpha_5$ -subunit co-occurred in the same nuclear family, and p.E243K in the  $\alpha_5$ -subunit was accompanied by a deleterious frameshift mutation in *GABRG2* in another family (figures 2a, 3a). Variants with altered receptor function were all located in the N-terminus containing GABA binding sites or in the pore region. p.M1I suppresses the start codon such that translation starts six amino acids later, which shortens the signalling peptide consisting of the first 20 amino acids. While the peptide is removed and not part of the mature GABA<sub>A</sub> receptor in the plasma membrane, this alteration could

still affect the protein biogenesis and lead to reduced expression of functional receptors. p.R3S, which also affects the signalling peptide, and p.P453L, located in the functionally less relevant C-terminus, did not lead to a significant change in receptor function. p.R3S recurred in three French-Canadian families and p.P453L was detected in only one of several affected members of a larger family indicating that they might represent benign polymorphisms.

## Discussion

Our results show an excess of rare missense variants in GABA<sub>A</sub> receptor subunit encoding genes in three independent cohorts of altogether >1000 familial and sporadic GGE index cases. Five selected variants in two genes, *GABRB2* and *GABRA5*, previously not associated with GGE (i) clearly changed receptor function and (ii) co-segregated in nuclear families, suggesting an important contribution to the disease phenotype and inheritance in those pedigrees. Previous studies in smaller cohorts failed to show a significant excess of variants in cases versus controls either in a test for the set of all ion channel encoding genes<sup>8</sup> or in single-gene collapsing tests based on whole exomes<sup>9</sup>. Our findings indicate that the enrichment of rare genetic variants in a set of inhibitory GABA receptors does play a significant role in the pathogenesis of GGE. The difference between these previous studies and ours could be explained by (i) a larger sample size in our study across all cohorts and (ii) by testing different gene-sets that had not been considered before. In a parallel study<sup>10</sup>, a similar effect could be shown for ultra-rare deleterious variants in gene-sets comprising known epilepsy genes or genes associated with epileptic encephalopathies. Due to our smaller sample size and the associated low number of ultra-rare variants, we here chose a different approach considering all variants with a MAF<0.005, which proved to yield significant results in other studies<sup>34-36</sup>. Both studies (i.e. Epi4k and ours) failed to identify single genes with a genome-wide significant burden of rare variants in individuals with GGE. It will be interesting in future studies to combine different cohorts to increase sample size and power for such analyses to shed further light on the complex genetic architecture of GGE.

One limitation of the current study is that the cohorts, due to funding restrictions of the individual projects, were sequenced at different locations using different technologies. Combining and analysing such data in an unbiased way is still a major challenge in large genetic sequencing projects. An a priori selection bias for the targeted genes yielding a false significance can also not be completely ruled out. The careful choice of gene sets was based on purely biological and published evidence and did not change the selection afterwards. This approach should minimize any potential selection bias and associated false-positive findings. We further addressed these issues by using a stringent QC and consistent processing of all datasets (appendix), and by using two different GGE cohorts to replicate our data in independent case and control datasets.

One of the variants detected and functionally examined in our study (p.V316I in *GABRB2*) has been described in the meantime as a *de novo* mutation in a different dataset of cases with severe

developmental and epileptic encephalopathies, in which whole genome sequencing of parent-patient trios was used<sup>32</sup>. This finding clearly corroborates the pathogenicity of this variant. The association of genetic variants with different phenotypes is well-known as the phenomenon of pleiotropy, and has also been described in other GABA<sub>A</sub> receptor encoding genes<sup>3,24</sup> including large phenotypic variability within one extended pedigree<sup>3</sup>.

We have also recently characterized the variant p.T336M in *GABRA3* – which was detected in our discovery cohort (table S9) – as part of another study in which we identified *GABRA3* as a new epilepsy gene associated with highly heterogeneous epileptic phenotypes including asymptomatic variant carriers<sup>37</sup>. This variant also causes a severe loss-of-function effect but does not co-segregate in the respective pedigree, so that other factors must contribute to the GGE at least in two family members. While co-segregation is a strong indicator for the pathogenicity of genetic variants, we have to be aware that GGE is a common disease with complex inheritance. Variants in GABA<sub>A</sub> receptor encoding genes could therefore still contribute to the disease in the carriers, whereas other family members not carrying the respective variants must have other genetic causes of their epilepsy. Similarly, copy number variations often do not co-segregate within nuclear families but have been replicated several times as a significant risk factor for GGE<sup>38-41</sup>. Given the reproducibility of our results in three independent datasets together with co-segregation and functional evidence for GABA<sub>A</sub> receptor dysfunction, many but not all of the detected variants may contribute to the etiology of GGE in our three cohorts. This disease-relevant contribution may range from a major gene effect – as observed in ‘monogenic’ Mendelian epilepsies – to relatively small effect sizes in the variant carriers, depending on the amount of the electrophysiological dysfunction and other unknown factors, such as the genetic background. Overall, we therefore consider the detected increase in GABA<sub>A</sub> receptor variants in cases vs. controls as a significant risk factor to develop GGE.

