Ultra-Rare Genetic Variation in the Epilepsies: A Whole-Exome Sequencing Study of 17,606 Individuals

Epi25 Collaborative*,*

Sequencing-based studies have identified novel risk genes associated with severe epilepsies and revealed an excess of rare deleterious variation in less-severe forms of epilepsy. To identify the shared and distinct ultra-rare genetic risk factors for different types of epilepsies, we performed a whole-exome sequencing (WES) analysis of 9,170 epilepsy-affected individuals and 8,436 controls of European ancestry. We focused on three phenotypic groups: severe developmental and epileptic encephalopathies (DEEs), genetic generalized epilepsy (GGE), and non-acquired focal epilepsy (NAFE). We observed that compared to controls, individuals with any type of epilepsy carried an excess of ultra-rare, deleterious variants in constrained genes and in genes previously associated with epilepsy; we saw the strongest enrichment in individuals with DEEs and the least strong in individuals with NAFE. Moreover, we found that inhibitory $GABA_A$ receptor genes were enriched for missense variants across all three classes of epilepsy, whereas no enrichment was seen in excitatory receptor genes. The larger gene groups for the GABAergic pathway or cation channels also showed a significant mutational burden in DEEs and GGE. Although no single gene surpassed exome-wide significance among individuals with GGE or NAFE, highly constrained genes and genes encoding ion channels were among the lead associations; such genes included *CACNA1G*, *EEF1A2*, and *GABRG2* for GGE and *LGI1*, *TRIM3*, and *GABRG2* for NAFE. Our study, the largest epilepsy WES study to date, confirms a convergence in the genetics of severe and less-severe epilepsies associated with ultra-rare coding variation, and it highlights a ubiquitous role for GABAergic inhibition in epilepsy etiology.

Introduction

Epilepsy is a group of disorders characterized by repeated seizures caused by excessive electrical activity in the brain and is one of the most common neurological conditions; epilepsy affects 5–7 of every 1,000 individuals worldwide.[1,2] Human genetics research has established that a genetic basis contributes to the susceptibility to epilepsy for a majority of the cases.[3–6] However, the multifactorial condition of epilepsy that subsumes a variety of epilepsy types, seizures, levels of severity, and comorbidity has made it a core challenge to disentangle the genetic architecture for different types of epilepsy and to determine the specific genetic risks for each individual with epilepsy.

In recent years, our understanding of the genetic risk factors for epilepsy has substantially expanded thanks to the rapid advancement in sequencing technology. Currently, gene identification from sequencing-based studies has been primarily limited to rare, monogenic forms of epilepsy, and much of the focus has been on a group of severe epilepsy syndromes known as the developmental and epileptic encephalopathies (DEEs [MIM: 308350]).[7–11] DEEs typically begin early in life and are characterized by intractable seizures and profound to mild developmental impairment. It was found that one in every 2,000 infants develops severe epilepsy with onset under 18 months.[12] For these severe epilepsies, dozens of genes with *de novo* pathogenic variants have been identified, and the number continues to grow. The other major epilepsy types broadly encompass genetic generalized epilepsy (GGE [MIM: 600669]) and non-acquired focal epilepsy (NAFE [MIM: 604364, 245570]), the former characterized by seizures involving both hemispheres of the brain, the latter a localized cortical region. The incidence of these groups is not well established, but they are recognized as the more-common, less-severe forms of epilepsy, and epidemiological studies have estimated that generalized and focal epilepsies each account for 20%–40% of incident epilepsies.[13–16] As with DEEs, there are several specific electroclinical syndromes within the classes of GGE and NAFE, but the genetic etiology is more complex. Genetic investigations into GGE or NAFE thus far both support both a role for an oligogenic or polygenic component[17–20] and provide some evidence for monogenic causes for a minority of affected individuals.[5] Despite a significant heritability consistently demonstrated from twin, family, and genome-wide association studies (GWAS),[4,19–22] the discovery of individual genes associated with GGE and NAFE has remained scarce. Most genes identified to date come from monogenic families of focal epilepsies, and attempts to identify risk genes associated with GGE have been largely unsuccessful.[23–25] For most of the GGE- and NAFE-affected individuals with non-familial onsets, the specific pathogenic variants are not yet known, and gene findings from small-scale studies have often not been reproducible.[26–28]

Two recent whole-exome sequencing (WES) case-control studies leveraged hundreds of familial cases and provided clear evidence of specific gene groups linked to the risk of GGE and NAFE.[24,25] Specifically, the authors showed that ultra-rare genetic variation in genes associated with DEEs was enriched in GGE and NAFE and that enrichment of missense variants in all genes encoding $GABA_A$ receptors

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was observed for the first time in GGE. These findings highlight that genes commonly implicated in epilepsy can span a wider range of epilepsy phenotypes than previously postulated. Studying rare genetic variation involving severe to milder electroclinical syndromes of epilepsy can help researchers to better understand the extent of phenotypic pleiotropy and variable expressivity that could inform treatment strategies. On the other hand, the extensive phenotypic and genetic heterogeneity of epilepsy, especially for GGE and NAFE, underscores the need to enlarge the scale of such studies beyond familial cases.

Here, we evaluate a WES case-control study of epilepsy from the Epi25 Collaborative—an ongoing global effort to collect an unprecedented number of patient cohorts primarily for the three major classes of non-lesional epilepsies: DEEs, GGE, and NAFE.[29] We aimed to pinpoint the distinct and overlapping genetic risk of ultra-rare coding variants for these different phenotypic groups by evaluating the burden at the individual gene level and in candidate gene sets in order to understand the role of rare genetic variation and identify specific associated genes across the severity spectrum for epilepsy syndromes.

Material and Methods

Study Design and Participants

We collected DNA from and detailed phenotyping data on individuals with epilepsy from 37 sites in Europe, North America, Australasia, and Asia (see Supplemental Data and Table S1). Here we analyzed subjects with GGE (also known as idiopathic generalized epilepsy; n = 4,453), NAFE (n = 5,331), and DEEs (n = 1,476); and a small number of other epilepsies were also included in the initiative (Table S1). A subset of the data is available on dbGaP: phs001489. Control samples were aggregated from local collections at the Broad Institute or obtained from dbGaP, and these controls consisted of 17,669 individuals of primarily European ancestry who were not screened for neurological or neuropsychiatric conditions (see Table S2 and Supplemental Data).

Phenotyping Procedures

Epilepsies were diagnosed by experienced epileptologists on clinical grounds based on the criteria given in the next paragraph (see below for criteria GGE, NAFE, and DEEs, respectively) and consistent with International League Against Epilepsy (ILAE) classification at the time of diagnosis and recruitment. De-identified (non-PHI [protected health information]) phenotyping data were entered into the Epi25 Data repository (hosted at the Luxembourg Centre for Systems Biomedicine) via detailed online case record forms based on the RedCAP platform. Where subjects were part of previous coordinated efforts with phenotyping on databases (e.g., the Epilepsy Phenome/Genome Project[30] and the EpiPGX project [Web Resources]), deidentified data were accessed and transferred to the new platform. Phenotyping data underwent review for uniformity among sites and quality control (QC) by automated data checking, followed by manual review if required. Where doubt remained about eligibility, cases were reviewed by the phenotyping committee, and sometimes further data were requested from the source site before a decision was made.

Case Definitions

Diagnosis of GGE required a convincing history of generalized seizure types (generalized tonic-clonic, absence, or myoclonic seizures) and generalized epileptiform discharges on EEG. We excluded cases with evidence of focal seizures or with moderate-to-severe intellectual disability and those with an epileptogenic lesion found on neuroimaging (although neuroimaging was not obligatory). If a diagnostic source EEG was not available, then only cases with archetypal clinical histories as judged by the phenotyping committee (e.g., morning myoclonus and generalized tonic-clonic seizures for a diagnosis of juvenile myoclonic epilepsy) were accepted.

Diagnosis of NAFE required a convincing history of focal seizures; an EEG with focal epileptiform or normal findings (since routine EEGs are often normal in focal epilepsy); and neuroimaging showing no epileptogenic lesion except hippocampal sclerosis. (MRI was preferred, but CT was accepted.) Exclusion criteria were a history of generalized onset seizures or moderate-to-severe intellectual disability.

The DEE group was comprised of subjects with severe refractory epilepsy of unknown etiology, with developmental plateau or regression, with no epileptogenic lesion on MRI, and with epileptiform features found on EEG. Because this is the group with the largest number of gene discoveries to date, we encouraged inclusion of those with non-explanatory epilepsy gene panel results, but we did not exclude those who had not undergone prior testing (Table S7).

Informed Consent

Adult subjects, or in the case of children, their legal guardians, provided signed informed consent at the participating centers according to local national ethical requirements. Samples had been collected over a 20 year period in some centers, so the consent forms reflected standards at the time of collection. Samples were only accepted if the consent did not exclude data sharing. For samples collected after January 25, 2015, consent forms required specific language according to the National Institutes of Health's Genomic Data Sharing policy (see Web Resources).

Whole-Exome Sequencing Data Generation

All samples were sequenced at the Broad Institute of Harvard and the Massachusetts Institute of Technology (MIT) on the Illumina HiSeq X platform, with the use of 151 bp paired-end reads. Exome capture was performed with Illumina Nextera Rapid Capture Exomes or TruSeq Rapid Exome enrichment kit (target size 38 Mb), except for three control cohorts (MIGen ATVB, MIGen Ottawa, and Swedish SCZ controls) for which the Agilent SureSelect Human All Exon Kit was used (target size 28.6 Mb–33 Mb). Sequence data in the form of BAM files were generated via the Picard data-processing pipeline and contained well-calibrated reads aligned to the GRCh37 human genome reference. Samples across projects were then jointly called via the Genome Analysis Toolkit (GATK) best-practice pipeline[31] for data harmonization and variant discovery. This pipeline detected single-nucleotide variants (SNVs) and small insertion or deletion (indel) variants from exome sequence data.

Quality Control

Variants were pre-filtered so that only those passing the GATK VQSR (variant quality score recalibration) metric and those lying outside of low-complexity regions remained.[32] Genotypes with

genotype quality (GQ) <20 and heterozygous genotype calls with allele balance >0.8 or <0.2 were set to missing. To control for capture platform difference, we retained variants that resided in GENCODE coding regions where 80% of Agilent and Illumina-sequenced samples show at least 10-× coverage. This resulted in the removal of ~50% of the called sites (23% of the total coding variants and 97% of the total non-coding variants) but effectively reduced the call rate difference between cases and controls (Figure S1). To further identify potential false positive sites due to technical variation, we performed single-variant association tests (for variants with a minor-allele frequency [MAF] >0.001) among the controls, treating one platform as the pseudo-case group with adjustment for sex and the first ten principal components (PCs). We removed variants that were significantly associated with capture labels (p value < 0.05). We also excluded variants with a call rate <0.98, case-control call-rate difference >0.005, or Hardy-Weinberg Equilibrium (HWE) test p value $<1\times10^{-6}$ on the basis of the combined case and control cohort.

Samples were excluded if they had a low average call rate (<0.98), low mean sequence depth (<30; Figure S2), low mean genotype quality (<85), high freemix contamination estimate (>0.04), or high percent chimeric reads (>1.4%). We performed a series of principal-component analyses (PCAs) to identify ancestral backgrounds and to control for population stratification; we kept only individuals of European (EUR) ancestry classified by Random Forest with 1000 Genomes data (Figure S3). Within the EUR population, we removed controls not well matched with cases on the basis of the top two PCs, and we removed individuals with an excessive or a low count of synonymous singletons—a number that increases with the north-to-south axis (Figure S4). We also removed one sample from each pair of related individuals (proportion identity by descent >0.2), and we removed those whose genetically imputed sex was ambiguous or did not match with self-reported sex. Outliers (>4 SD from the mean) of transition/transversion ratio, heterozygous/homozygous ratio, or insertion/deletion ratio within each cohort were further discarded (Figures S5–S7). At the phenotype level, we removed individuals with epilepsy phenotype to be determined or marked as "excluded" from further review.

The number of variant and sample dropouts at each step is detailed in Tables S3 and S4.

Variant Annotation

Annotation of variants was performed with Ensembl's Variant Effect Predictor (VEP)[33] for human genome assemble GRCh37. On the basis of the most severe consequence, we used relevant terms and SnpEff[34] impact to define four mutually exclusive functional classes of variants (Table S5): protein-truncating variant (PTV), damaging missense variant (predicted by PolyPhen-2 and sorting intolerant from tolerant [SIFT]), benign missense variant (predicted by PolyPhen-2 and SIFT), and synonymous variant. To further discriminate those missense variants that were most likely deleterious from benign missense variants, we applied an *in silico* missense deleteriousness predictor (missense badness, PolyPhen-2, and regional constraint [MPC] score)[35] that leverages regional constraint information to annotate a subset of missense variants that are highly deleterious (MPC ≥ 2). The MPC ≥ 2 group accounts for a small proportion of the total damaging and benign missense variants annotated by PolyPhen-2 and SIFT. Because many of our control samples were obtained from external datasets used in the Exome Aggregation Consortium (ExAC)[36] (Table S2), we used the DiscovEHR cohort—an external population allele frequency reference cohort that contains 50,726 whole-exome sequences from a largely European and non-diseased adult population[37]—to annotate whether a variant is absent in the general population (Figure S8).

Gene-Set Burden Analysis

To estimate the excess of rare, deleterious protein-coding variants in individuals with epilepsy, we conducted burden tests across the entire exome, for biologically relevant gene sets, and at the individual gene level. We focused on two definitions of "ultra-rare" genetic variants (URVs) for the primary analyses: (1) variants not seen in the DiscovEHR database and observed only once among the combined case and control test cohort (allele count [AC] = 1) or (2) variants absent in DiscovEHR and observed no more than three times in the test cohort (AC ≤ 3). These URVs have been observed previously[24,38] and in our study to contain the strongest burden of deleterious pathogenic variants compared to less-stringent allele frequency thresholds (Figures S9 and S10). We performed these case-control comparisons separately for each of the three primary epilepsy disorders (DEEs, GGE, and NAFE) and again for all epilepsy-affected individuals combined.

We implemented gene-set burden tests by using logistic regression to examine the enrichment of URVs in individuals with epilepsy versus controls. We performed the test by regressing case-control status on certain classes of URVs aggregated across a target gene set in an individual and adjusting for sex, the top ten PCs, and exome-wide variant count. This analysis tested the burden of URVs separately for five functional coding annotations: synonymous, benign missense as predicted by PolyPhen-2 and SIFT, damaging missense as predicted by PolyPhen-2 and SIFT, PTVs, and missense with MPC ≥ 2 (Table S5). To help determine whether our burden model was well calibrated, we used synonymous substitutions as a negative control where significant burden effects would more likely indicate insufficient control of population stratification or exome-capture differences. The inclusion of overall variant count as a covariate—which tracks with ancestry—made our test conservative but allowed for better control of residual population stratification not captured by PCs and effectively reduced inflation of signals in synonymous variants (Figure S11). We collected and tested 11 different gene sets, including constrained genes that are intolerant to loss-of-function (LoF) mutations (pLI > 0.9 and pLI > 0.995[39]) or missense variation (mis-Z > 3.09[39]), brain-enriched genes that are expressed at a level more than 2-fold greater in brain tissues than in other tissues according to Genotype-Tissue Expression Consortia (GTEx) data,[40] and genes reported to be associated with epilepsy in a dominant fashion[10,24] or with epilepsy-related mechanisms[25] (Table S6).Because, unlike the gene-based burden tests, most of the gene-set tests were not independent, for multiple testing we used a false discovery rate (FDR) correction that accounted for the number of functional categories (five), gene sets (11), and epilepsy phenotypes (four), totaling 220 tests, and defined a significant enrichment at FDR < 0.05.

Gene-Based Collapsing Analysis

For gene-based tests, we restricted our testing to deleterious URVs annotated as PTVs, missense variants with MPC ≥ 2, or in-frame insertions and deletions. For each gene, individuals who each

had at least one copy of these deleterious variants were counted as carriers, and we used a two-tailed Fisher's exact test (FET) to assess whether the proportion of carriers among epilepsy subgroup cases was significantly higher than among controls. Instead of assuming a uniform distribution for p values under the null, we generated empirical p values by permuting case-control labels 500 times, ordering the FET p values of all genes for each permutation, and taking the average across all permutations to form a rank-ordered estimate of the expected p value distribution. We did this by modifying functions in the "QQperm" R package (see Web Resources). To avoid potential false discoveries, we defined a stringent exome-wide significance at a p value $< 6.8 \times 10^{-7}$ and used Bonferroni correction to account for 18,509 consensus coding sequence genes tested and the four individual case-control comparisons.

Considering that recessive pathogenic variants were implicated in a number of epilepsy-associated genes, mostly identified from individuals with a DEE phenotype,[8] we conducted a secondary gene-based FET by using a recessive model and comparing the proportion of carriers that are homozygous for the minor allele between cases and controls. The recessive model was assessed for PTVs, missense (MPC $\geq$ 2) variants, and in-frame indels separately. For this analysis, we did not restrict to non-DiscovEHR variants, and we relaxed the allele frequency up to MAF < 0.01 to account for the sparse occurrences.