Lastly, our results indicate a genetic overlap among rare and common forms of epilepsy, since there is increasing evidence that *de novo* variants in GABA<sub>A</sub> receptor encoding genes cause severe forms of epileptic encephalopathies<sup>22-26,37,42</sup> and they re-iterate a central role of GABAergic mechanisms in generalized epilepsies<sup>3-5,23-25,27-31,43,44</sup>.

## Contributions

HL, RoIK, PC, FZa, SM, PN, PM and SG designed the study. FB, SW, PL-T, CM MG, SB, PS, HC, AS, KE, RoIK, RSM, HH, HM, IH, WK, YGW, SW, SMS, SS, RN, AC, MSV, DK-NT, BB, UO, NB, KMK, FR, DKN, FD, CP, LC, AL, RD, J-FC, CC-W, GA, TS, ELG, BPCK, FZa, PC, HL, GJS, PA, BF, MRJ, AGM, BBe, JWS, JWS, AA, MMc, GLC, ND, MB, CD, MK, FZi, MNi, and the Epicure, EuroEPINOMICS CoGIE and the EpiPGX consortia recruited and phenotyped subjects. PM, SG, DRB, JS, CDK, MT, AP, MI, RGL, SB, AB, CM, HT, JA, KJ, A-KR, WJ, DL,

ELG, JvR, RK, AI, AGU, JMS, BPCCK, AP, A-EL, MN, PN, FZa, PC, RolK, and HL performed or supervised genetic studies (Sanger or whole exome sequencing, or data analysis), MH, CER, JK, RR, SC, BT, IDC, CAR, SS, StP, MM, SM and HL performed or supervised functional studies, PM, SG, MH, DRB, JS, SW, and HL wrote the manuscript. All authors revised the manuscript.

### **Declarations of interests**

HL received personal fees for consulting, speaking or travel support from Bial, Biomarin, Desitin, Eisai or UCB Pharma, and research support from Bial, all outside the submitted work; he received funding from DFG, BMBF, EU, DGfE and the foundation noepilep related in part to this work (see acknowledgements). KMK reports personal fees from UCB Pharma, personal fees from Novartis Pharma AG, personal fees from Eisai, outside the submitted work. SP reports other from Praxis Precision Medicine, grants from RogCon, other from Pairnomix, outside the submitted work; In addition, SP has a patent U.S. Provisional Application 62/450,025 pending. FR reports personal fees and non-financial support from UCB Pharma, personal fees from Shire, personal fees from EISAI, personal fees from Desitin Arzneimittel, personal fees from Bial, personal fees from cerbomed, GW-Pharma, Bayer Vital, Sandoz, University of Munich (LMU), and from Verband der forschenden Arzneimittelindustrie, non-financial support from Novartis Japan, , grants from European Union, FP7, grants from Hessisches Ministerium für Wissenschaft und Kunst (LOEWE-Programme), personal fees from University of Munich (LMU), grants from Detlev-Wrobel Fonds for Epilepsy Research, outside the submitted work. AA is employed by UCB Pharma SPRL, Belgium, as Associate Director. AGM was awarded grants from GSK, Eisai, and UCB Pharma, which funded the National Audit of Seizure Management in Hospitals. JWS has served on scientific advisory boards for UCB Pharma and Eisai; has served on speaker's bureaus for UCB Pharma, Eisai, Bial and Janssen; has received research support from UCB Pharma, Eisai, The Marvin Weil Epilepsy Research Fund, and NL Nationaal Epilepsie Fonds; and his current position is endowed by the UK Epilepsy Society. AC was awarded a grant from EISAI and personal fees for speaking from Eisai, outside of the submitted work. GJS has received personal fees for consulting and/or speaking from UCB Pharma and Eisai, all outside the submitted work. The other authors declare no competing financial interests.