Additionally, to evaluate the contribution of low-frequency deleterious variants to epilepsy risk, we explored the gene burden of all protein-truncating and damaging missense variants for those that had an MAF <0.01 using SKAT[41] and including sex and the top ten PCs as covariates in the analysis. We performed the tests with the default weighting scheme [dbeta(1,25)].

Single-Variant Association

We estimated associations of common and low-frequency variants (MAF > 0.001) with epilepsy by Firth's method to perform logistic regression and correcting for sex and the first ten PCs.

QC, annotation, and analysis were largely performed with Hail (see Web Resources), open-source software for scalable genomic data analysis, in conjunction with R (version 3.4.2).

Results

Whole-Exome Sequencing, QC, and Sample Overview

We performed WES on an initial dataset of over 30,000 epilepsy-affected and control individuals. After stringent QC, we identified a total of 9,170 individuals with epilepsy and 8,436 controls without reported neurological or neuropsychiatric-related conditions; none of these individuals were related, and all were of European descent. Among the individuals with epilepsy, 1,021 were diagnosed with a DEE, 3,108 with GGE, 3,597 with NAFE, and 1,444 with other epilepsy syndromes (lesional focal epilepsy, febrile seizures, and others). We carefully matched affected individuals and controls on the basis of genetic ancestry to eliminate the possibility that, as can occur in studies of individuals from differing ancestries, population stratification or effects of variable MAF resolution would result in false positive findings. Because there was a lack of cosmopolitan controls from non-European populations, affected individuals who were identified from PCA and who had non-European ancestry were removed. Furthermore, to ensure that the distribution of rare variants was balanced between cases and controls,[42] we removed a subset of cohorts that included only affected individuals or only control individuals (from Sweden, Finland, Cyprus, and Turkey) where the mean synonymous singleton count that significantly deviated from the overall average was the consequence of incomplete ancestry matching (Figure S4). We called a total of 1,844,644 sites in 18,509 genes in the final dataset, comprising 1,811,325 SNVs and 33,319 indels, 48.5% of which were absent in the DiscovEHR database.[37] Among the non-DiscovEHR sites, 85% were singletons (defined as only one instance of that variant), and 99% had a minor AC not more than three (equivalent to MAF $\leq$ 0.01%; Figure S8); the missense with MPC $\geq$ 2 annotation accounted for 2.0% of the total missense variants (5.5% of the damaging and 1.0% of the benign missense variants predicted by PolyPhen-2 and SIFT). In our primary burden analyses, we focused on the URVs that are unique to the 17,606 individuals under study and are seen either only once (AC = 1) or no more than three times (AC $\leq$ 3) in our dataset. These URVs were shown to confer the largest risk of epilepsy in comparison to singletons observed in DiscovEHR, doubletons, or beyond (Figures S9 and S10). As previously described, epilepsy enrichment signals diminished with an increase in allele frequency.[24]

Enrichment of Ultra-Rare Deleterious Variants in Constrained Genes in DEEs and GGE

We first tested the burden of singleton URVs for each epilepsy subgroup, as well as for all epilepsy-affected individuals combined, versus controls among gene sets collected on the basis of current understanding and hypothesis of epilepsy causation. These included genes under evolutionary constraint, genes highly expressed in the brain, genes previously associated with epilepsy, $GABA_A$ receptor subunit-encoding genes, genes delineating GABAergic pathways, genes encoding excitatory neuronal receptors, and cation channel-encoding genes (Table S6). To evaluate the burden in constrained genes, we defined "LoF-intolerant" genes as those with either a pLI score[36] > 0.9 (3,488 genes) or separately a pLI score > 0.995 (1,583 genes), and we defined "missense-constrained" genes as those with a missense Z score > 3.09 (1,730 genes).[39] Genes marked by these specific cutoffs have been shown to be extremely intolerant to LoF or missense variation and thus help to identify specific classes of variants with a higher burden in diseased individuals.[36,43,44] We used a version of the scores derived from the non-neuropsychiatric subset of the ExAC samples. Because some of our control cohorts are also in ExAC (Table S2), we restricted our constrained gene burden tests to controls outside of the ExAC cohort (n = 4,042).

Consistent with a recent study that evaluated *de novo* burden in autism,[44] burden signals of PTVs were mostly

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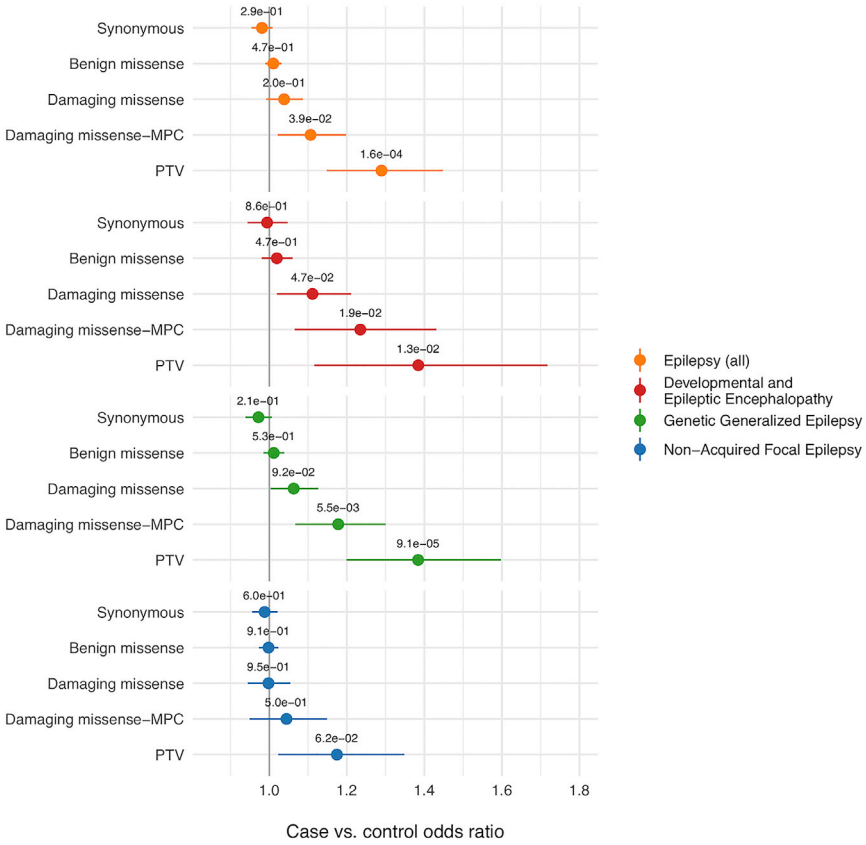

Figure 1. Burden of Ultra-Rare Singletons in LoF-Intolerant Genes (pLI > 0.995)
This analysis was restricted to 4,042 non-ExAC controls for comparison with individuals who have epilepsy. We focused on "ultra-rare" variants not observed in the DiscovEHR database. Significance of association was displayed in false discovery rate (FDR)-adjusted p values; errors bars indicated 95% confidence intervals (CIs) of the corresponding odds ratios. Odds ratios and 95% CIs were not multiplicity adjusted. The five functional coding annotations were defined as described in Table S5. PTV denotes protein-truncating variants; the "damaging missense" and "benign missense" categories were predicted by PolyPhen-2 and SIFT, and "damaging missense-MPC" was a group of missense variants with a missense badness, PolyPhen-2, and regional constraint (MPC) score ≥2. From top to bottom are the results based on all epilepsies: DEEs, GGE, and NAFE. Compared to controls (FDR < 0.05), individuals with epilepsy, except for individuals with NAFE, carried a significant excess of ultra-rare PTV and damaging missense (MPC ≥ 2) variants. PTV burden was higher than missense (MPC ≥ 2) burden across epilepsy types.

contained in genes with a pLI > 0.995, as opposed to pLI > 0.9 (Figures S12 and S13). Focusing on pLI > 0.995 in the all-epilepsy case-control analysis, both protein-truncating and damaging missense (MPC[35] ≥ 2) URVs in LoF-intolerant genes showed a mutational burden with an odds ratio (OR) of 1.3 (FDR-adjusted p value [*adj.p*] = 1.6×10^{-4}) and 1.1 (*adj.p* = 0.039), respectively. Breaking this down by epilepsy types, we found a significant excess of these deleterious URVs among individuals with DEEs ($OR_{PTV} = 1.4$, $adj.p_{PTV} = 0.013$; $OR_{MPC} = 1.2$, $adj.p_{MPC} = 0.019$), as expected. This enrichment was also seen in individuals with GGE at a magnitude comparable to that in individuals with DEEs ($OR_{PTV} = 1.4$, $adj.p_{PTV} = 9.1 \times 10^{-5}$; $OR_{MPC} = 1.2$, $adj.p_{MPC} = 5.5 \times 10^{-3}$) but was not significant in individuals with NAFE ($OR_{PTV} = 1.2$, $adj.p_{PTV} = 0.062$; $OR_{MPC} = 1.0$, $adj.p_{MPC} = 0.37$; Figure 1). There was no evidence of excess burden in synonymous URVs, suggesting that enrichment of deleterious pathogenic variants was unlikely to be the result of un-modeled population stratification or technical artifact. Among *in silico* missense predictors, MPC ≥ 2 annotations consistently showed a higher burden than those predicted by PolyPhen-2 and SIFT. The burden among missense-constrained genes exhibited a similar pattern: PTVs showed a higher burden in DEEs than in GGE and NAFE (Figure S14). In addition, both large gene sets were more enriched for PTVs than for damaging missense variants.

Burden in Candidate Genetic Etiologies Associated with Epilepsy

Among URVs in previously reported genes associated with epilepsy, we found an expected and pronounced difference in the number of singleton protein-truncating URVs in DEE-affected individuals relative to controls. PTVs were associated with an increased DEE risk in 43 genes known to carry mutations that cause dominant epilepsy disorders[24] (OR = 6.3, $adj.p = 2.1 \times 10^{-8}$), 50 known genes associated with dominant DEE syndromes[10] (OR = 9.1, $adj.p = 7.8 \times 10^{-11}$), and 33 genes with *de novo* burden in neurodevelopmental disorders with epilepsy[10] (OR = 14.8, $adj.p = 1.7 \times 10^{-12}$). Evidence for an excess of ultra-rare PTVs was also observed in individuals with GGE, at an OR ranging from 2 to 4. No enrichment of PTVs was observed among people with NAFE (Figure 2A; Table S9). In contrast, the burden of singleton missense (MPC ≥ 2) URVs was more pervasive across epilepsy types. In comparison to controls, there was a 3.6-fold higher rate of these missense URVs in established epilepsy-associated genes in individuals with DEEs ($adj.p = 1.6 \times 10^{-10}$), a 2.3-fold elevation in individuals with GGE ($adj.p = 6.4 \times 10^{-7}$), and a 1.9-fold elevation in individuals with NAFE ($adj.p = 2.8 \times 10^{-4}$).

Burden in Genes Encoding for Cation Channels and Neurotransmitter Receptors

Among brain-enriched genes—those defined as genes whose expression in brain tissues was at least 2-fold greater

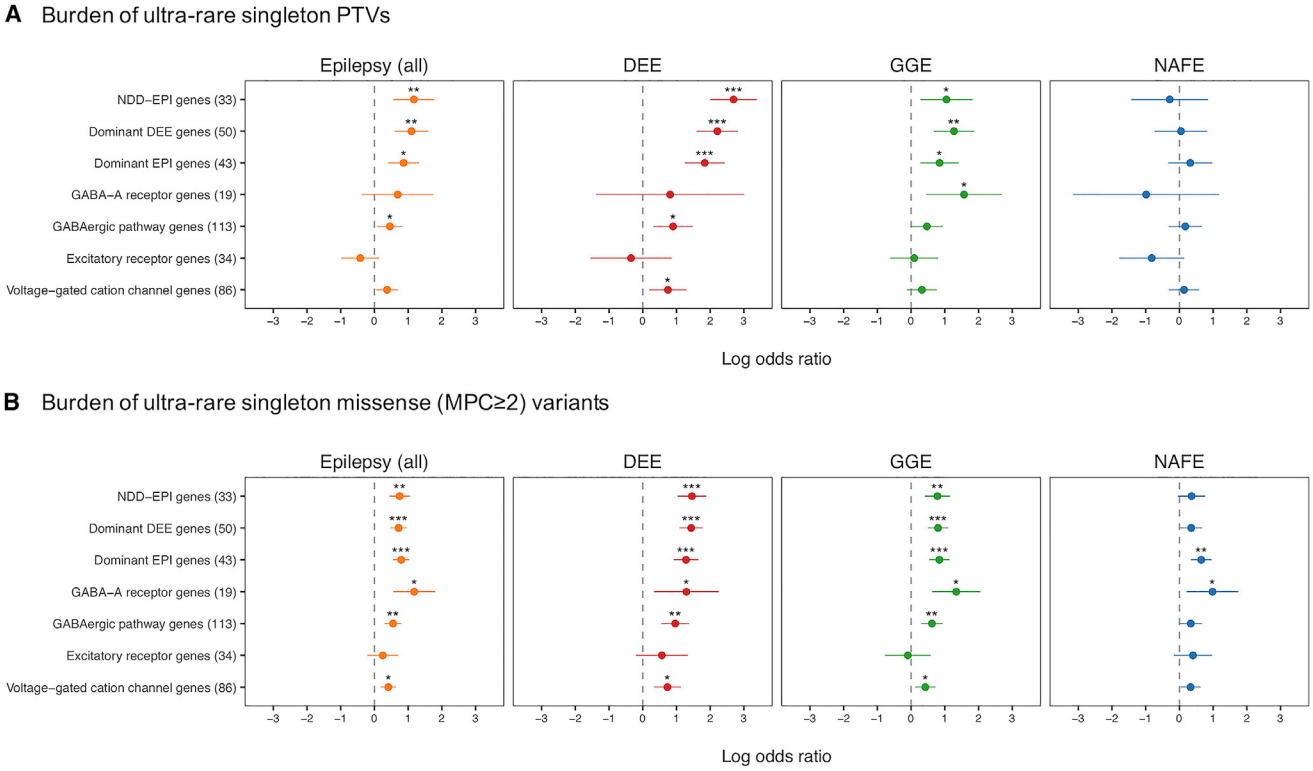

Figure 2. Burden of Ultra-Rare Singletons Annotated as Protein-Truncating Variants or Damaging Missense (MPC ≥ 2) Variants
"Ultra-rare" variants (URVs) were defined as not observed in the DiscovEHR database. Gene sets were defined in Table S6 and the number of genes was specified in the parenthesis. DEE stands for individuals with developmental and epileptic encephalopathies, GGE for genetic generalized epilepsy, NAFE for non-acquired focal epilepsy, and EPI for all epilepsy; NDD-EPI genes are genes with *de novo* burden in neurodevelopmental disorders with epilepsy. Asterisks indicate significance after FDR control (* for FDR-adjusted p value < 0.05, ** for adjusted p value $< 1 \times 10^{-3}$, and *** for adjusted p value $< 1 \times 10^{-5}$). Effects were displayed in odds ratios with the corresponding 95% confidence intervals (CIs).
(A) Protein-truncating variants (PTVs) were enriched in candidate epilepsy-associated genes for individuals with DEEs relative to other epilepsy subgroups but did not show a strong signal in inhibitory, excitatory receptors, or voltage-gated cation channel genes.
(B) The burden of damaging missense (MPC ≥ 2) variants, on the other hand, was stronger across these gene sets than was that of PTVs, especially for $GABA_A$ receptor genes and genes involved in GABAergic pathways. Relative to other epilepsy types, individuals with NAFE consistently showed the least burden of deleterious URVs. No enrichment was observed from excitatory receptors.