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## **EpiPGX Consortium**

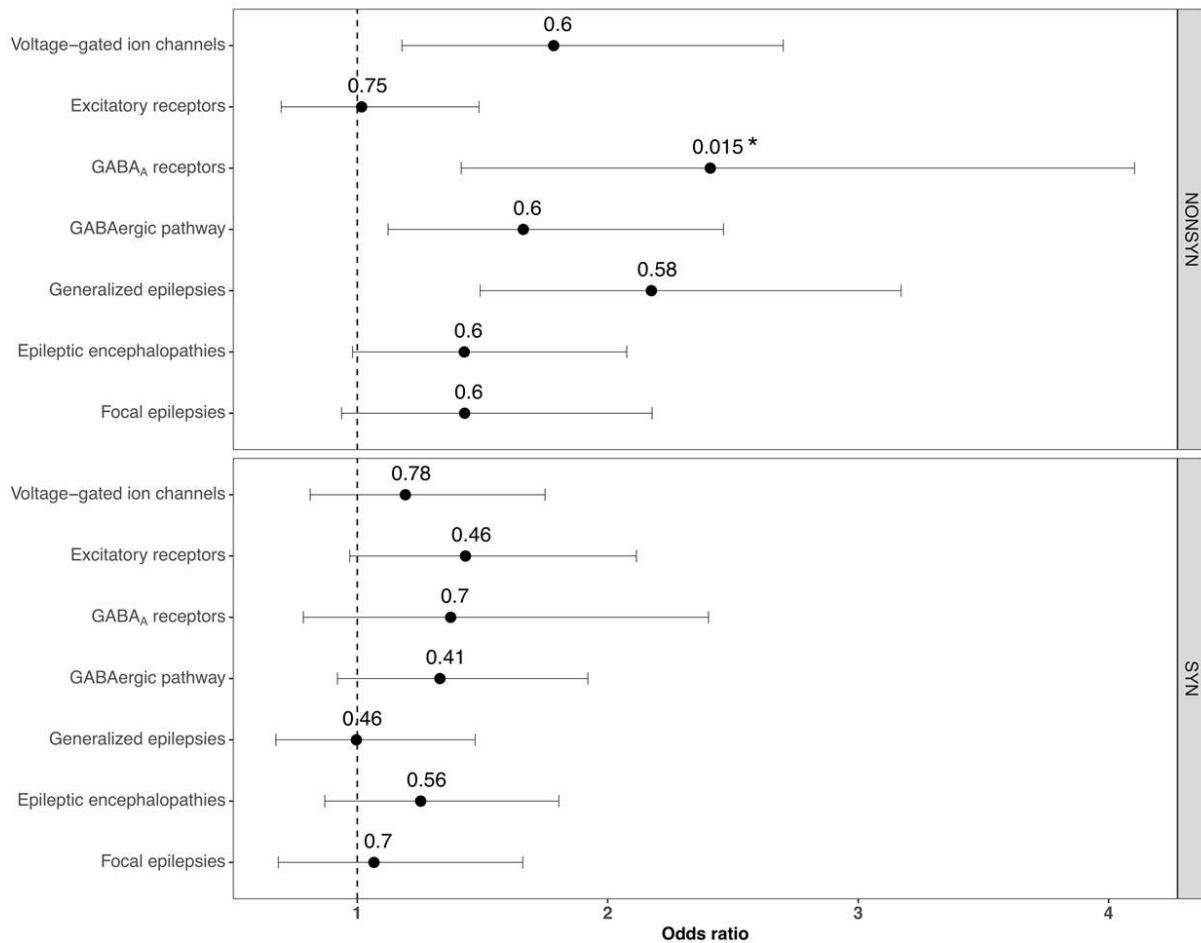
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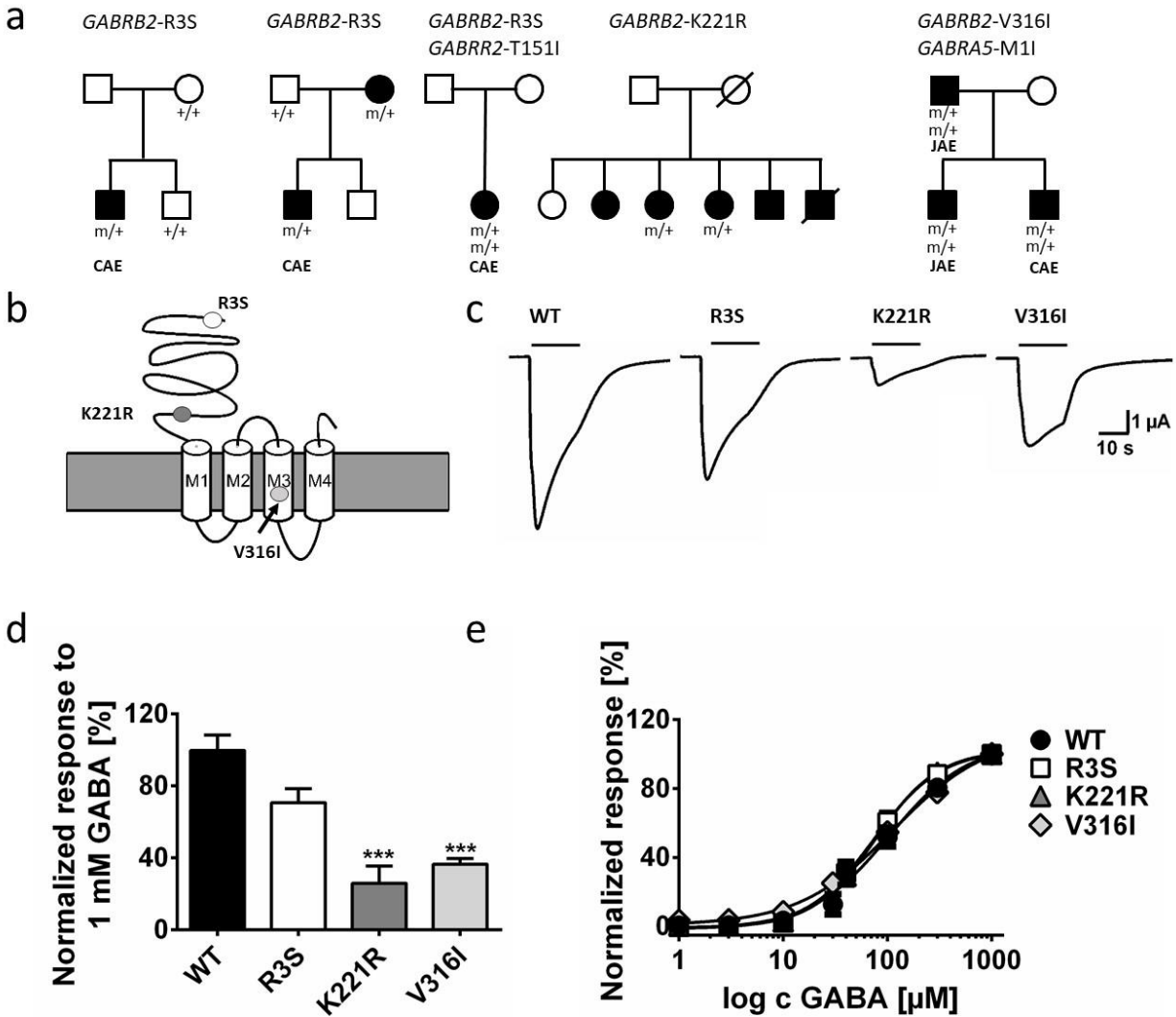
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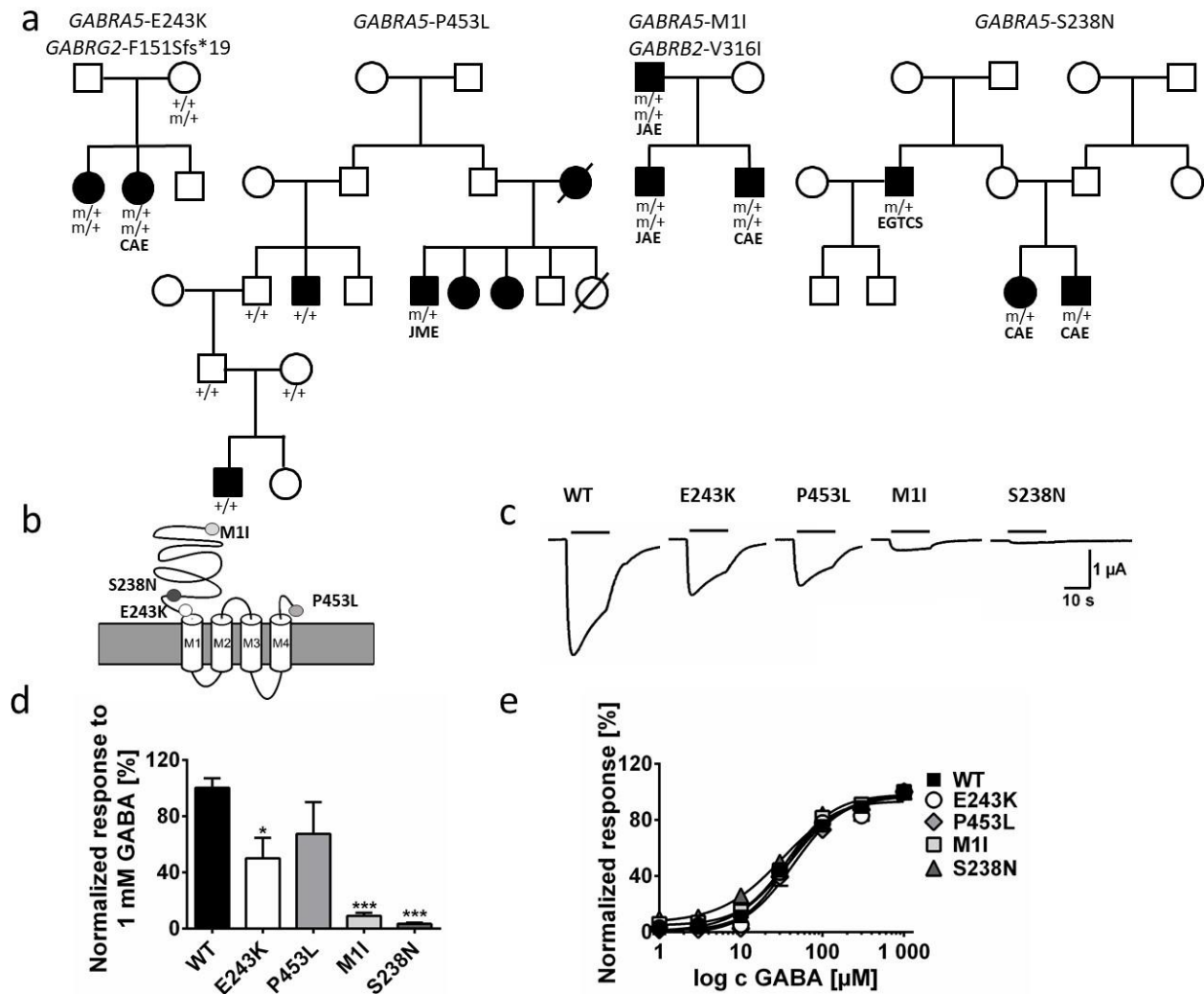
**Figure 1: Rare variant gene-set odds ratios and burden enrichment for rare variants in the whole-exome sequencing GGE discovery cohort**