than their average expression across tissues on the basis of GTEx data[40]—both protein-truncating and damaging missense (MPC ≥ 2) URVs were significantly enriched in individuals with epilepsy versus controls, and the missense burden was much higher than the PTV burden (Figure S15). We then investigated the burden in four smaller gene sets previously implicated as mechanisms driving the etiology of epilepsy; these included 19 genes encoding $GABA_A$ receptor subunits, 113 genes involved in GABAergic pathways, 34 genes encoding excitatory receptors (ionotropic glutamate receptor subunits and nicotinic acetylcholine receptor subunits), and 86 voltage-gated cation channel genes (e.g., sodium, potassium, calcium—full list in Table S6).[25] We discovered that, relative to that of damaging missense variants, the distribution of PTVs in most of these gene sets did not differ significantly between epilepsy cases and controls (Figure 2A; Table 1). The PTV signals that remained significant after FDR correction included, for individuals with a DEE, an increased burden in GABAergic pathway genes and voltage-gated cation channels, and noticeably for individuals with GGE, an increased burden in the inhibitory $GABA_A$ receptors (OR = 4.8, $adj.p$ = 0.021). No PTV burden was detected for individuals with NAFE. In contrast, the enrichment of missense (MPC ≥ 2) URVs was more extensive in these gene sets across all epilepsy-to-control comparisons (Figure 2A; Table 1). The burden of these damaging missense pathogenic variants was seen in $GABA_A$ receptor genes ($OR_{DEE} = 3.7$, $adj.p_{DEE} = 0.028$; $OR_{GGE} = 3.8$, $adj.p_{GGE} = 1.4 \times 10^{-3}$; $OR_{NAFE} = 2.7$, $adj.p_{NAFE} = 0.039$), GABAergic pathway genes ($OR_{DEE} = 2.6$, $adj.p_{DEE} = 4.7 \times 10^{-5}$; $OR_{GGE} = 1.9$, $adj.p_{GGE} = 9.9 \times 10^{-4}$; $OR_{NAFE} = 1.4$, $adj.p_{NAFE} = 0.11$), and voltage-gated cation channel genes ($OR_{DEE} = 2.1$, $adj.p_{DEE} = 1.7 \times 10^{-3}$; $OR_{GGE} = 1.5$, $adj.p_{GGE} = 0.023$; $OR_{NAFE} = 1.4$, $adj.p_{NAFE} = 0.081$). However, no enrichment was detected in genes encoding excitatory receptors. For individuals with NAFE, the burden signals were consistently the weaker across gene sets compared than in individuals with the other epilepsy phenotypes. None of the gene sets was enriched for putatively neutral variation, except for a slightly elevated synonymous burden in $GABA_A$ receptor genes (Table S9). These

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Table 1. Enrichment of Ultra-Rare Protein-Truncating or Damaging Missense (MPC ≥ 2) Singletons in Epilepsy

	Carriers (N)					
Epilepsy Type	**Affected Individuals**	**Controls**	**OR**	**95% CI**	***p* Value**	**FDR-Adjusted *p***
Dominant Epilepsy Disorders (43)						
PTV (95)						
EPI	67	27	2.37	(1.50–3.74)	2.0×10^{-4}	1.2×10^{-3}
DEE	24	27	6.28	(3.48–11.3)	1.0×10^{-9}	2.1×10^{-8}
GGE	22	27	2.33	(1.32–4.11)	3.6×10^{-3}	1.4×10^{-2}
NAFE	15	27	1.38	(0.72–2.66)	3.4×10^{-1}	4.7×10^{-1}
EPI	235	98	2.21	(1.74–2.81)	1.1×10^{-10}	2.8×10^{-9}
MPC ≥ 2 (335)						
DEE	47	98	3.60	(2.50–5.19)	5.0×10^{-12}	1.6×10^{-10}
GGE	85	98	2.31	(1.71–3.12)	4.4×10^{-8}	6.4×10^{-7}
NAFE	80	98	1.91	(1.41–2.60)	3.3×10^{-5}	2.8×10^{-4}
Dominant DEE Syndromes (50)						
PTV (89)						
EPI	68	21	3.00	(1.82–4.95)	1.8×10^{-5}	1.6×10^{-4}
DEE	27	21	9.13	(4.93–16.9)	2.1×10^{-12}	7.8×10^{-11}
GGE	25	21	3.57	(1.95–6.54)	3.7×10^{-5}	3.0×10^{-4}
NAFE	10	21	1.05	(0.48–2.29)	9.1×10^{-1}	9.3×10^{-1}
EPI	224	101	2.05	(1.61–2.60)	6.5×10^{-9}	1.2×10^{-7}
MPC ≥ 2 (327)						
DEE	54	101	4.20	(2.97–5.95)	6.0×10^{-16}	1.3×10^{-13}
GGE	85	101	2.22	(1.64–3.00)	2.0×10^{-7}	2.6×10^{-6}
NAFE	63	101	1.42	(1.02–1.97)	3.7×10^{-2}	8.8×10^{-2}
Neuro-Developmental Disorders with Epilepsy (33)						
PTV (63)						
EPI	49	14	3.22	(1.75–5.90)	1.6×10^{-4}	9.9×10^{-4}
DEE	29	14	14.77	(7.4–29.49)	2.3×10^{-14}	1.7×10^{-12}
GGE	14	14	2.86	(1.32–6.17)	7.7×10^{-3}	2.7×10^{-2}
NAFE	4	14	0.75	(0.24–2.34)	6.2×10^{-1}	7.2×10^{-1}
MPC ≥ 2 (215)						
EPI	149	65	2.11	(1.57–2.84)	9.4×10^{-7}	1.1×10^{-5}
DEE	36	65	4.30	(2.81–6.57)	1.8×10^{-11}	5.1×10^{-10}
GGE	54	65	2.18	(1.50–3.17)	4.2×10^{-5}	3.2×10^{-4}
NAFE	41	65	1.43	(0.96–2.15)	8.0×10^{-2}	1.6×10^{-1}
$GABA_A$ Receptors (19)						
PTV (17)						
EPI	12	5	1.99	(0.69–5.74)	2.0×10^{-1}	3.2×10^{-1}
DEE	1	5	2.25	(0.25–20.2)	4.7×10^{-1}	6.0×10^{-1}
GGE	9	5	4.81	(1.57–14.7)	5.9×10^{-3}	2.1×10^{-2}
NAFE	1	5	0.37	(0.04–3.27)	3.7×10^{-1}	5.0×10^{-1}

(*Continued on next page*)

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Table 1. ***Continued***

Epilepsy Type	Carriers (N)		OR	95% CI	*p* Value	FDR-Adjusted *p*
	Affected Individuals	Controls				
MPC ≥ 2 (62)						
EPI	49	13	3.25	(1.74–6.07)	2.1×10^{-4}	1.2×10^{-3}
DEE	7	13	3.65	(1.39–9.54)	8.3×10^{-3}	2.8×10^{-2}
GGE	21	13	3.81	(1.86–7.81)	2.5×10^{-4}	1.4×10^{-3}
NAFE	15	13	2.67	(1.23–5.77)	1.3×10^{-2}	3.9×10^{-2}
GABAergic Pathway (113)						
PTV (127)						
EPI	81	44	1.58	(1.10–2.28)	1.4×10^{-2}	4.4×10^{-2}
DEE	16	44	2.46	(1.37–4.39)	2.4×10^{-3}	1.0×10^{-2}
GGE	28	44	1.60	(0.99–2.57)	5.3×10^{-2}	1.1×10^{-1}
NAFE	24	44	1.19	(0.73–1.95)	4.9×10^{-1}	6.1×10^{-1}
MPC ≥ 2 (287)						
EPI	185	101	1.73	(1.35–2.22)	1.6×10^{-5}	1.6×10^{-4}
DEE	34	101	2.62	(1.74–3.95)	4.5×10^{-6}	4.7×10^{-5}
GGE	68	101	1.86	(1.35–2.56)	1.6×10^{-4}	9.9×10^{-4}
NAFE	58	101	1.40	(1.00–1.95)	4.7×10^{-2}	1.1×10^{-1}
Excitatory Receptors (34)						
PTV (54)						
EPI	22	32	0.66	(0.37–1.15)	1.4×10^{-1}	2.5×10^{-1}
DEE	3	32	0.71	(0.21–2.35)	5.7×10^{-1}	6.7×10^{-1}
GGE	11	32	1.10	(0.54–2.23)	8.0×10^{-1}	8.4×10^{-1}
NAFE	5	32	0.44	(0.17–1.15)	9.5×10^{-2}	1.8×10^{-1}
MPC ≥ 2 (80)						
EPI	47	33	1.28	(0.81–2.02)	2.9×10^{-1}	4.3×10^{-1}
DEE	9	33	1.76	(0.81–3.81)	1.5×10^{-1}	2.6×10^{-1}
GGE	12	33	0.91	(0.46–1.79)	7.8×10^{-1}	8.3×10^{-1}
NAFE	20	33	1.50	(0.84–2.65)	1.7×10^{-1}	2.8×10^{-1}
Voltage-Gated Cation Channels (86)						
PTV (163)						
EPI	100	63	1.45	(1.05–2.01)	2.5×10^{-2}	7.0×10^{-2}
DEE	18	63	2.11	(1.21–3.66)	8.2×10^{-3}	2.8×10^{-2}
GGE	31	63	1.38	(0.88–2.16)	1.6×10^{-1}	2.7×10^{-1}
NAFE	30	63	1.15	(0.73–1.81)	5.5×10^{-1}	6.7×10^{-1}
MPC ≥ 2 (329)						
EPI	206	121	1.51	(1.20–1.90)	4.7×10^{-4}	2.4×10^{-3}
DEE	34	121	2.08	(1.40–3.10)	3.1×10^{-4}	1.7×10^{-3}
GGE	73	121	1.52	(1.12–2.07)	6.6×10^{-3}	2.3×10^{-2}
NAFE	74	121	1.39	(1.03–1.88)	3.1×10^{-2}	8.1×10^{-2}

This analysis compared the burden of deleterious pathogenic variants between cases and controls using logistic regression and adjusting for sex, the first ten principal components, and overall variant count. FDR correction was based on a full list of burden tests shown in Table S9. Tested epilepsy types included all epilepsies (EPI; n = 9,170), developmental and epileptic encephalopathies (DEE; n = 1,021), genetic generalized epilepsy (GGE; n = 3,108), and non-acquired focal epilepsy (NAFE; n = 3,597). All were compared against 8,436 control samples. Figure 2 shows the enrichment pattern of PTVs and MPC ≥ 2 variants across the seven gene sets listed here.