Cases from the CoGIE discovery cohort, matched with controls from the Rotterdam study. Gene-set collapsing analysis by use of a SKAT-O test was performed on seven epilepsy-related gene sets for missense (NONSYN) and synonymous (SYN) variants. The gene sets are described in the appendix. The star denotes the enriched gene-set collapsing p-value after Holm correction.



**Figure 2: Characterization of *GABRB2* missense variants associated with GGE**

*GABRB2* mutations associated with GGE. (a) Pedigree of the families. (b) Schematic representation of the  $\beta_2$  subunit of the GABA<sub>A</sub>R and predicted positions of the R3S and K221R mutations located in the N-terminal domain and V316I located in the transmembrane domain 3. (c) Examples of GABA-induced currents after 1 mM GABA application for WT, R3S, K221R and V316I mutations. (d) Current responses normalized to 1 mM GABA application for WT (n=30), R3S (n=24), K221R (n=21) and V316I (n = 16); \*\*\*p<0.001, Kruskal Wallis test, with Dunn's comparison test. (e) Dose-response curve for  $\alpha_1\beta_2\gamma_2s$  WT (n=30), R3S (n=14), K221R (n=10), V316I (n=7) obtained using application of different GABA concentrations and normalization to the maximal GABA response for each cell.



**Figure 3: Characterization of *GABRA5* missense variants associated with GGE**

(a) Pedigree of the families. (b) Schematic representation of the  $\alpha_5$  subunit of the GABA<sub>A</sub>R and predicted positions of the M1I, S238N and E243K mutations located in the N-terminal domain and P453L located in the C-terminal domain. (c) Examples of GABA-induced currents after application of 1 mM GABA for WT, M1I, S238N, E243K and P453L mutations. (d) Normalized current responses to 1 mM GABA application for WT (n=43), M1I (n=10), S238N (n=13), E243K (n=14) and P453L (n=11); \* $p < 0.05$ , \*\*\* $p < 0.0001$ , Kruskal Wallis test, with Dunn's comparison test. (e) Dose-response curve for  $\alpha_1\beta_2\gamma_2\delta$  WT (n=37), M1I (n=15), S238N (n=11), E243K (n=8) and P453L (n=8) obtained after application of different GABA concentrations and normalization to the maximal GABA response for each cell.