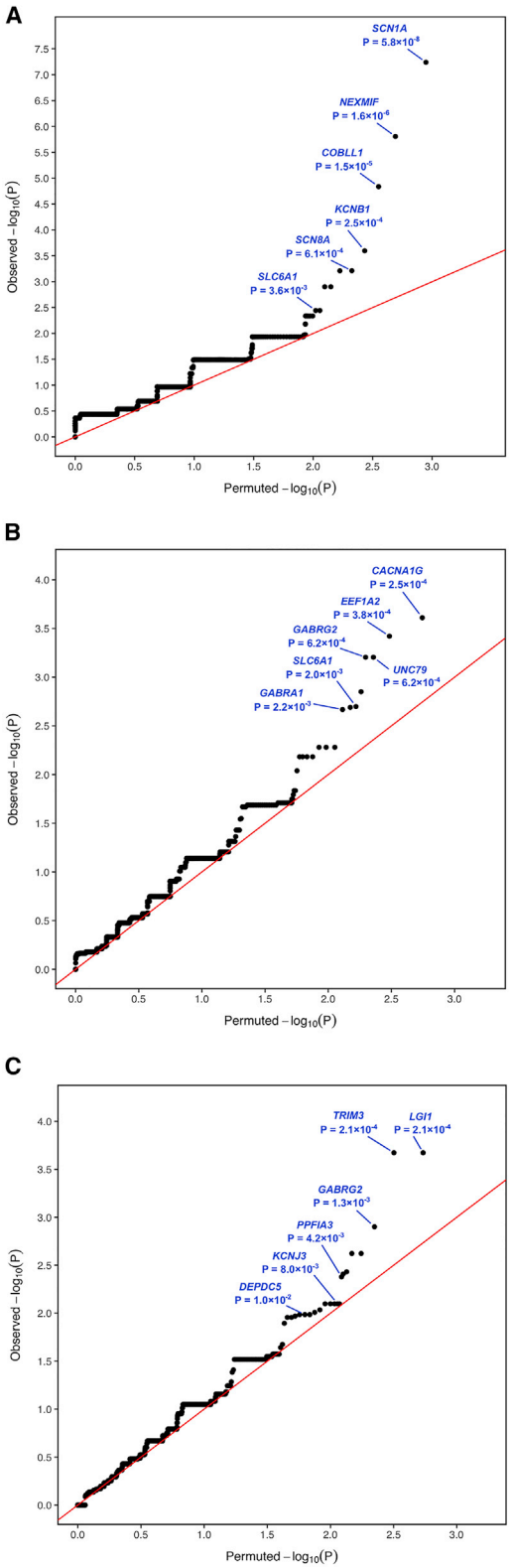

Figure 3. Gene Burden for Individuals Diagnosed with Developmental and Epileptic Encephalopathies, Genetic Generalized Epilepsy, or Non-Acquired Focal Epilepsy

This analysis focused on ultra-rare (non-DiscovEHR) singleton variants annotated as protein-truncating variants (PTVs), damaging missense variants (MPC $\geq$ 2), or in-frame insertions and deletions and used Fisher's exact test (FET) to identify genes with a differential carrier rate of these ultra-rare deleterious variants in individuals with epilepsy in comparison to controls. Exome-wide significance was defined by a p value $< 6.8 \times 10^{-7}$ after Bonferroni correction (see Materials and Methods). Only *SCN1A* achieved exome-wide significance for individuals with developmental and epileptic encephalopathies (DEEs). Panels refer to individuals diagnosed with (A) developmental and epileptic encephalopathies, (B) genetic generalized epilepsy, and (C) non-acquired focal epilepsy.

results support a recent finding where rare missense variation in $GABA_A$ receptor genes conferred a significant risk to GGE[25] and together implicate the relative importance and involvement of damaging missense variants in abnormal inhibitory neurotransmission in both severe and less severe forms of epilepsy.

For gene sets other than the three lists of previously associated genes (Table S6; 74 non-overlapping genes in total), we evaluated the residual burden of URVs after correcting for events in the 74 known genes. For the gene sets of cation channel and neurotransmitter receptor genes, the adjusted burden signals of singleton deleterious URVs was largely reduced, although some weak associations remained in $GABA_A$ receptor-encoding or GABAergic genes among individuals with DEEs or GGE. For the larger gene groups of constrained genes and brain-enriched genes, burden signals were attenuated, but many remained significant, especially the strong enrichment of missense MPC $\geq$ 2 variants in brain-enriched genes across all three classes of epilepsy (Figure S16). These findings suggest that although most gene burden is driven by previously identified genes, more associations could be uncovered with larger sample sizes.

Gene-Based Collapsing Analysis Recaptures Known Genes Associated with DEEs

For gene discovery, because both protein-truncating and damaging missense (MPC $\geq$ 2) URVs showed an elevated burden in epilepsy cases, we aggregated both together as deleterious pathogenic variants along with in-frame insertions and deletions in our gene-collapsing analysis. This amassed to a total of 46,917 singleton URVs and 52,416 URVs with AC $\leq$ 3. Surprisingly, for individuals diagnosed with DEEs, we re-identified several of the established candidate genes associated with DEEs as top associations (Figure 3A). Although screening was not performed systematically, using clinical gene panels prior to enrollment produced negative results for many participants with DEEs (Table S7). According to the results from singleton URVs, *SCN1A* (MIM: 182389) was the only gene that reached exome-wide significance (OR = 18.4, $p = 5.8 \times 10^{-8}$); other top-ranking known genes included *NEXMIF* ([MIM: 300524] previously known as *KIAA2022*; OR > 99, $p = 1.6 \times 10^{-6}$), *KCNB1* ([MIM: 600397] OR = 20.8, $p = 2.5 \times 10^{-4}$), *SCN8A* ([MIM: 600702] OR = 13.8, $p = 6.1 \times 10^{-4}$), and *SLC6A1* ([MIM: 137165] OR = 11.1, $p = 3.6 \times 10^{-3}$) (Table S11). Some carriers of deleterious URVs in lead genes were affected individuals with normal results for gene panel testing; for example, these included two out of the three carriers of qualifying URVs for *PURA*

(MIM: 600473) and two out of five for *KCNB1* (Table S7). This is primarily because gene panels ordered for a particular diagnosis usually do not screen all of the genes commonly implicated in DEEs (e.g., one of the carriers of qualifying URVs in *KCNB1* was diagnosed with West syndrome [MIM: 308350] and screened with a customized panel that did not include *KCNB1*). Overall, more than 50 different gene panels were used across sample-contributing sites, which adds to the heterogeneity in screening procedures and interpretation. The gene burden results held up when URVs with AC $\leq$ 3 were considered and these results often showed even stronger associations; two other well-studied genes, *STXBP1* ([MIM: 602926] OR = 13.3, $p = 1.4 \times 10^{-5}$) and *WDR45* ([MIM: 300526] OR > 49, $p = 1.2 \times 10^{-3}$), emerged on top, and both of these have been implicated in DEEs and developmental disorders (Table S12).

Channel and Transporter Genes Implicated in GGE and NAFE

When evaluating gene burden in the GGE and NAFE epilepsy subgroups, we did not identify any genes of genome-wide significance. However, several candidate genes previously associated with epilepsy made up the lead associations; such genes included ion channel and transporter genes, mutations of which are known to cause rare forms of epilepsy. For the GGE case-control analysis in singleton deleterious URVs, the lead associations included four previously associated genes (*EEF1A2* [MIM: 602959], OR = 32, $p = 3.8 \times 10^{-4}$; *GABRG2* [MIM: 137164], OR = 19.0, $p = 6.2 \times 10^{-4}$; *SLC6A1* [MIM: 616421], OR = 7.3, $p = 2.0 \times 10^{-3}$; and *GABRA1* [MIM: 137160], OR = 9.5, $p = 2.2 \times 10^{-3}$), and two genes (*CACNA1G* [MIM: 604065], OR = 9.1, $p = 2.5 \times 10^{-4}$ and *UNC79* [MIM: 616884], OR = 19.0, $p = 6.2 \times 10^{-4}$) that were not previously linked to epilepsy but are both highly expressed in the brain and under evolutionary constraint (Figures 3B; Table S13). Although evidence has been mixed, *CACNA1G* was previously implicated as a potential susceptibility gene associated with GGE in mutational analysis[45] and was reported to modify mutated sodium channel (*SCN2A* [MIM: 182390]) activity in epilepsy.[46] *UNC79* is an essential part of the UNC79-UNC80-NALCN (MIM: 612636, MIM: 611549) channel complex that influences neuronal excitability by interacting with extracellular calcium ions,[47] and this channel complex has been previously associated with infantile encephalopathy.[48] Notably, all of these lead genes were more enriched for damaging missense (MPC $\geq$ 2) than for protein-truncating URVs despite the lower rate of MPC $\geq$ 2 variants relative to PTVs (Table S13).

For individuals with NAFE, the analysis of singleton deleterious URVs identified *LGI1* (MIM: 604619) and *TRIM3* (MIM: 605493) as the top two genes carrying a disproportionate number of deleterious URVs; however, neither reached exome-wide significance (OR > 32, $p = 2.1 \times 10^{-4}$). *GABRG2*, a lead association in individuals with GGE, was among the top ten most enriched genes, along with two brain-enriched, constrained genes (*PPFIA3* [MIM: 603144], OR = 8.2, $p = 4.2 \times 10^{-3}$ and *KCNJ3* [MIM: 601534], OR = 16.4, $p = 1.2 \times 10^{-3}$). *GABRG2* has previously been reported to show an enrichment of variants compared to controls in a cohort of individuals with Rolandic epilepsy ([MIM: 245570, 300643] childhood epilepsy with centrotemporal spikes) or related phenotypes, the most common group of focal epilepsies of childhood.[49] Two other genes previously associated with epilepsy, *DEPDC5* (MIM: 614191) and *SCN8A* (both OR = 5.5, $p = 0.01$), were among the top 20 associations (Figures 3C; Table S14). *LGI1* and *DEPDC5* are established genes associated with focal epilepsy, and *DEPDC5* was the only hit of exome-wide significance in the Epi4K WES study for familial NAFE cases.[24] *TRIM3* has not been previously implicated in epilepsy, but evidence from a study involving a mouse model implicates it in regulation of $GABA_A$ receptor signaling and thus in modulation of seizure susceptibility.[50] The single-gene burden for both GGE and NAFE remained similar when URVs with an allele count up to AC $\leq$ 3 were considered (Tables S14 and S16). Gene burden tests collapsing all epilepsy phenotypes recapitulated the lead genes in each of the subgroup-specific analyses, but none of the genes achieved exome-wide significance (Tables S17 and S18). It is worth noting that some of the genes were enriched for deleterious URVs among the controls; this is clearly driven by non-neuropsychiatric disease ascertainment for many of the available controls (e.g., *LDLR* [MIM: 606945] in Table S17; most control carriers were individuals with cardiovascular diseases from the MIGen cohorts in Table S2). Thus, these should not be interpreted as potential protective signals associated with epilepsy.

Recessive Model, SKAT Gene Test, and Single-Variant Association

The secondary gene-based test of a recessive model did not identify genes that differed significantly in the carrier rate of homozygous deleterious variants between epilepsy-affected individuals and controls (Table S19). Even if we considered variants up to MAF < 0.01, for most of the lead genes, only one case carrier was identified. For the DEE cohort, these genes included previously implicated recessive genes, such as *ARV1* (MIM: 611647), *BRAT1* (MIM: 614506), and *CHRDL1* (MIM: 300350)[51] with a homozygous PTV and *OPHN1* (MIM: 300127)[51] with a recessive missense (MPC $\geq$ 2) variant (Table S19A). For the GGE and NAFE cohorts, in the lead gene associations, a few studied recessive epilepsy-associated genes were also observed, such as *SLC6A8*[51] ([MIM: 300036] a homozygous PTV) for GGE (Table S19B), and *SLC6A8* (a homozygous missense MPC) and *SYN1*[51] ([MIM: 313440] a homozygous PTV) for NAFE (Table S19C). One GGE-affected individual was found to be homozygous for an in-frame deletion on *CHD2* (MIM: 602119), a gene previously reported to carry autosomal dominant pathogenic variants in

persons with DEE[51] (Table S19B). These findings suggest that an even larger cohort will be needed to clearly identify recessive risk variants for different groups of epilepsy.

Beyond URVs, we used SKAT to study the contribution of low-frequency deleterious variants (MAF < 0.01) to epilepsy risk. Top associations for individuals with DEEs included known genes such as missense-enriched *STXBP1* ($p = 9.3 \times 10^{-9}$), *KCNA2* ([MIM: 176262] $p = 1.0 \times 10^{-5}$; Figure S18), PTV-enriched *NEXMIF* ($p = 7.1 \times 10^{-8}$), and *SCN1A* ($p = 3.9 \times 10^{-4}$; Figure S19). However, no significant gene enrichment was observed in the GGE and NAFE cohorts or when all epilepsy-affected individuals were combined. The tests for PTVs and missense variants with MPC ≥ 2 were mostly underpowered as a result of sparse observations (Figures S18 and S19). No individual low-frequency variant (MAF > 0.001) was significantly associated with overall epilepsy or with any of the studied epilepsy phenotypes (Figure S20). The primary gene-based test results and single-variant associations are available on our Epi25 WES browser (see Web Resources).

Discussion

In this, the largest exome study of epilepsies to date, we show that ultra-rare deleterious coding variation—variation absent in a large population-based exome database—is enriched across the severity spectrum for epilepsy syndromes when individuals with these syndromes are compared to ancestrally matched controls. When all genes were considered in the tested gene sets, PTVs showed a more significant signal than did missense variants with an MPC ≥ 2, and enrichment in deleterious URVs was more pronounced in individuals diagnosed with DEEs and GGE than in those diagnosed with NAFE. Although no single gene surpassed exome-wide statistical significance to be associated with GGE or NAFE, specific gene sets that had previously been associated with epilepsy or with encoding biologically interesting entities showed a clear enrichment of deleterious URVs. Specifically, we observed a significant excess of deleterious URVs in constrained genes, established epilepsy-associated genes, and $GABA_A$ receptor subunit genes, a larger group of genes delineating the GABAergic pathway, and also in all cation-channel-encoding genes. Our results thus support the concept that defects in GABAergic inhibition underlie various forms of epilepsy. The epilepsy-associated excess of deleterious URVs in our study most likely comprises signals from both inherited and *de novo* variants; these *de novo* variants were enriched by the restriction of variant inclusion to a combination of study-specific singletons and by their absence in a population reference cohort (DiscovEHR).[38,43] These findings, based on a more than 5-fold increase in sample size over previous exome-sequencing studies,[24–26,52] clearly support observations that have been hypothesized for GGE and NAFE from studies of rare, large monogenic families and confirm that the same genes are relevant in both settings. Thus, a further increase in sample size will continue to unravel the complex genetic architecture of GGE and NAFE. The evidence that URVs contribute, in part, to GGE and NAFE is clear, but what remains unclear is the extent to which the excess rate of URVs observed in individuals with epilepsy is a consequence of a small subset of affected individuals carrying highly penetrant mutations or a result of URVs that confer risk but, instead of rising to the level of Mendelian acting mutations, simply contribute to an overall polygenic risk for these syndromes. Interestingly, no enrichment was seen in genes encoding the excitatory glutamate and acetylcholine receptors. For GGE, this difference between variants in inhibitory versus excitatory receptor genes could be real: excitatory receptor variants have not been shown so far in single subjects or families. In NAFE, however, we suspect it is probably due to a lack of power and/or genetic heterogeneity because genetic variants in specific subunits of nicotinic acetylcholine and NMDA receptors have been described extensively in different types of non-acquired familial focal epilepsies.[53]

Notably, our overall finding of a mild to moderate burden of deleterious coding URVs in NAFE (Figures 1 and 2) contrasts with results reported in the Epi4K WES study, where the familial NAFE cohort showed a strong enrichment signal of ultra-rare functional variation in genes commonly implicated in epilepsy and ion-channel genes.[24] In addition, our findings for GGE showed a genetic risk comparable to or even stronger than that in the Epi4K familial GGE cohort. The strong signal in our GGE cohort most likely reflects the larger sample size, whereas the weaker signal in our NAFE cohort is most likely due to differences in patient ascertainment. In Epi4K, the cohort was deliberately enriched with familial cases, most of whom had an affected first-degree relative and were ascertained in sibling or parent-child pairs or multiplex families, and familial NAFE is relatively uncommon. In the Epi25 collaboration, a positive family history of epilepsy was not a requirement, and only 9% of individuals with DEEs, 12% of those with GGE, and 5% of those with NAFE had a known affected first-degree relative. Removing these familial cases led to no change in gene-set burden (Figure S17) and to a slightly attenuated association for some of the lead genes in the GGE and NAFE cohorts (Table S20). Indeed, our results were consistent with those from the Epi4K sporadic NAFE cohort, where no signals of enrichment were observed.[24,54] This difference might reflect the substantial etiological and genetic heterogeneity of epilepsy even within subgroups, especially in NAFE. In particular, the dramatically weaker genetic signals, per sample, observed in individuals with NAFE studied here in comparison with those in the previous Epi4K study illustrate a pronounced difference in the genetic signals associated with familial and non-familial NAFE. The reasons for this striking difference remain to be elucidated. Our comparisons of GGE and NAFE showed a larger genetic burden from URVs for GGE relative to

NAFE, which could be due to heterogeneity in electroclinical syndromes within each class and should not be viewed as conclusive. On the other hand, in the latest GWAS of common epilepsies, which was comprised of 15,212 cases and 29,677 controls from the ILAE Consortium,[20] fewer GWAS hits were discovered, and less heritability was explained by common genetic variation for the focal epilepsy cohort (9.2%) compared to the GGE cohort (32.1%), suggesting that current evidence from both common and rare-variant studies is converging on a larger genetic component underlying the etiology of non-familial cases of GGE relative to NAFE, as originally postulated.

We found that ultra-rare missense variants with an MPC score[35] $\geq$ 2 (2.0% of missense variants) were enriched in individuals with epilepsy at an effect size approaching the results from PTVs in the investigated gene groups. For GGE and NAFE, the burden of these missense variants (MPC $\geq$ 2) was even more prominent than the burden of PTVs in known genes associated with epilepsy and GABAergic genes (Figure 2). At the gene level, some of the most commonly implicated channel genes (e.g., *GABRG2*, *CACNA1G*) carried a higher number of missense variants (MPC $\geq$ 2) than PTVs in people with epilepsy. For instance, in the gene-based collapsing analysis considering all epilepsies, 15 *GABRG2* pathogenic variants were found in epilepsy-affected individuals (including seven with GGE and seven with NAFE; Tables S13, S15, and S17) versus only one pathogenic variant in controls; among the case-specific pathogenic variants, one was a splice-site mutation, and the other 14 were all missense variants (MPC $\geq$ 2) (Figure S21) that linked to an impaired channel function. This is in line with findings from a recent exome-wide study of 6,753 individuals with neurodevelopmental disorder with and without epilepsy;[10] that study detected an association of missense *de novo* variants with the presence of epilepsy, particularly when considering only ion-channel genes. An association of missense variants, rather than PTVs, with disease points to a pathophysiological mechanism of protein alteration (e.g., gain-of-function or dominant-negative effects) rather than haploinsufficiency, but ultimately, only functional tests can elucidate these mechanisms. A recent study on the molecular basis of six *de novo* missense variants in *GABRG2* identified in DEEs reported that the overall inhibitory function of *GABRG2* was reduced as a result of decreased cell surface expression or GABA-evoked current amplitudes, suggesting GABAergic disinhibition as the underlying mechanism.[55] Surprisingly, two of those recurrent *de novo* missense variants (c.316G>A [p.Ala106Thr] and c.968G>A [p.Arg323Gln]) were seen in two GGE-affected individuals in our study, and another recently reported variant in *GABRB2* (c.946G>A [p.Val316Ile]) also occurred both *de novo* in DEEs[56] and as an inherited variant in a GGE family showing a loss of receptor function.[25] This suggests that changes in protein function from the same missense pathogenic variant might contribute not only to severe epilepsy syndromes but also to epilepsy phenotypes with milder presentations, similar to what is known about variable expressivity in large families carrying *GABRG2* variants.[53,57–59] Reduced receptor function due to *GABRG2* variants has been also been shown previously[49,59] for childhood epilepsy with centrotemporal spikes, symptoms that belong to the NAFE group in this study. Moving forward, discovering how variant-specific perturbations of the neurotransmission and signaling system in a gene can link to a spectrum of epilepsy syndromes will require in-depth functional investigation.

Although we have increased the sample size from the Epi4K and EuroEPINOMICS WES studies for both GGE and NAFE subgroups by more than 5-fold, the phenotypic and genetic heterogeneity of these less-severe forms of epilepsy—on par with other complex neurological and neuropsychiatric conditions—will require many more samples to achieve statistical power for identifying exome-wide significant genes. We estimated that at least 8,000 cases and 20,000 controls would be required in order for some of the lead genes from the GGE and NAFE cohorts to exome-wide significance (Table S8). Furthermore, although we implemented stringent QC to effectively control for the exome capture differences between individuals with epilepsy and controls, this concomitantly resulted in a loss of a substantial number of the called sites and reduced our detection power to identify associated variants. As sample sizes grow, the technical variation across projects and sample collections will remain a challenge in large-scale sequencing studies that rely on a global collaborative effort.

With this, the largest epilepsy WES study to date, we demonstrated a strong replicability of existing gene findings in an independent cohort. $GABA_A$ receptor genes affected by predicted-pathogenic missense variants were enriched across the three subgroups of epilepsy. An ongoing debate in epilepsy genetics is the degree to which generalized and focal epilepsies segregate separately and whether their genetic determinants are largely distinct or sometimes shared.[4,22] Although clinical evidence for general separation of pathophysiological mechanisms in these two forms is strong, and most monogenic epilepsy families have either generalized or focal syndromes, the distinction is not absolute. Here, the finding of rare variants in $GABA_A$ receptor genes in both forms adds weight to the case for shared genetic determinants.

Our results suggest that clinical presentations of GGE and NAFE with complex inheritance patterns have a combination of both common and rare genetic risk variants. The latest ILAE epilepsy GWAS of over 15,000 affected individuals and 25,000 controls identified 16 genome-wide significant loci for common epilepsies,[20] mapped these loci to ion-channel genes, transcriptional factors, and pyridoxine metabolism, and implicated these loci as having a role in epigenetic regulation of gene expression in the brain. A combination of rare and common genetic association studies with large sample sizes, along with the growing evidence from studies of copy-number variation

and tandem repeat expansions in epilepsy,[23,60,61] will further decipher the genetic landscape of GGE and NAFE. The ongoing effort of the Epi25 Collaborative is expected to double the patient cohorts in upcoming years; the goal is to elucidate shared and distinct gene discoveries associated with severe and less-severe forms of epilepsy and ultimately facilitatate precision medicine strategies in the treatment of epilepsy.

Accession Numbers

The accession number for the Epi25 Year1 whole-exome sequence data reported in this paper is dbGaP: phs001489.

Supplemental Data

Supplemental Data can be found online at https://doi.org/10.1016/j.ajhg.2019.05.020.

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Declaration of Interests

B.M.N. has roles in the following genomics companies: Deep Genomics (scientific advisory board), Camp4 Therapeutics Corporation

(scientific advisory board, consultant), Takeda Pharmaceutical (consultant), Biogen-Consultant Genomics Analytics (advisory panel). D.B.G. has equity interest in Praxis Therapeutics and Q State Biosciences, companies focused on precision medicine in neurodevelopmental diseases. S.F.B. is a consultant to Praxis Therapeutics.

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Web Resources

DiscovEHR cohort, http://www.discovehrshare.com
Epi25 Collaborative, http://epi-25.org/
Epi25 WES results browser, http://epi25.broadinstitute.org/
EpiPGX project, http://www.epipgx.eu
Exome Aggregation Consortium (ExAC), http://exac.broadinstitute.org
Hail, https://github.com/hail-is/hail
NIH Genomic Data Sharing Policy, https://osp.od.nih.gov/scientific-sharing/policies/
Online Mendelian Inheritance in Man, https://www.omim.org
QQperm: Permutation-Based QQ Plot and Inflation Factor Estimation. R package version 1.0.1., https://CRANR-projectorg/package=QQperm

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